SUMOylation regulates Kv2.1 and modulates pancreatic β-cell excitability

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Summary

The covalent attachment of small ubiquitin-like modifier (SUMO) proteins regulates protein localization and function. SUMOylation has recently been shown to modulate ion-channel function; however, the extent to which this affects native currents and cellular excitability remains to be determined. The voltage-dependent K^+ (Kv) channel Kv2.1 regulates pancreatic β-cell excitability and insulin secretion. We found that YFP-tagged SUMO1 (SUMO1-YFP) can be immunoprecipitated with Kv2.1 when these two proteins are coexpressed in HEK 293 cells. Furthermore, direct infusion of recombinant SUMO1 peptide or coexpression of SUMO1-YFP inhibited current through cloned Kv2.1 by 80% and 48%, respectively. Insulin-secreting cells express SUMO variants 1 and 3, and expression of the SUMO1-YFP in human β-cells or insulinoma cells inhibited native Kv currents (by 49% and 33%, respectively). Inhibition of the channel resulted from an acceleration of channel inactivation and an inhibition of recovery from inactivation, resulting in the widening of β-cell action potentials and a decreased firing frequency. Finally, these effects on channel function and excitability were augmented by the conjugating enzyme Ubc9 and rescued by the SUMO protease SENP1. Thus, protein SUMOylation can exert a strong inhibitory action on the voltage-dependent K^+ channel Kv2.1 and can regulate cellular excitability in native β-cells.

Key words: Kv2.1, SUMO, Insulin, Ion channels, Islets of Langerhans, Voltage-dependent K^+ channels

Introduction

The small ubiquitin-like modifier (SUMO) proteins regulate a diverse array of cellular functions, including mitosis, gene transcription, DNA repair, nucleocytoplasmic transport and subnuclear targeting (Verger et al., 2003; Seeler and Dejean, 2003; Muller et al., 2004). There are three known functional SUMO isoforms (SUMO1, SUMO2 and SUMO3), which, through a well-established series of reactions, are conjugated to target proteins to exert post-translational modulation of protein function (reviewed by Johnson, 2004). These effects are reversible through the action of SUMO-specific proteases. Recently, post-translational SUMOylation has been suggested to cause a depolarizing shift in the steady-state inactivation of the voltage-dependent K^+ (Kv) channel Kv1.5 (Benson et al., 2007) and to silence the K2P1 K^+ leak channel (Rajan et al., 2005), although the latter finding is controversial (Feliciangeli et al., 2007). Thus, SUMOylation of ion channels might represent a unique mechanism for the modulation of cellular electrical excitability.

Pancreatic islet β-cells secrete insulin in response to glucose-stimulated electrical activity (Rorsman, 1997). Kv2.1 is the major Kv channel responsible for action potential repolarization in these cells (MacDonald et al., 2001; MacDonald et al., 2002; Yan et al., 2004; Herrington et al., 2006; Jacobson et al., 2007). The human Kv2.1 sequence contains three to six potential cytoplasmic consensus SUMOylation motifs. We have therefore examined the ability of SUMOylation to regulate the cloned human Kv2.1 channel and native Kv currents in insulinoma cells and primary human β-cells.

We find that expression or infusion of SUMO1 inhibits both cloned Kv2.1 and native Kv currents. This can be reversed by the SUMO protease SENP1. SUMOylation is associated with both an acceleration of time-dependent inactivation and delayed recovery from inactivation. Finally, we show that SUMOylation modulates the excitability of insulin-secreting cells in a manner consistent with the observed Kv current inhibition. Thus, SUMOylation is able to regulate native Kv currents and modulate β-cell excitability, and this might represent an important mechanism regulating insulin secretion.

Results and Discussion

SUMOylation inhibits Kv2.1 current

We coexpressed human Kv2.1 in HEK 293 cells together with a human SUMO1-YFP construct, and either the SUMO conjugating enzyme Ubc9 or the SUMO protease SENP1. SUMO1-YFP can effectively conjugate with its targets (Harder et al., 2004), and when coexpressed with Kv2.1 is co-immunoprecipitated with an anti-Kv2.1 antibody (Fig. 1A). The anti-GFP-positive signal was present at a range of molecular weights, suggesting that several SUMOylated proteins are pulled-down with the channel. We also consistently detected a high molecular mass band (>250 kDa) in the immunoprecipitate that is positive for both the channel and SUMO1-YFP (yellow in the overlay, Fig. 1), which could result from multi-SUMOylation of the channel or from association of the channel with an SDS-resistant protein complex. However, it seems clear that, as suggested previously (Benson et al., 2007), only a minority (<1%) of channel proteins might be directly SUMOylated. Nonetheless, we find that SUMO1-YFP is co-immunoprecipitated with the Kv2.1 channel. Furthermore, this interaction is enhanced by coexpression of Ubc9 and lost upon coexpression of SENP1 (Fig. 1A).

As SUMOylation of either Kv2.1 or its binding partners might regulate the channel, we examined the effect of SUMO1-YFP on

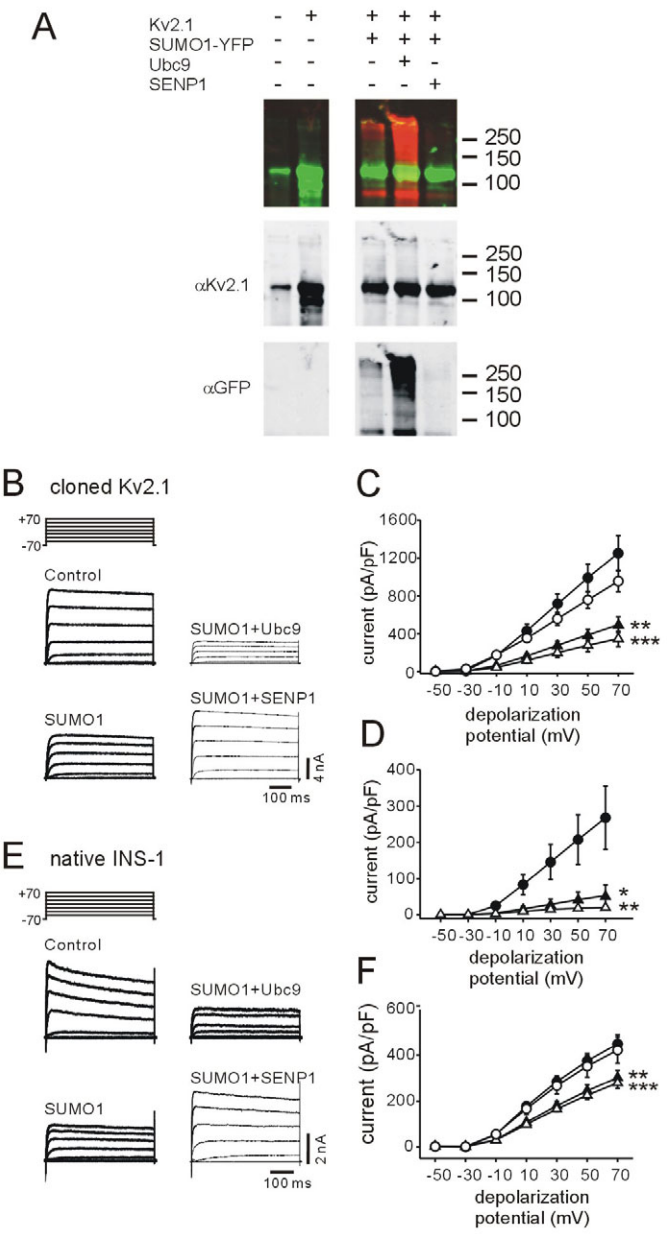

Fig. 1. SUMOylation regulates Kv2.1 and native INS-1 Kv current. (A) HEK 293 cells were transfected with the indicated cDNAs. Immunoprecipitation was performed using an anti-Kv2.1 antibody and analyzed by immunoblotting using anti-Kv2.1 and anti-GFP antibodies as indicated, and identified by the green and red signals, respectively, in the colored panel. Colocalization of these is shown in yellow. (B,C) Representative current traces and current-voltage relationships from HEK 293 cells expressing Kv2.1 alone (control, black circles) or together with SUMO1-YFP (SUMO1, black triangles), SUMO1-YFP+Ubc9 (white triangles) or SUMO1-YFP+SENP1 (white circles). (D) Current-voltage relationships from HEK 293 cells expressing Kv2.1 and infused with recombinant GST (black circles), SUMO1 (black triangles) or SUMO1+Ubc9 (open triangles) peptides. (E,F) Representative current traces and current-voltage relationships from INS1 832/13 cells expressing EGFP (control, black circles), SUMO1-YFP (SUMO1, black triangles), SUMO1-YFP+Ubc9 (white triangles) or SUMO1-YFP+SENP1 (open circles). **P*<0.05, ***P*<0.01 and ****P*<0.001 compared with control; error bars, s.e.m.

Kv2.1 function. Expression of SUMO1-YFP inhibits Kv2.1 current by 48% (n=21, P<0.05) (Fig. 1B,C) compared with control cells expressing Kv2.1 and EGFP. Coexpression of the SUMO ligase Ubc9 inhibited current by 64% (n=15, P<0.001), whereas coexpression of the SUMO protease SENP1 reversed the inhibitory effect of SUMO1-YFP (n=16) (Fig. 1B,C). These effects were probably not due to altered channel expression because whole-cell Kv2.1 protein levels did not change in concert with channel inhibition. Furthermore, acute infusion of recombinant human SUMO1 peptide (5 μM), alone or with recombinant Ubc9 (10 μg/ml), through the patch pipette, also inhibited Kv2.1 current (80% and 92% inhibition, n=14 and 13, respectively, P<0.001) (Fig. 1D).

Kv2.1 is the major Kv channel in insulin secreting β-cells where it regulates action potential repolarization and insulin secretion (MacDonald et al., 2001; Yan et al., 2004; Jacobson et al., 2007). We detected the expression of mRNA encoding SUMO1 and SUMO3, but not SUMO2, by RT-PCR of INS-1 832/13 cells, mouse islet and human islet cDNA (not shown). Expression of the SUMO1-YFP in INS-1 832/13 cells, a common insulinoma model (Hohmeier and Newgard, 2004), inhibited endogenous Kv currents by 33% (n=27, P<0.05). Coexpression of Ubc9 had little additional inhibitory effect in the INS-1 832/13 cells, although this could perhaps be owing to differing levels of endogenous SUMO-conjugating enzymes in the two models. Nonetheless, and similar to our observations in HEK 293 cells overexpressing Kv2.1, the inhibitory effect was prevented by coexpression of SENP1 (n=12) (Fig. 1E,F). The overall inhibitory effect of SUMO1-YFP was less than observed in HEK 293 cells, and this is probably because of the mixed nature of the native currents in INS-1 cells which also express several additional Kv isoforms (Su et al., 2001).

SUMOylation regulates native Kv currents in human β-cells

We were interested in whether SUMO1 can regulate Kv currents in primary human β-cells because as much as 60% of these are contributed by Kv2.1 (Herrington et al., 2005). Expression of the SUMO1-YFP in primary human β-cells from three healthy donors inhibited native Kv currents (Fig. 2A-D). Overall, combining results from the donors, SUMO1-YFP resulted in a 49% (n=27, P<0.05) reduction in native Kv current compared to controls expressing EGFP alone (Fig. 2E). Coexpression of SENP1 was able to largely prevent the inhibition of native Kv current by SUMO1-YFP (n=7) (Fig. 2E), demonstrating the importance of SUMO conjugation for the inhibitory effect. Furthermore, the direct infusion of recombinant human SUMO1 peptide (5 μM) or SUMO1 with Ubc9 (10 μg/ml) in β-cells from two additional donors inhibited native Kv currents by 41% and 58% respectively (n=16 and 9, P<0.05) (Fig. 2F,G).

SUMOylation regulates Kv2.1 inactivation

In response to a 10-second depolarization, cloned Kv2.1 inactivated slowly [time constant (τ)=11.7±1.4 seconds, n=16] (Fig. 3A,B). Coexpression of SUMO1-YFP quickened the time-dependent inactivation of Kv2.1 (τ=8.2±1.2 seconds, n=19, P<0.05). This effect was enhanced by the additional presence of Ubc9 (τ=5.3±0.7 seconds, n=12, P<0.01) compared with SUMO1 alone (P<0.05), and was reversed by SENP1 (τ=9.6±1.4 seconds, n=14) (Fig. 3A,B). Similar results were obtained from human β-cells, where inactivation of Kv current was fit to an exponential decay with both fast (τ_1) and slow (τ_2) time constants (MacDonald et al., 2003; Herrington et al., 2005). In human β-cells, τ_1 was 0.6±0.2 seconds (n=10) and this was significantly decreased by coexpression of SUMO1-YFP and Ubc9 (0.2±0.1 seconds, n=13, P<0.05). Similarly, τ_2 in the human β-cells, most probably represents the contribution

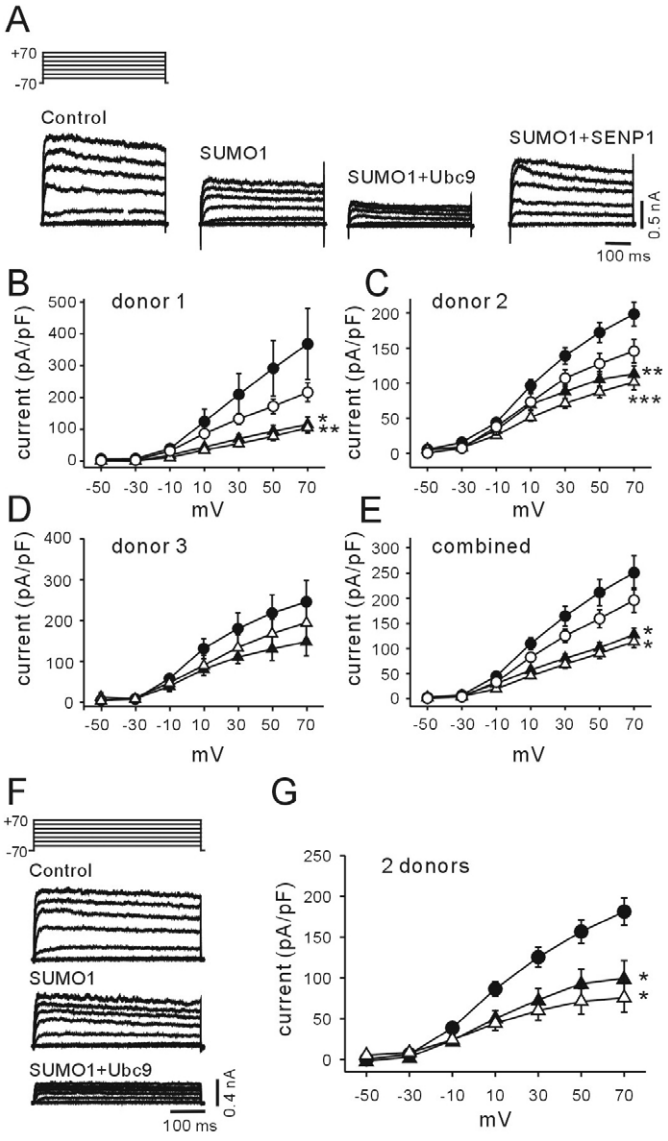

Fig. 2. SUMOylation regulates native human β-cell Kv current. (A) Representative current traces from human β-cells expressing EGFP alone (control) or SUMO1-YFP (SUMO1) with Ubc9 and/or SENP1 as indicated. (B-D) Current-voltage relationships were from β-cells from three human donors expressing EGFP (black circles), SUMO1-YFP (black triangles), SUMO1-YFP+Ubc9 (white triangles) or SUMO1-YFP+SENP1 (open circles). (E) Same as B-D, but data from all donors was pooled. (F,G) Representative current traces and current-voltage relationships from human β-cells (two donors) infused with recombinant GST (black circles), SUMO1 (black triangles), or SUMO1+Ubc9 (open triangles). **P*<0.05, ***P*<0.01 and ****P*<0.001 compared with control; error bars, s.e.m.

of native Kv2.1 (Braun et al., 2008), and was reduced from 3.5±0.6 seconds (n=10) to 2.1±0.2 seconds (n=13, P<0.05) by expression of SUMO1-YFP together with Ubc9.

Additionally, we found that the recovery of Kv2.1 from inactivation is impaired by SUMOylation (Fig. 3C-E). The recovery from inactivation at 65.5 seconds (Fig. 3D) was complete in control cells (99.7±0.3% recovery, n=7) and occurred with a time-constant ($\tau_{recovery}$) of 7.4±1.3 seconds (n=7). In cells coexpressing SUMO1-YFP, recovery was delayed ($\tau_{recovery}$=15.7±2.9 seconds, n=10, P<0.05) and incomplete (82.2±7.4%, n=10, P<0.05). When Ubc9 was coexpressed together with SUMO1-YFP, recovery at 65.5 seconds was 77.5±0.9% (n=8, P<0.01) and $\tau_{recovery}$ was 16.2±3.3 seconds (n=8, P<0.05). These effects were reversed by the coexpression of SENP1 (99.9±2.0% recovery, n=3; $\tau_{recovery}$=5.5±1.1 seconds, n=3). Recovery in the cells expressing SUMO1-YFP and SUMO1-YFP plus Ubc9 (+Ubc9) remained incomplete after more than two minutes (not shown).

In contrast to the recent study examining the effect of SUMOylation on cloned Kv1.5 current (Benson et al., 2007), we find that SUMOylation had no effect on the voltage-dependence of steady-state inactivation of either cloned Kv2.1 (Fig. 3F) or native INS-1 832/13 or human β-cell Kv currents. Half-inactivation of the cloned Kv2.1, INS-1 Kv currents and human β-cell Kv currents was –25.3±0.3 (n=14), –33.8±0.3 (n=12) and –39.1±1.3 mV (n=15), respectively, and these were unchanged by expression of SUMO1-YFP alone or together with Ubc9 or SENP1. Thus, the exact mechanism for channel regulation might differ somewhat between the Kv1.5 and 2.1 isoforms. Our present results suggest that SUMOylation inhibits Kv2.1 by quickening channel inactivation and slowing the recovery from this inactivation. The reduced current observed in the sequential depolarizations in Figs 1 and 2 probably results from cumulative channel inactivation and an associated failure to recover from this during the interpulse interval. This would have important consequences on repetitive action potential firing.

SUMOylation modifies β-cell excitability

We examined action potential generation in the INS-1 832/13 cells (Fig. 4A). Action potentials were elicited in the whole-cell current-clamp mode by a 10 pA current injection (Fig. 4A). In control cells, action potential firing occurred with a frequency of 15.7±3.2 Hz (n=16) and was significantly reduced by expression of SUMO1-YFP (to 6.5±2.2 Hz, n=12, P<0.05) (Fig. 4A,B). Firing was almost completely ablated by coexpression of SUMO1-YFP and Ubc9 (2.0±1.4 Hz, n=7, P<0.01) (Fig. 4A,B). Similar to effects on the cloned and native Kv currents, action potential firing was restored by the coexpression of the SENP1 (to 15.1±1.7 Hz, n=4).

Action potential half-width was increased by expression of SUMO1-YFP, from 7.7±1.3 mseconds (n=10) to 24.7±4.6 ms (n=6, P<0.001), and this was reversed by the coexpression of SENP1 (12.5±1.3 mseconds, n=4) (Fig. 4C). This is consistent with the SUMOylation-dependent inhibition of Kv current and probably explains the observed effects on firing frequency. We also observed a reduced amplitude of the action potential peak upon expression of SUMO1-YFP, from 7.8±2.1 mV (n=10) to –2.4±3.8 mV (n=6, P<0.05), which was also rescued by coexpression of SENP1 (4.9±3.1 mV, n=4). This latter finding is somewhat surprising and probably not explained by inhibition of Kv current. It seems possible that inhibition of voltage-dependent Ca^{2+} or Na^{+} channels might underlie the changes in action potential peak amplitude, but this remains unexplored. Nonetheless, the present results demonstrate that channel SUMOylation can exert strong regulatory effects on β-cell excitability that are consistent with Kv current inhibition.

Conclusions

Reversible protein SUMOylation has a well-recognized role in several cellular functions (Verger et al., 2003; Seeler and Dejean, 2003; Muller et al., 2004). Although a role in the acute regulation of cellular excitability was recently suggested (Rajan et al., 2005; Benson et al., 2007), it remains controversial as to whether direct SUMOylation can modulate ion-channel function (Feliciangeli et al., 2007). In the present work we have demonstrated that

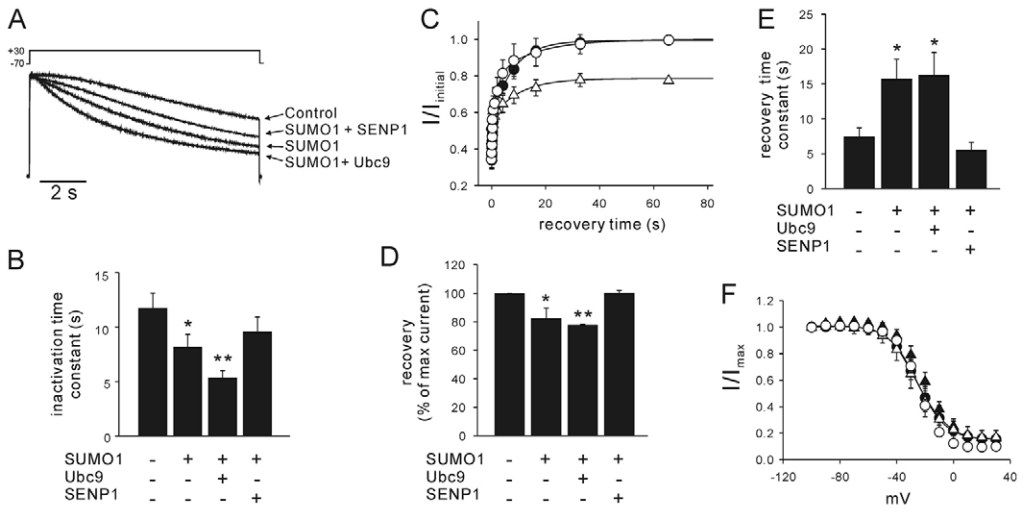

Fig. 3. SUMOylation regulates inactivation and recovery of Kv2.1 channels. (A) Representative current traces from HEK 293 cells expressing Kv2.1 alone (control) or with SUMO1-YFP (SUMO1), Ubc9 and/or SENP1 as indicated and normalized to peak current. (B) Inactivation time constants determined by fitting a single exponential decay function to the currents in panel A. (C) Recovery from inactivation following a 5-second depolarization to +30 mV in HEK 293 cells expressing Kv2.1 alone (black circles) or with SUMO1-YFP+Ubc9 (white triangles) or SUMO1-YFP+SENP1 (white circles). The Kv2.1+SUMO1-YFP curve was between the control and SENP1 curves and the Ubc9 curve (not shown). (D,E) The percent recovery to initial maximum current at 65.5 seconds and the recovery time constant determined by fitting the recovery curve to a single exponential function. (F) Voltage-dependence of steady-state inactivation of Kv2.1 expressed in HEK 293 cells either alone (black circles), with SUMO1-YFP (black triangles), with SUMO1-YFP+Ubc9 (white triangles) or with SUMO1-YFP+SENP1 (white circles). **P*<0.05 and ***P*<0.01 compared with control; error bars, s.e.m.

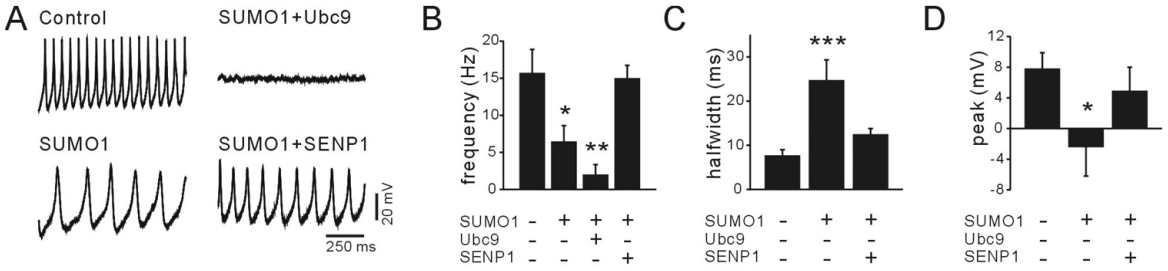

Fig. 4. SUMOylation regulates the excitability of INS1 832/13 β-cells. (A) Representative action potential traces induced by injection of a 10 pA current in INS1 832/13 cells expressing EGFP (control) or SUMO1-YFP (SUMO1), Ubc9 and/or SENP1 as indicated. (B-D) Action potential firing frequency, half-width and peak amplitudes for the groups shown in panel A. The half-width and peak amplitudes for the cells expressing SUMO1-YFP+Ubc9 were not analyzed because action potentials were rarely observed in this group. **P*<0.05, ***P*<0.01 and ****P*<0.001 compared with control; error bars, s.e.m.

SUMOylation is indeed able to regulate Kv2.1 and native Kv currents of insulinoma and human β-cells. This occurs as a result of distinct effects on the rate of, and recovery from, channel inactivation. Although it remains unclear as to whether this results from SUMOylation of the channel or of a channel binding partner, we now show that SUMOylation processes can exert a strong modulatory effect on cellular excitability and action potential firing that is consistent with the inhibition of Kv2.1. Finally, the present work suggests that SUMOylation processes might represent a novel and important mechanism for regulating pancreatic β-cell insulin secretion.

Materials and Methods

Recombinant constructs, cells and cell culture

The human SUMO1-YFP (Harder et al., 2004) was in the pEYFP-C3 vector (Clontech, Palo Alto, CA). The human SUMO1 (untagged), Ubc9 and SENP1 constructs in the pCMV6-XL4 vector were from Origene Technologies (Rockville, MD). The human Kv2.1 construct (MacDonald et al., 2002) was in the pcDNA3.1+ vector (Invitrogen Canada, Burlington, Canada) and was originally from Rolf H. Joho (University of Texas Southwestern Medical Center, Dallas, TX). The pIRES-EGFP vector (Clontech) was used for control transfections. Recombinant human SUMO1 and Ubc9 peptides were from GeneTex (San Antonio, TX).

HEK 293 cells were cultured in DMEM with 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% CO_2. INS-1 832/13 cells (a kind gift from Christopher B. Newgard, Duke University, NC) were cultured in RPMI 1640 medium containing 11.1 mM glucose and supplemented with 10% fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C and 5% CO_2. Human islets from five healthy donors were provided by James Shapiro (University of Alberta, Alberta, Canada) and the ABCC Human Islet Distribution Program at the University of Alberta. Islets were dispersed to single cells by shaking in Ca^{2+}-free buffer. Primary β-cells were positively identified following patch-clamp by immunostaining for insulin. Cell lines and primary cells were transfected using Lipofectamine 2000 (Invitrogen Canada). All experiments on human cells were approved by the Human Research Ethics Board at the University of Alberta.

Western blotting and immunoprecipitation

Cells were homogenized in lysis buffer, which contained (in mM): 20 HEPES (pH 7.4 with KOH), 100 NaCl, 40 KCl, 1 EDTA, 20 NEM, 10 NaF, and 1 Na_3VO_4 with 10% glycerol, 1% Triton X-100, and protease inhibitor cocktail (1:100 dilution, Sigma-Aldrich Canada, Oakville, Canada). Cell lysates were subjected to 1 hour pre-clearing with protein G-Sepharose (Zymed Laboratories, San Francisco, CA) followed by incubation with anti-Kv2.1 antibody (Alomone Labs, Jerusalem, Israel) overnight at

4°C and with protein G-Sepharose for 4 hours at 4°C. The immunoprecipitated material was analyzed by immunoblotting with anti-Kv2.1 (1:500) and anti-GFP (1:500, Clontech, Mountain View, CA). Anti-mouse IRDye 800CW and anti-rabbit IRDye 680 secondary antibodies were used at 1:5000. Images were obtained using an Odyssey infrared imaging system (LI-COR, Lincoln, NE).

Electrophysiology

Whole-cell patch-clamp was performed with an EPC10 patch-clamp amplifier controlled with PatchMaster software (HEKA Electronik, Lambrecht, Germany). Patch pipettes were pulled from thin-walled borosilicate glass tubes and had a resistance between 3 and 5 MΩ when fire-polished, coated with Sylgard and filled with intracellular solution. The intracellular solution was composed of (in mM): 140 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 EGTA and 3 ATP-Mg (pH 7.3 with KOH). The bath solution was composed of (in mM): 135 NaCl, 5.4 KCl, 1 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES and 5 glucose (pH 7.3 with NaOH). Experiments on cloned channels were at room temperature and experiments on native currents were at 32-35°C.

Current-voltage relationships were generated by sequential 500-msecond depolarizations from a holding potential of –70 mV with a 6-second intersweep interval. Time-dependent inactivation time constants were determined during a 10-second depolarization to +30 mV. Recovery from inactivation was determined by varying the recovery time (at –70 mV) between sequential 5-second and 500-msecond depolarizations to +30 mV. Steady-state inactivation was determined by a 500-msecond depolarization to +30 mV from holding potentials between –100 and +30 mV. Action potential firing was elicited in the whole-cell current-clamp mode by injection of a 10 pA current. Data was analyzed using FitMaster (HEKA Electronik) and SigmaPlot 10 (Systat Software, Point Richmond, CA) and compared by multiple ANOVA and Student's *t*-test. Data is expressed as mean ± s.e.m. and *P*-values of less than 0.05 were considered significant.

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