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A ROLE OF THE UBIQUITIN-PROTEASOME DEGRADATION PATHWAY IN THE BIOGENESIS EFFICIENCY OF β -CELL ATP-SENSITIVE POTASSIUM CHANNELS

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Abstract

ATP-sensitive potassium (K_{ATP}) channels of pancreatic β -cells mediate glucose-induced insulin secretion by linking glucose metabolism to membrane excitability. The number of plasma membrane K_{ATP} channels determines the sensitivity of β -cells to glucose stimulation. The K_{ATP} channel is formed in the endoplasmic reticulum (ER) upon co-assembly of four inwardly rectifying potassium channel Kir6.2 subunits and four sulfonylurea receptor 1 (SUR1) subunits. Little is known about the cellular events that govern the channel's biogenesis efficiency and expression. Recent studies have implicated the ubiquitin-proteasome pathway in modulating surface expression of several ion channels. In this work, we investigated whether the ubiquitin-proteasome pathway plays a role in the biogenesis efficiency and surface expression of K_{ATP} channels. We provide evidence that, when expressed in COS cells, both Kir6.2 and SUR1 undergo ER-associated degradation (ERAD) via the ubiquitin-proteasome system. Moreover, treatment of cells with proteasome inhibitors MG132 or lactacystin leads to increased surface expression of K_{ATP} channels by increasing the efficiency of channel biogenesis. Importantly, inhibition of proteasome function in a pancreatic β -cell line, INS-1, that express endogenous K_{ATP} channels also results in increased channel number at the cell surface, as assessed by surface biotinylation and whole-cell patch-clamp recordings. Our results support a role of the ubiquitin-proteasome pathway in the biogenesis efficiency and surface expression of β -cell K_{ATP} channels.

Introduction

The primary function of pancreatic β -cells is to maintain glucose homeostasis by releasing insulin when the blood glucose level rises. Key to this glucose-insulin secretion coupling process is a membrane protein complex called the ATP-sensitive potassium (K_{ATP}) channel. K_{ATP} channels link plasma glucose levels to the insulin secreting machinery by virtue of their sensitivities to intracellular nucleotides ATP and ADP, whose levels fluctuate as a result of glucose metabolism (1,4). ATP promotes channel closure whereas Mg^{2+} -complexed ADP promotes channel opening. In this way, they translate metabolic signals to electrical signals, which in turn control insulin secretion. The extent to which K_{ATP} channels control β -cell membrane potential is critically dependent on the level of channel expression at the cell surface. Reduced expression leads to inability of the β -cell to shut down insulin secretion when plasma glucose falls (8,23,35,42), while increased expression is expected to stabilize the cell near its resting membrane potential and raise the concentration of glucose required to elicit an insulin response.

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Biogenesis of the β -cell K_{ATP} channel, defined here as formation of functional channel complexes that properly traffic from the ER to the plasma membrane, requires initial coassembly of the inwardly rectifying potassium channel Kir6.2 and the sulfonylurea receptor SUR1 into an octamer in a 4:4 stoichiometry (2,3,24). Upon successful assembly in the ER, the channel travels through the Golgi, where N-linked glycosylation of SUR1 is modified, before reaching the plasma membrane (12,43). Several molecular signals built into the channel proteins have been implicated in monitoring proper folding, assembly, and trafficking of the channel. These include two N-linked glycosylation sites in SUR1, a tripeptide -RKR- ER retention/retrieval motif present in both SUR1 and Kir6.2, as well as a putative forward trafficking signal present in the C-terminus of SUR1 (14,32,43). Deletion of the N-linked glycosylation sites or the C-terminal putative forward trafficking signal in SUR1 dramatically reduce channel expression at the cell surface (14,32), while inactivation of the RKR retention/retrieval motif in SUR1 or Kir6.2 leads to surface expression of unassembled individual channel subunits or partially assembled channel complexes that are physiologically nonfunctional (37,43). Aside from these molecular signals, what cellular events might influence the efficiency of channel biogenesis, thereby the level of channel expression at the cell surface, remain largely unexplored.

Recent studies suggest the biogenesis efficiency and/or surface expression of certain ion channels, including aquaporin, connexin and AChR, can be modulated via the ubiquitin-proteasome pathway (10,26,39), which plays a major role in ER-associated degradation (ERAD) of proteins that fail to fold or assemble properly (6,11,20). In this work, we sought to determine whether the ubiquitin-proteasome degradation pathway contributes to the biogenesis and surface expression efficiencies of K_{ATP} channels. We found in COS cells both Kir6.2 and SUR1 are polyubiquitinated, and degradation of both channel subunits is slowed by proteasome inhibitors. Importantly, inhibition of proteasome function leads to an increase in the number of surface K_{ATP} channels both in COS cells and in a rat pancreatic β -cell line, INS-1, that expresses endogenous K_{ATP} channels. Our results suggest that degradation of nascent K_{ATP} channel subunits by the ubiquitin-proteasome pathway plays a role in setting the biogenesis efficiency, thereby the level of surface expression, of K_{ATP} channels in pancreatic β -cells.

Materials and Methods

Expression Constructs -- FLAG epitope (DYKDDDDK) tagged hamster SUR1 cDNA (referred to as fSUR1 hereinafter) in the pECE plasmid was constructed as described previously (8). Rat Kir6.2 cDNA (in pcDNA3) and hamster SUR1 cDNA with a V5 epitope (GKPIPNPLLGLDST) sequence added to the 3'-end (in pcDNA3.1, referred to as SUR1_{V5}) were kindly provided by Dr. Carol A. Vandenberg.

Immunoblotting - COSm6 cells (referred to as COS cells hereinafter) grown in 35 mm dishes were transfected with 0.6 μ g fSUR1 (or SUR1_{V5}) and 0.4 μ g rat Kir6.2 using FuGene6 (Roche Applied Science, Indianapolis, IN). Cells were lysed 48-72 hours later in 20 mM HEPES, pH 7.0/5 mM EDTA/150 mM NaCl/1% Nonidet P-40 (IGAPEL) with Complete^{TR} protease inhibitors (Roche Applied Science, Indianapolis, IN). Proteins in the cell lysate were separated by SDS/PAGE (7.5% for SUR1 and 12% for Kir6.2), transferred to nitrocellulose membrane, and analyzed by incubation with appropriate primary antibodies followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Amersham Biosciences, Piscataway, NJ), and visualized by enhanced chemiluminescence (Super Signal West Femto; Pierce, Rockford, IL). The primary antibodies used are: M2 mouse monoclonal anti-FLAG antibody for fSUR1 (Sigma, St. Louis, MO), mouse monoclonal anti-V5 for SUR1_{V5} (Invitrogen, Carlsbad, CA), rabbit polyclonal anti-Kir6.2 for Kir6.2, and mouse monoclonal anti-ubiquitin for ubiquitin (both from Santa Cruz Biotechnology, Santa Cruz, CA).

Metabolic Labeling and Immunoprecipitation - COS cells were transfected with SUR1 and Kir6.2 as described above. Forty-eight hours post transfection, cells were starved for 30 min in methionine/cysteine-free Dulbecco's minimum essential medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 5% dialyzed fetal bovine serum (FBS) and 2 mM glutamine prior to labeling for 30 min with 150-250 μ Ci/ml Trans³⁵S-Label (MP Biomedicals, Irvine, CA) in the same medium. Labeled cultures were chased for various times in regular medium (DMEM plus 10% fetal bovine serum) supplemented with 10 mM L-methionine. At the end of the chase, cells were lysed in 500 μ l lysis buffer (50 mM Tris-HCl, pH 7.0/150 mM NaCl/1% IGAPEL, with Complete^{TR} protease inhibitors) on ice for 30 minutes. Note to ensure solubilization of channel proteins that might have aggregated as a result of proteasome inhibitor treatment, 1% SDS was included in the lysis buffer, which was later diluted (to 0.1% SDS) prior to immunoprecipitation. Cell lysate was centrifuged at 16,000 x g for 5 min at 4°C and supernatant immunoprecipitated by incubation with 10 μ g anti-FLAG M2 antibodies (or 1 μ l anti-V5 antibody, or 1 μ l anti-Kir6.2 antibody) per sample for 2 hours at 4°C, and then with protein A-Sepharose 4B (Bio-Rad, Hercules, CA; ~2mg/sample) for 2 hours at 4°C. The precipitate was washed three times in lysis buffer and proteins eluted by incubation in SDS sample buffer at room temperature for 10 minutes. Eluted proteins were separated by SDS-PAGE and the gel subjected to fluorography. Protein bands were quantified using a Storm PhosphorImager (Amersham Biosciences, Piscataway, NJ).

Chemiluminescence Assay -- COS cells in 35 mm dishes were fixed with 2% paraformaldehyde for 20 min at room temperature 48-72 hours post transfection. Fixed cells were pre-blocked in PBS+0.1% BSA for one hour, incubated in M2 anti-FLAG antibody (10 μ g/ml) for an hour, washed 4 x 30 min in phosphate buffered saline (PBS) + 0.1% bovine serum albumin (BSA), incubated in HRP-conjugated anti-mouse secondary antibodies (Amersham, 1:1000 dilution) for 20 min, washed again 4 x 30 min in PBS +0.1% BSA, and 2 x 5 min in PBS. Chemiluminescence signal was read in a TD-20/20 luminometer (Turner Designs) following 10 sec incubation in Power Signal Elisa luminol solution (Pierce, Rockford, IL). For measuring channel internalization rate, cells were preincubated with anti-FLAG antibody at 4°C for 30 min to label surface fSUR1. After brief washing, cells were returned to 37°C to allow protein trafficking to resume for various times. Residual surface label at each time point was then determined following the standard chemiluminescence assay protocol described above. Results of each experiment are the average of 2-3 dishes and unless specified, data point shown in figures is the average of 3-5 independent experiments.

Surface Biotinylation and Detection of Biotinylated fSUR1 - COS cells transiently expressing fSUR1 and Kir6.2 were washed twice with ice-cold HEPES buffer (20 mM HEPES, 150 mM NaCl, 1.8 mM CaCl₂, pH 7.4). Biotinylation of surface proteins was carried out by incubating cells with 1mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Pierce) in HEPES buffer for 30 min on ice. The reaction was terminated by incubating cells for 5 min with HEPES buffer containing 20 mM glycine followed by three washes in ice-cold PBS. Cells were either lysed immediately in Triton lysis buffer (20 mM HEPES, 125 mM NaCl, 4 mM EDTA, 1 mM EGTA, 1% Triton X-100, pH 7.4; 30 min at 4°C with rotation) or incubated further at 37°C in regular culture medium for another 30 or 60 min (to monitor surface channel stability) before lysing. Cell lysate was cleared by centrifugation at 21,000x g for 20 min at 4°C, and biotinylated proteins pulled down by incubation with Neutravidin-agarose beads (Pierce) for three hours at 4°C with rotation. The beads were washed three times with lysis buffer and proteins eluted by incubation with SDS sample buffer containing 2.5% β -mercaptoethanol for 30 min at room temperature. Eluted proteins were then separated by SDS-PAGE, and fSUR1 detected by Western blots using anti-FLAG antibody. Surface biotinylation experiments in INS-1 cells expressing endogenous K_{ATP} channels used similar procedures with the following modifications. INS-1 cells clone 832/13 [a gift from Dr. Chris Newgard, (21)] were cultured in RPMI-1640 with 11.1 mM D-glucose (Invitrogen) supplemented with 10% FBS, 100U/ml penicillin, 100 μ g/ml

streptomycin, 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, and 50 μ M β -mercaptoethanol. Surface protein biotinylation and pull-down was carried out at 4°C as described above. Due to the absence of good antibodies for endogenous SUR1, we have used anti-Kir6.2 antibody to detect surface biotinylated K_{ATP} channel complex. Note in this case, surface Kir6.2 itself is not biotinylated due to lack of extracellular lysine in the protein, but it is pulled down by Neutravidin beads via association with biotinylated surface SUR1. In control experiments where cells were not surface biotinylated, no Kir6.2 was detected, confirming that the Kir6.2 signal as shown in Fig.5B was indeed surface Kir6.2.

Whole-cell Patch-Clamp Recording --INS-1 cells were plated onto coverslips. Micropipettes were pulled on a horizontal puller (Sutter Instrument, Co., Novato, CA). The pipette solution consists of 140 mM KCl, 10 mM K-HEPES, 1 mM K-EGTA, and 1mM MgCl₂, pH 7.3. Recordings were made at room temperature with cells perfused with Tyrode's solution (137 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.5 mM MgCl₂, 5 mM HEPES, 3 mM NaHCO₃, and 0.16 mM NaH₂PO₄). Giga-Ohm seals were formed prior to break-in, after which the cell was held at -75mV and stimulated every 2 seconds with alternate $\pm$ 10 mV pulses, each for 100 ms, to monitor the current. K_{ATP} currents were derived by subtracting the steady-state K⁺ current observed in 500 μ M tolbutamide from that observed in 250 μ M diazoxide. Whole cell K_{ATP} current was then normalized to cell surface area (measured as cell capacitance) to obtain the K_{ATP} current density, represented as pS/pF. In pharmacological experiments, cells received drug treatment 4-6 hours prior to being subjected to patch-clamp recording.

Results

K_{ATP} Channel Subunits are Polyubiquitinated -- Most membrane and secretory proteins that fail to mature or assemble properly in the ER are degraded through an ER-associated degradation process known as ERAD (6). Typical ERAD substrates are tagged by polyubiquitination, retrotranslocated into the cytosol, and targeted to the proteasome for degradation (11,20,28,41), although exceptions that appear to follow a proteasome-independent pathway have been reported (7,17,29). To test whether the ubiquitin-proteasome pathway is involved in degradation of nascent K_{ATP} channel subunits, we first examined whether SUR1 and Kir6.2 transiently expressed in COS cells are polyubiquitinated. We have chosen to use a SUR1 tagged with a FLAG epitope at its extracellular N-terminus (referred to as fSUR1) for these experiments to facilitate detection; the construct has been previously shown to give rise to channels with properties indistinguishable from wild-type (WT) channels (8). The fSUR1 or Kir6.2 expressed in COS cells were subject to immunoprecipitation using anti-FLAG or anti-Kir6.2 antibodies (see Experimental Procedures), the precipitated proteins were then analyzed by Western blot using antibodies against the channel proteins or ubiquitin. In COS cells expressing fSUR1 alone, ubiquitin labeling that appeared as a high molecular weight "smear" was already apparent (Fig.1A, *right*). If the ubiquitinated SUR1 is targeted to degradation in the proteasome, then treating cells with proteasome inhibitors, such as lactacystin or MG132, may lead to accumulation of polyubiquitinated SUR1. Indeed, a more intense signal in the anti-ubiquitin blot was observed in cells treated with MG132 (10 μ M, 4 hours). Similar observations were made in cells co-expressing fSUR1 and Kir6.2 (Fig.1A). Likewise, Kir6.2 was also found polyubiquitinated, either when expressed alone or together with fSUR1. In the anti-ubiquitin blot of Kir6.2 immunoprecipitates, discrete bands, which likely represent Kir6.2 with different numbers of ubiquitin moieties attached, can be discerned (Fig.1B). These results demonstrate that both SUR1 and Kir6.2 are substrates for polyubiquitination, like most proteins that are targeted for proteasome-mediated ERAD. Note in this and subsequent experiments, results concerning fSUR1 have been confirmed using a SUR1 with a V5 tag placed at the cytoplasmic C-terminus (SUR1_{V5}). This is to ensure that any conclusion we draw is not dependent on the position and/or the nature of the epitope tag, although for space consideration only one construct is described or shown in figures.

Proteasome Inhibitors Slow the Degradation Rate of K_{ATP} Channel Proteins -- To further demonstrate that proteasome is involved in degradation of nascent K_{ATP} channel subunits, we examined the effects of proteasome inhibitors on degradation of SUR1 using pulse-chase analysis. In cells expressing SUR1_{V5} (or fSUR1) alone, treatment of cells with 10 μ M MG132 throughout the pulse-chase period significantly slowed the degradation of the protein (Fig. 2A). The amount of labeled SUR1_{V5} increased by ~30% at the end of 1-hour chase. This initial increase is expected since the partially translated, labeled SUR1_{V5} which would have been degraded during the first hour of chase is now allowed to complete translation due to inhibition of degradation and join the pool of labeled full-length SUR1_{V5}. Similarly, degradation of Kir6.2 expressed alone in COS cells was significantly slowed by proteasome inhibitors (Fig. 2B). Note for Kir6.2, cells were pretreated for two hours with 10 μ M MG132 prior to the pulse-chase analysis; this is so that any effects of MG132 would not be missed since degradation of Kir6.2 is more rapid. We also performed experiments where SUR1 and Kir6.2 were co-expressed. MG132 again slowed the overall degradation rates of newly synthesized channel subunits (see below; the degradation profile of fSUR1 is shown in Fig. 4A). Together, these data demonstrate that nascent K_{ATP} channel subunits are subject to degradation by the ubiquitin-proteasome pathway.

Inhibition of Proteasome Function Increases Surface Expression of K_{ATP} Channels by increasing channel biogenesis efficiency in COS Cells -- Although a general function of ERAD is to remove unwanted proteins in the ER, recent studies show that it can play a regulatory role in protein expression by controlling the degradation of WT proteins (20,26,39). Having established that newly synthesized K_{ATP} channel proteins undergo ERAD *via* the ubiquitin-proteasome pathway, we sought to determine whether inhibition of proteasome function would increase surface expression of K_{ATP} channels. To monitor channel expression at the cell surface, we first performed surface biotinylation experiment in which cell surface proteins were biotinylated using a membrane impermeable biotinylation reagent Sulfo-NHS-SS-Biotin, biotinylated proteins were then pulled down by Neutravidin-agarose beads and fSUR1 detected by Western blots using anti-FLAG antibody. As shown in Fig. 3A, the signal of surface biotinylated fSUR1 is stronger in cells treated with MG132 (10 μ M for four hours) than that in control cells, indicating increased channel expression at the cell surface. To better quantify and characterize the effects of proteasome inhibitors, we used a chemiluminescence assay that is much more sensitive than biochemical assays (8,27). Exposure of COS cells to the proteasome specific inhibitor MG132 (150 nM or 10 μ M) or lactacystin (6 μ M) (19) for 6 hours increased surface expression of the channel by ~30-40% (Fig. 3B). In contrast, incubation of cells with lysosomal inhibitors (chloroquine at 100 μ M or NH₄Cl at 20 mM) or the calpain inhibitor ALLM (at 10 or 100 μ M) had no or little effects on surface expression.

Treatment with proteasome inhibitors could potentially increase surface expression of K_{ATP} channels by stabilizing channels in the plasma membrane, as has been observed for β -adrenergic receptors, growth hormone receptors, and AMPA-type glutamate receptors (13, 33,38), or by promoting channel biogenesis, as seen in AChRs (10). To distinguish between these possibilities, we performed metabolic pulse-chase experiments in cells co-expressing fSUR1 and Kir6.2 to follow the efficiency at which nascent fSUR1 is converted to the mature complex-glycosylated form (upper band), which reflects the efficiency of channel biogenesis. In these experiments, MG132 treatment began one hour prior to the pulse-chase to ensure that the inhibitor has sufficient time to enter the cell and exert its effect. As expected, the overall degradation rate of fSUR1 was slowed in MG132 treated cells (similar results were obtained for Kir6.2; not shown). Importantly, the percentage of initially labeled fSUR1 that was converted to the upper band was consistently higher (~10%) than in control cells (Fig. 4A). In calculating the efficiency of conversion, the upper band fSUR1 signal in control or drug-treated cells were divided by the initial label of the respective groups, thus correcting for potential effects of MG132 at the pre-translational or translational level. We next compared the stability

of surface channels in control cells and cells treated with proteasome inhibitors. In surface biotinylation pulse-chase experiments, the signal of surface fSUR1 in both control cells and cells pretreated with MG132 (10 μ M for 4 hours) decreased rapidly in a 60 minutes chase period with similar rates (Fig.4B, *left panel*). To better quantify the rate of surface channel degradation, we performed chemiluminescence assays using a similar pulse-chase experimental paradigm. Consistent with the surface biotinylation results, the signal of FLAG-antibody labeled channels decreased to ~50% of the initial value in 20 minutes, and no significant difference was observed between control and MG132 treated cells (Fig.4B, *right panel*). Taken together, the above results led us to conclude that proteasome inhibitors increase surface expression of K_{ATP} channels by increasing the biogenesis efficiency of the channel without affecting the stability of surface channels.

Endogenous K_{ATP} Channel Subunits are also affected by the ubiquitin-proteasome pathway -- The studies described so far were conducted on K_{ATP} channels heterologously expressed in COS cells where protein overexpression might be a concern. To determine whether ubiquitin-proteasome pathway mediated ERAD also plays a role in the biogenesis efficiency of native K_{ATP} channel subunits expressed in insulin secreting cells, we performed experiments using a rat β -cell line, INS-1E clone 832/13, which closely resembles native β -cells in glucose-stimulated insulin secretion response (21). To first confirm that endogenous channel subunits are also substrates for polyubiquitination, Kir6.2 in INS-1 cells was immunoprecipitated and its ubiquitination probed by immunoblotting using an anti-ubiquitin antibody. To enhance the signal of ubiquitinated Kir6.2, cells were treated with the proteasome inhibitor MG132 (10 μ M for 3 hours), which allows accumulation of polyubiquitinated proteins. As shown in Fig. 5A, polyubiquitinated Kir6.2 was already detectable in control cells, and proteasome inhibitors dramatically increased this signal, implicating involvement of the ubiquitin-proteasome pathway in degradation of endogenous K_{ATP} channels. We next examined whether proteasome inhibitors also affect surface expression of the channel in INS-1 cells by probing the amount of surface Kir6.2, which could be pulled down by Neutravidin-agarose beads *via* association with biotinylated surface SUR1 (see Materials and Methods). Using this approach, we found the level of surface Kir6.2 to be consistently higher in cells treated with MG132 (10 μ M for 3 hours) than in control cells, demonstrating that inhibition of proteasome function also increases surface expression of endogenous K_{ATP} channels.

Inhibition of Proteasome Function Increases K_{ATP} Current Density in INS-1 Cells - To better quantify the effect of proteasome inhibitors on surface channel expression in INS-1 cells, we employed a functional assay that measures K_{ATP} current density by whole-cell patch-clamp recordings since no antibodies that recognize the extracellular domain of native SUR1 or Kir6.2 are currently available to allow quantification by chemiluminescence assays. Increased surface channel expression is expected to result in increased K_{ATP} currents. To elicit maximal K_{ATP} currents, cells were dialyzed with zero ATP pipette solution and perfused with 250 μ M of the K_{ATP} channel opener diazoxide. Under this condition, the current gradually increased to a maximal level (usually ~80-90 seconds after break-in; Fig.6A). The maximal K^+ current seen in zero ATP and the presence of diazoxide was taken as the K_{ATP} current, after subtraction of background K^+ currents obtained by perfusing cells with 500 μ M of the K_{ATP} channel inhibitor tolbutamide. This whole-cell K_{ATP} current was then divided by the whole-cell capacitance to obtain current density, which reflects the density of K_{ATP} channels in the plasma membrane. The averaged data from a total of 12-13 recordings from three independent experiments show that treatment of cells for four hours with 150 nM MG132 led to a significant increase (~40%) in K_{ATP} current density. By contrast, inhibition of lysosome function by NH_4Cl (12.5 mM) or inhibition of calpain by ALLM (10 or 100 μ M) had little effects on K_{ATP} current density in INS-1 cells, demonstrating the specificity of the proteasome inhibitor effect. Control experiments were performed using inside-out patch-clamp recording to ensure that MG132 does not have acute effect on K_{ATP} channel open probability or other background K^+ currents

(data not shown). Note due to its documented effect on K_{ATP} channel gating, chloroquine was not used as a lysosome inhibitor in these whole-cell recording experiments (18). The electrophysiological data presented above are consistent with biochemical data and support a role of the ubiquitin-proteasome degradation pathway in determining the biogenesis and surface expression efficiency of native K_{ATP} channels.

Do Proteasome Inhibitors Promote Surface Expression of Mutant Channels with Biogenesis/Trafficking Defects? - Several SUR1 mutations identified in the insulin secretion disease congenital hyperinsulinism have been shown to cause K_{ATP} channel biogenesis/surface expression defects (5,8,9,30,35,36,42). Having established that proteasome inhibitors promote the biogenesis efficiency and surface expression of WT K_{ATP} channels, we sought to determine whether inhibition of proteasome function could overcome the surface expression defects caused by disease mutations. Three SUR1 mutations, A116P, V187D, and $\Delta F1388$, which we have previously shown to result in ER retention and surface expression defects of K_{ATP} channels, were tested. Treatment of cells with 10 μM MG132 for 6 hours did not cause a statistically significant increase in surface expression of the mutants ($p > 0.01$), unlike that seen in WT channels (Fig.7A). The result suggests that the effectiveness of proteasome inhibitors on promoting channel biogenesis/surface expression may depend on the ability of the channel proteins to fold and assemble correctly. To further test this, we examined the combined effect of glibenclamide and MG132 on surface expression of the A116P and V187D mutants. Sulfonylureas such as glibenclamide have previously been shown to significantly increase surface expression of the A116P and V187D mutants, presumably by acting as pharmacological chaperones to help mutant SUR1 fold more efficiently (42). We found that pretreatment of COS cells expressing the A116P or the V187D mutant with 5 μM glibenclamide led to a significant increase in mutant channel surface expression ($p < 0.01$) upon subsequent exposure to the proteasome inhibitor MG132 (Fig.7B). These findings support the notion that the effectiveness of proteasome inhibitors on enhancing channel surface expression is correlated with the ability of the channel proteins to fold and assemble correctly.

Discussion

Proteins retained in the ER are degraded mostly through the ubiquitin-proteasome ERAD pathway. While a generally recognized function of this pathway is to prevent mutant or unassembled proteins from accumulating, it has also been shown to play an active role in regulating expression of normal proteins. Examples include T-cell-receptor complex, HMG-CoA reductase, connexin, aquaporin-1, and most recently the nicotinic acetylcholine receptor (6,10,20,26,39). The data presented in this study demonstrate that nascent K_{ATP} channel subunits are subject to degradation by the ubiquitin-proteasome pathway and that inhibition of this pathway leads to significantly increased expression of K_{ATP} channels at the cell surface. Involvement of the ubiquitin-proteasome pathway in K_{ATP} channel degradation is not unique to the COS cell system, where protein overexpression might be a concern (40). Endogenous K_{ATP} channel subunits expressed in INS-1 cells are also substrates for polyubiquitination and surface channel expression is increased by inhibition of proteasome function. Based on these findings, we propose that the ubiquitin-proteasome pathway mediated ERAD of K_{ATP} channel subunits likely contributes to the biogenesis efficiency, thereby functional expression, of K_{ATP} channels in native β -cells.

Multiple lines of evidence suggest that proteasome inhibitors promote K_{ATP} channel surface expression by increasing channel biogenesis efficiency rather than affecting other cellular events. First, metabolic pulse-chase experiments demonstrate directly that the efficiency of channel biogenesis, as reflected by the efficiency of which fSUR1 is converted from the core-glycosylated form to the complex-glycosylated form, is higher in cells treated with proteasome inhibitors than in control cells (Fig.4A). Second, pharmacological agents targeting other major

cellular degradation pathways including the lysosome and the cytosolic proteases calpains had little or no effect on surface K_{ATP} channel expression, excluding indirect effects of the proteasome inhibitors on channel expression *via* inhibition of other degradation pathways. Third, the stability of surface K_{ATP} channels was unaltered by proteasome inhibitors, as assessed by two independent assays (Fig.4B). One model consistent with our data is that the biogenesis efficiency of K_{ATP} channels is a balance between the rate of channel subunit degradation by the proteasome and the rate of channel assembly. Inhibition of proteasome activity slows the degradation of nascent channel proteins, causing their accumulation in the ER, thereby shifting the equilibrium towards channel assembly. A similar mechanism has recently been proposed for the hetero-pentameric nicotinic AChRs (10). Interestingly, metabolic pulse-chase experiments suggest that the biogenesis efficiency of K_{ATP} channels in COSm6 cells is rather low under our experimental conditions (fSUR1: Kir6.2 cDNA ratio ~1). Although one might argue that the low efficiency could be due to protein overexpression, the fact that endogenous K_{ATP} channel subunits are polyubiquitinated and that surface expression of K_{ATP} channels is also increased by proteasome inhibitors in INS-1 cells lends support to the idea that there exists an excess pool of channel subunits in β -cells that can be rendered available for channel assembly when their degradation *via* the proteasome is inhibited.

To assess whether proteasome inhibitors can overcome K_{ATP} channel surface expression defects caused by disease mutations, we examined the response of three SUR1 mutants known to abolish or reduce surface channel expression (8,42). Among them, Δ F1388 has been proposed to cause severe folding defects while A116P and V187D appear to have milder defects that can be partially overcome by sulfonylurea treatment. None of the mutant channels showed a statistically significant increase in surface expression upon proteasome inhibitor treatment. However, upon pretreatment with glibenclamide, the A116P and V187D mutant channels responded to proteasome inhibitors with a statistically significant increase in surface expression (Fig.7B). These results suggest that in order for proteasome inhibitors to be effective, the channel proteins have to be competent in adopting correct conformation. For mutant proteins that are unable to fold correctly, inhibition of proteasome function may instead render accumulation of the mutant protein either in the ER or in intracellular inclusion bodies resembling aggregates (25), as we have observed for the Δ F1388 mutant (Cartier and Shyng, unpublished). Interestingly, a recent study found that while some mutations in the *Shaker* potassium channel protein lead to rapid degradation *via* proteasome-dependent ERAD, others lead to degradation that is insensitive to the proteasome inhibitor lactacystin (29). We cannot rule out that such a pathway may also contribute to degradation of the mutant channel proteins we have studied here, partially accounting for why proteasome inhibitors did not improve their surface expression.

While not the focus of this study, in examining the effect of proteasome inhibitors on the stability of nascent channel subunits and surface channel proteins, two results came to light that warrant further discussion, as they differ from previous reports published by others. First, we found that degradation of SUR1 (both fSUR1 and SUR1_{V5}) expressed alone in COSm6 cells is biphasic (Fig.2A), with a fast component (~80%, half-life ~1 hour) and a slow component (~20%, half-life ~18 hours). This is in contrast to that reported by Crane and Aguilar-Bryan, who found unassembled SUR1 to be very stable, with a single half-life of ~25.5 hours (15). Criteria used for degradation curve fitting, as well as several differences in experimental details including epitope tag, detergent used in the lysis buffer, and the amount of cDNA used for transfection could potentially account for this apparent discrepancy. We are currently investigating these variables systematically to resolve this issue. Regardless, while the intrinsic metabolic stability of SUR1 in the absence of Kir6.2 may have implications on the channel assembly process, it does not impact on the conclusion of the present study especially considering that the effect of proteasome inhibitors is observed in INS-1 cells expressing endogenous channels. Another result worth noting is the rapid degradation of

surface K_{ATP} channel proteins we observed in COSm6 cells. Both surface biotinylation experiments and chemiluminescence assays yielded similar results indicating that the half-life of surface channel proteins is ~20 minutes (Fig.4B). This result is different from that reported by Hu *et al.* who found little internalization of surface K_{ATP} channels expressed in COS-7 cells in a 30 minute period using chemiluminescence assays (22). Difference in the cell used for transfection and the Kir6.2 construct (Hu *et al.* used a Kir6.2 tagged with a HA-epitope in the extracellular domain) could potentially contribute to the difference in results. Again, the absolute stability of surface channels does not affect our conclusion as long as MG132 has no effect on this stability.

Modulation of protein expression by the ubiquitin-proteasome system has been reported under certain physiological conditions. For example, aquaporin-1 water channel surface expression is increased by hypertonic stress *via* inhibition of protein degradation by the ubiquitin-proteasome pathway (26). Upregulation of gap junctions at the cell surface is observed when cells are challenged with mild oxidative or thermal stress, which reduce retro-translocation of newly synthesized connexin back to the cytosol for proteasome degradation (39). Conversely, downregulation of multiple postsynaptic density proteins due to increased degradation *via* the ubiquitin-proteasome pathway has been reported during activity-dependent synapse remodeling (16). It will be interesting to determine in the future whether K_{ATP} channel degradation through the ubiquitin-proteasome pathway can also be modulated to alter surface channel expression *in vivo* during development or under stressful physiological conditions. Such modulation would be expected to alter the sensitivity of β -cells to glucose stimulation and affect insulin secretion. It is worth noting that during submission of our paper, Tanaka *et al.* reported similar upregulation of cardiac K_{ATP} channels (SUR2A/Kir6.2) in cells treated with proteasome inhibitors (34), suggesting the mechanism likely applies to other K_{ATP} channel subtypes.

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References

1. Aguilar-Bryan L, Bryan J. Molecular biology of adenosine triphosphate-sensitive potassium channels. *Endocr Rev* 1999;20:101–135. [PubMed: 10204114]
2. Aguilar-Bryan L, Clement JPt, Gonzalez G, Kunjilwar K, Babenko A, Bryan J. Toward understanding the assembly and structure of KATP channels. *Physiol Rev* 1998;78:227–245. [PubMed: 9457174]
3. Aguilar-Bryan L, Nichols CG, Wechsler SW, Clement JPt, Boyd AE 3rd, Gonzalez G, Herrera-Sosa H, Nguy K, Bryan J, Nelson DA. Cloning of the beta cell high-affinity sulfonylurea receptor: a regulator of insulin secretion. *Science* 1995;268:423–426. [PubMed: 7716547]
4. Ashcroft SJ. The beta-cell K(ATP) channel. *J Membr Biol* 2000;176:187–206. [PubMed: 10931971]
5. Babenko AP, Bryan J. SUR domains that associate with and gate KATP pores define a novel gatekeeper. *J Biol Chem* 2003;26:26.
6. Bonifacino JS, Weissman AM. Ubiquitin and the control of protein fate in the secretory and endocytic pathways. *Annu Rev Cell Dev Biol* 1998;14:19–57. [PubMed: 9891777]
7. Cabral CM, Choudhury P, Liu Y, Sifers RN. Processing by endoplasmic reticulum mannosidases partitions a secretion-impaired glycoprotein into distinct disposal pathways. *J Biol Chem* 2000;275:25015–25022. [PubMed: 10827201]
8. Cartier EA, Conti LR, Vandenberg CA, Shyng SL. Defective trafficking and function of KATP channels caused by a sulfonylurea receptor 1 mutation associated with persistent hyperinsulinemic hypoglycemia of infancy. *Proc Natl Acad Sci U S A* 2001;98:2882–2887. [PubMed: 11226335]

9. Chan KW, Zhang H, Logothetis DE. N-terminal transmembrane domain of the SUR controls trafficking and gating of Kir6 channel subunits. *Embo J* 2003;22:3833–3843. [PubMed: 12881418]
10. Christianson JC, Green WN. Regulation of nicotinic receptor expression by the ubiquitin-proteasome system. *Embo J*. 2004
11. Ciechanover A, Schwartz AL. The ubiquitin-proteasome pathway: the complexity and myriad functions of proteins death. *Proc Natl Acad Sci U S A* 1998;95:2727–2730. [PubMed: 9501156]
12. Clement, JPt; Kunjilwar, K.; Gonzalez, G.; Schwanstecher, M.; Panten, U.; Aguilar-Bryan, L.; Bryan, J. Association and stoichiometry of K(ATP) channel subunits. *Neuron* 1997;18:827–838. [PubMed: 9182806]
13. Colledge M, Snyder EM, Crozier RA, Soderling JA, Jin Y, Langeberg LK, Lu H, Bear MF, Scott JD. Ubiquitination regulates PSD-95 degradation and AMPA receptor surface expression. *Neuron* 2003;40:595–607. [PubMed: 14642282]
14. Conti LR, Radeke CM, Vandenberg CA. Membrane Targeting of ATP-sensitive Potassium Channel. EFFECTS OF GLYCOSYLATION ON SURFACE EXPRESSION. *J Biol Chem* 2002;277:25416–25422. [PubMed: 11994306]
15. Crane A, Aguilar-Bryan L. Assembly, maturation, and turnover of K(ATP) channel subunits. *J Biol Chem* 2004;279:9080–9090. [PubMed: 14699091]
16. Ehlers MD. Activity level controls postsynaptic composition and signaling via the ubiquitin-proteasome system. *Nat Neurosci* 2003;6:231–242. [PubMed: 12577062]
17. Fayadat L, Siffroi-Fernandez S, Lanet J, Franc JL. Degradation of human thyroperoxidase in the endoplasmic reticulum involves two different pathways depending on the folding state of the protein. *J Biol Chem* 2000;275:15948–15954. [PubMed: 10748076]
18. Gribble FM, Davis TM, Higham CE, Clark A, Ashcroft FM. The antimalarial agent mefloquine inhibits ATP-sensitive K-channels. *Br J Pharmacol* 2000;131:756–760. [PubMed: 11030725]
19. Groll M, Huber R. Inhibitors of the eukaryotic 20S proteasome core particle: a structural approach. *Biochim Biophys Acta* 2004;1695:33–44. [PubMed: 15571807]
20. Hampton RY. ER-associated degradation in protein quality control and cellular regulation. *Curr Opin Cell Biol* 2002;14:476–482. [PubMed: 12383799]
21. Hohmeier HE, Mulder H, Chen G, Henkel-Rieger R, Prentki M, Newgard CB. Isolation of INS-1-derived cell lines with robust ATP-sensitive K⁺ channel-dependent and - independent glucose-stimulated insulin secretion. *Diabetes* 2000;49:424–430. [PubMed: 10868964]
22. Hu K, Huang CS, Jan YN, Jan LY. ATP-sensitive potassium channel traffic regulation by adenosine and protein kinase C. *Neuron* 2003;38:417–432. [PubMed: 12741989]
23. Huopio H, Shyng SL, Otonkoski T, Nichols CG. K(ATP) channels and insulin secretion disorders. *Am J Physiol Endocrinol Metab* 2002;283:E207–216. [PubMed: 12110524]
24. Inagaki N, Gonoi T, Clement JPt, Namba N, Inazawa J, Gonzalez G, Aguilar-Bryan L, Seino S, Bryan J. Reconstitution of IKATP: an inward rectifier subunit plus the sulfonylurea receptor. *Science* 1995;270:1166–1170. [PubMed: 7502040]
25. Johnston JA, Ward CL, Kopito RR. Aggresomes: a cellular response to misfolded proteins. *J Cell Biol* 1998;143:1883–1898. [PubMed: 9864362]
26. Leitch V, Agre P, King LS. Altered ubiquitination and stability of aquaporin-1 in hypertonic stress. *Proc Natl Acad Sci U S A* 2001;98:2894–2898. [PubMed: 11226337]
27. Margeta-Mitrovic M, Jan YN, Jan LY. A trafficking checkpoint controls GABA(B) receptor heterodimerization. *Neuron* 2000;27:97–106. [PubMed: 10939334]
28. McCracken AA, Brodsky JL. Evolving questions and paradigm shifts in endoplasmic-reticulum-associated degradation (ERAD). *Bioessays* 2003;25:868–877. [PubMed: 12938176]
29. Myers MP, Khanna R, Lee EJ, Papazian DM. Voltage sensor mutations differentially target misfolded K⁺ channel subunits to proteasomal and non-proteasomal disposal pathways. *FEBS Lett* 2004;568:110–116. [PubMed: 15196930]
30. Partridge CJ, Beech DJ, Sivaprasadarao A. Identification and pharmacological correction of a membrane trafficking defect associated with a mutation in the sulfonylurea receptor causing familial hyperinsulinism. *J Biol Chem* 2001;276:35947–35952. [PubMed: 11457841]

31. Schwappach B, Zerangue N, Jan YN, Jan LY. Molecular basis for K(ATP) assembly: transmembrane interactions mediate association of a K⁺ channel with an ABC transporter. *Neuron* 2000;26:155–167. [PubMed: 10798400]
32. Sharma N, Crane A, Clement JPt, Gonzalez G, Babenko AP, Bryan J, Aguilar-Bryan L. The C terminus of SUR1 is required for trafficking of KATP channels. *J Biol Chem* 1999;274:20628–20632. [PubMed: 10400694]
33. Shenoy SK, McDonald PH, Kohout TA, Lefkowitz RJ. Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin. *Science* 2001;294:1307–1313. [PubMed: 11588219]
34. Tanaka H, Miake J, Notsu T, Sonyama K, Sasaki N, Iitsuka K, Kato M, Taniguchi S, Igawa O, Yoshida A, Shigemasa C, Hoshikawa Y, Kurata Y, Kuniyasu A, Nakayama H, Inagaki N, Nanba E, Shiota G, Morisaki T, Ninomiya H, Kitakaze M, Hisatome I. Proteasomal degradation of Kir6.2 channel protein and its inhibition by a Na(+) channel blocker aprindine. *Biochem Biophys Res Commun* 2005;331:1001–1006. [PubMed: 15882977]
35. Taschenberger G, Mougey A, Shen S, Lester LB, LaFranchi S, Shyng SL. Identification of a familial hyperinsulinism-causing mutation in the sulfonylurea receptor 1 that prevents normal trafficking and function of KATP channels. *J Biol Chem* 2002;277:17139–17146. [PubMed: 11867634]
36. Tornovsky S, Crane A, Cosgrove KE, Hussain K, Lavie J, Heyman M, Neshier Y, Kuchinski N, Ben-Shushan E, Shatz O, Nahari E, Potikha T, Zangen D, Tenenbaum-Rakover Y, de Vries L, Argente J, Gracia R, Landau H, Eliakim A, Lindley K, Dunne MJ, Aguilar-Bryan L, Glaser B. Hyperinsulinism of infancy: novel ABCC8 and KCNJ11 mutations and evidence for additional locus heterogeneity. *J Clin Endocrinol Metab* 2004;89:6224–6234. [PubMed: 15579781]
37. Tucker SJ, Gribble FM, Zhao C, Trapp S, Ashcroft FM. Truncation of Kir6.2 produces ATP-sensitive K⁺ channels in the absence of the sulphonylurea receptor. *Nature* 1997;387:179–183. [PubMed: 9144288]
38. van Kerkhof P, Govers R, Alves dos Santos CM, Strous GJ. Endocytosis and degradation of the growth hormone receptor are proteasome-dependent. *J Biol Chem* 2000;275:1575–1580. [PubMed: 10636847]
39. VanSlyke JK, Musil LS. Dislocation and degradation from the ER are regulated by cytosolic stress. *J Cell Biol* 2002;157:381–394. [PubMed: 11980915]
40. Ward CL, Kopito RR. Intracellular turnover of cystic fibrosis transmembrane conductance regulator. Inefficient processing and rapid degradation of wild-type and mutant proteins. *J Biol Chem* 1994;269:25710–25718. [PubMed: 7523390]
41. Ward CL, Omura S, Kopito RR. Degradation of CFTR by the ubiquitin-proteasome pathway. *Cell* 1995;83:121–127. [PubMed: 7553863]
42. Yan F, Lin CW, Weisiger E, Cartier EA, Taschenberger G, Shyng SL. Sulfonylureas correct trafficking defects of ATP-sensitive potassium channels caused by mutations in the sulfonylurea receptor. *J Biol Chem* 2004;279:11096–11105. [PubMed: 14707124]
43. Zerangue N, Schwappach B, Jan YN, Jan LY. A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K(ATP) channels. *Neuron* 1999;22:537–548. [PubMed: 10197533]

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Fig. 1.**K_{ATP} channel subunits expressed in COS cells are substrates for polyubiquitination.**

Untransfected COS cells, or cells expressing either fSUR1 or Kir6.2, or co-expressing both subunits were lysed and fSUR1 or Kir6.2 immunoprecipitated (IP) using the M2 anti-FLAG antibody or anti-Kir6.2 antibody. The immunoprecipitate was then probed by immunoblotting (IB) using antibodies against the channel protein or against ubiquitin. *A*, Cell lysates immunoprecipitated with anti-FLAG (for fSUR1) and immunoblotted with anti-FLAG (*left*) or anti-ubiquitin (*right*). Filled arrow indicates the core-glycosylated fSUR1 and open arrow the complex-glycosylated form. Some higher molecular weight forms of fSUR1 that may represent polyubiquitinated fSUR1 or fSUR1 aggregates are seen in the anti-FLAG blot. *B*, Cell lysates immunoprecipitated with anti-Kir6.2 and immunoblotted with anti-Kir6.2 (*left*; Kir6.2 indicated by the arrow) or anti-ubiquitin (*right*). In the anti-Kir6.2 blot, high molecular mass bands near 80 and 160 kDa are observed. These likely represent Kir6.2 dimers and tetramers, as have been reported previously by others (31,43). Note discrete bands representing different copies of ubiquitin attached can be more easily discerned for Kir6.2 than for SUR1, likely because the 12% gel used for Kir6.2 gave better resolution in the given molecular mass range. Also in both *A* and *B*, cells treated with the proteasome inhibitor MG132 (10 μ M for 4 hours) exhibit enhanced signals in the anti-ubiquitin blots, especially for Kir6.2.

Fig. 2.

Proteasome inhibitors slow the degradation of SUR1 and Kir6.2. *A*, Degradation of SUR1 in the presence of MG132. COS cells transiently expressing SUR1_{V5} were subject to pulse-chase analysis. In the drug-treated group, 10 μ M MG132 was added to the medium at the time of the pulse and was present throughout the chase. *Top panel*: Fluorogram of a representative experiment. *Bottom panel*: Residual label during chase quantified by phosphorimaging. Note the signal increased transiently at the beginning of the chase before decreasing with time. The residual label at each time point is higher in MG132-treated cells than in control cells. Similar results were obtained using fSUR1 (not shown). *B*, Degradation of Kir6.2 in the presence or absence of 10 μ M MG132. *Top panel*: Fluorogram of a representative experiment. *Bottom panel*: Residual label during chase quantified by phosphorimaging. Note for Kir6.2, cells were pretreated with 10 μ M MG132 for two hours prior to the pulse-chase analysis. For both SUR1 and Kir6.2, only bands corresponding to monomeric form of the proteins (as opposed to oligomeric forms, see Fig. 1B) were included for quantification. Each data point is the mean $\pm$ s.e.m. of 3-4 independent experiments. Control group is shown as filled squares and MG132 treated shown as open circles.

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Fig. 3.

Proteasome inhibitors increase surface expression of K_{ATP} channels in COS cells. *A*, Biotinylated surface fSUR1 in control cells and cells treated for 6 hours with 10 μ M MG132. Duplicates are shown for each group. In both cases, cells treated with MG132 exhibited more surface fSUR1 than control cells. Note only one form of fSUR1, the complex-glycosylated form (open arrow), was detected, since the biotinylation reagent was membrane impermeable and only reacted with surface proteins. *B*, COSm6 cells transiently expressing K_{ATP} channels were treated with the indicated reagents for 6 hours, and then processed for chemiluminescence assays to quantify channel expression level at the cell surface. The proteasome inhibitors, lactacystin (6 μ M) and MG132 (0.15 or 10 μ M), both significantly increased surface channel expression ($n = 4$, $p < 0.01$ by student t -test). By contrast, inhibition of lysosomal function by chloroquine (100 μ M) or NH_4Cl (20 mM), or inhibition of the protease calpain by ALLM (10 or 100 μ M; only 100 μ M is shown) had little effects.

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Fig. 4.**Proteasome inhibitors promote biogenesis of K_{ATP} channels without affecting the**

stability of surface channels. *A*, COS cells transfected with fSUR1 and Kir6.2 were pretreated with or without 10 μ M MG132 for 1 hour, pulse-labeled for 30 min with ^{35} S-Cys/Met and chased for the times indicated in the presence or absence of 10 μ M MG132. Labeled fSUR1 were immunoprecipitated and analyzed by SDS-PAGE followed by fluorography (a representative experiment shown in the *Left panel*). The core- and complex-glycosylated fSUR1 bands are indicated by the solid and open arrows, respectively. The amount of total (both lower and upper bands; dotted line) fSUR1 as well as the upper band fSUR1 (solid line) present at each time point was quantified and expressed as percentage of total protein labeled at time 0 and plotted over time (*Right panel*). In cells treated with MG132 (open circle), a higher percentage of immature core-glycosylated fSUR1 was converted to the mature form, indicating increased channel biogenesis efficiency. *B*, Stability of surface K_{ATP} channels. *Left panel*: Western blots of residual surface biotinylated fSUR1 detected at 0, 30, and 60 min of chase. *Right panel*: Stability of surface K_{ATP} channels monitored by chemiluminescence assays. In these experiments, surface channel complexes in control cells or cells pretreated with 10 μ M MG132 for 4 hours were labeled at 4°C with anti-FLAG antibody and chased at 37°C for 10 or 20 min. Residual surface FLAG-epitope at each time point was quantified by the chemiluminescence assay and normalized to that observed in control cells at time 0. Note in cells treated with MG132, the initial label was higher than that seen in control cells, consistent with that shown in Fig.3B. All data points are the mean $\pm$ s.e.m. of 3-5 independent experiments.

Yan et al. Fig. 5

Fig. 5.

Endogenous K_{ATP} channels in INS-1 cells are also subject to regulation by the ubiquitin-proteasome system. A, Endogenous Kir6.2 expressed in INS-1 cells is polyubiquitinated. INS-1 cells treated with or without 10 μ M MG132 for 3 hours were lysed and Kir6.2 immunoprecipitated. The immunoprecipitate was probed in Western blot using anti-Kir6.2 antibody (*Left panel*) or anti-ubiquitin antibody (*Right panel*). B, Proteasome inhibitors increase the level of surface Kir6.2. In these experiments, surface K_{ATP} channel complexes were biotinylated (*via* the SUR1 subunit, see *Experimental Procedures*) and pulled down by Neutravidin-agarose beads. Kir6.2 was then detected by Western blots. Results from two independent experiments are shown. In both experiments, cells treated with MG132 exhibited more surface Kir6.2 (shown is the monomeric form; arrow) than control cells. In both A and B, molecular mass markers (in kDa) are indicated on the left of the blots.

Fig. 6.

K_{ATP} current density in INS-1 cells is increased upon treatment with proteasome inhibitors.

A, Representative whole-cell recordings of K_{ATP} currents in INS-1 treated with (top) or without (bottom) MG132 (150 nM) for four hours. The lower concentration of MG132 was used in these experiments to avoid cytotoxicity; at this concentration, cell surface channel expression was increased by ~30% in COS cells (Fig.3B). K_{ATP} current amplitude was plotted against time of recording (seconds). The cell was held at -75 mV and a brief (100 ms) +10 mV pulse was applied every 2 seconds to monitor the current. A gradual increase in current is observed upon dialysis of the cell with ATP-free solution and simultaneous perfusion of the cell with a bath solution containing 250 μM diazoxide. After the current amplitude has peaked, 500 μM tolbutamide was applied in the bath solution to inhibit K_{ATP} currents and to obtain background K⁺ currents from non-K_{ATP} channels. **B**, The averaged K_{ATP} current density obtained in cells treated with the various pharmacological agents. K⁺ currents attributed to opening of K_{ATP} channels were first derived by subtracting background K⁺ current observed in the presence of tolbutamide from the maximal K⁺ current observed in the presence of diazoxide. This K_{ATP} current was then divided by the total cell capacitance to correct for variation in current amplitude caused by variation in cell surface area. The value of each bar is the mean ± s.e.m. of 12-13 cells from three independent experiments. Only the MG132 treated cells show significant difference compared with control cells ($p < 0.01$, Student *t*-test). For ALLM treatment, both 10 and 100 μM were tested; only 100 μM is shown. All data points are expressed as mean ± standard error of the mean (s.e.m.). To avoid potential variation in K_{ATP} current density due to difference in INS-1 cell passage number, the K_{ATP} current density of cells treated with pharmacological agents was normalized only to that of control cells from the same culture and same day of recording.

Yan et al. Fig. 7

Fig. 7.

Effects of proteasome inhibitors on surface expression of mutant K_{ATP} channels in COS cells. *A*, COSm6 cells transiently co-expressing Kir6.2 and WT fSUR1 or fSUR1 bearing the A116P-, V187D-, or ΔF1388 mutation were treated with or without 10 μM MG132 for 6 hours, and processed for chemiluminescence assays to quantify channel expression level at the cell surface. Each data point represents the average of 6-8 samples from 2-4 independent experiments. *p < 0.01 by student *t*-test. *B*, Cells expressing Kir6.2 and WT, A116P-, or V187D- fSUR1 were treated for 24 hours with (Glib) or without (Control) 5 μM glibenclamide. For cells treated with both glibenclamide and proteasome inhibitor (Glib + MG132), 10 μM MG132 was added at 18 hours after the initiation of glibenclamide treatment and incubated further for 6 hours. All data points are the average of 6-10 samples from 3-5 independent experiments. *p < 0.01 by student *t*-test (comparison between Glib and Glib + MG132).