SUMO1 enhances cAMP-dependent exocytosis and glucagon secretion from pancreatic α-cells

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Key points:

- SUMOylation is the reversible modification of proteins by attachment of small ubiquitin-like modifier (SUMO) peptides, which in pancreatic β-cells inhibits insulin exocytosis and glucagon-like peptide-1 receptor (GLP-1-R) signaling.
- We find in glucagon-secreting pancreatic α-cells that SUMOylation increases excitability and enhances exocytosis by increasing L-type Ca^{2+} currents.
- The ability of SUMOylation to facilitate α-cell exocytosis is cAMP-dependent, leading to enhanced adrenaline-stimulated glucagon secretion.
- SUMO1 prevents inhibition of α-cell Na^{+} current and exocytosis by a GLP-1-R agonist, but does not prevent GLP-1-R-dependent inhibition of glucagon secretion.
- SUMOylation modifies α-cell responses to cAMP-dependent signaling and, in contrast to it's inhibitory effects in β-cells, enhances α-cell exocytosis and glucagon secretion.

(106 words)

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Abstract

Post-translational modification by the small ubiquitin-like modifier-1 (SUMO1) limits insulin-secretion from β-cells by inhibiting insulin exocytosis and glucagon-like peptide-1 (GLP-1) receptor signaling. The secretion of glucagon from α-cells is regulated in manner opposite to insulin; it is inhibited by elevated glucose and GLP-1, and increased by adrenergic signaling. We therefore sought to determine whether SUMO1 modulates mouse and human α-cell function. Action potentials (APs), ion channel function, and exocytosis in single α-cells from mice and humans, identified by glucagon immunostaining, and glucagon secretion from intact islets were measured. The effects of SUMO1 on α-cell function and the respective inhibitory and stimulatory effects of exendin 4 and adrenaline were examined. Up-regulation of SUMO1 increased α-cell AP duration, frequency and amplitude, in part due to increased Ca^{2+} channel activity that led to elevated exocytosis. The ability of SUMO1 to enhance α-cell exocytosis was cAMP-dependent and results from an increased L-type Ca^{2+} current and a shift away from exocytosis dependent on non-L-type channels; an effect that was mimicked by knockdown of the deSUMOylating enzyme sentrin/SUMO-specific protease-1 (SENP1). Finally, while SUMO1 prevented GLP-1 receptor-mediated inhibition of α-cell Na^+ channels and single-cell exocytosis, it failed to prevent the exendin-4-mediated inhibition of glucagon secretion. Consistent with its cAMP-dependence however, SUMO1 enhanced α-cell exocytosis and glucagon secretion stimulated by adrenaline. Thus, in contrast to its inhibitory role in β-cell exocytosis, SUMO1 is a positive regulator of α-cell exocytosis and glucagon secretion under conditions of elevated cAMP. --- *239 words*

Abbreviations AP, action potential; GLP-1, glucagon-like peptide-1; GFP, green fluorescent protein; GST, glutaghione-S-transferase; SENP, sentrin/SUMO-specific protease; SUMO, small ubiquitin-like modifier; Kv, voltage-dependent K^+; Na_v, voltage-dependent Na^+; VDCC, voltage-dependent Ca^{2+} channel.

Introduction

Insulin and glucagon are the two major hormones regulating glucose storage and mobilization, respectively. Their secretion from the pancreatic islets of Langerhans is likewise regulated in opposing manners by metabolic and hormonal inputs. Like β-cells, the function of α-cells depends on action potential (AP) firing, which is observed at low glucose concentrations and is coupled to the exocytosis of glucagon-containing secretory granules (Barg *et al.*, 2000). Several ion channels are required for appropriate α-cell AP firing: Voltage-dependent Na^+ (Na_v) channels are active at physiological membrane potentials in α-cells, and contribute to the AP upstroke (Göpel *et al.*, 2000; Vignali *et al.*, 2006). Inhibition of these decreases AP amplitudes and blunts glucagon secretion (Göpel *et al.*, 2000; Ernst *et al.*, 2009). Voltage-gated K^+ (Kv) channels contribute to α-cell AP repolarization, and inhibition of these blunts glucagon secretion (Ramracheya *et al.*, 2010; Spigelman *et al.*, 2010). Voltage-dependent Ca^{2+} channels (VDCCs) also contribute to the initiation and peak of α-cell APs, and are required for glucagon secretion and α-cell exocytosis. Under low glucose conditions, α-cell exocytosis and glucagon secretion can be blocked by ω-conotoxin (Macdonald *et al.*, 2007), suggesting a coupling to N-type or perhaps P/Q-type VDCCs (Rorsman *et al.*, 2012).

The non-L-type Ca^{2+} channels contribute <20% of α-cell Ca^{2+} current, although adrenergic stimulation is suggested to shift α-cell exocytosis towards a greater dependence upon Ca^{2+} entry through L-type VDCCs (De Marinis *et al.*, 2010; Rorsman *et al.*, 2012). Conversely, the intestinal incretin hormone glucagon-like peptide-1 (GLP-1) inhibits glucagon release (Baggio & Drucker, 2007). GLP-1 analogs and inhibitors of native GLP-1 degradation are now important tools in the clinical management of diabetes (Lovshin & Drucker, 2009; Hare *et al.*, 2010; Henry *et al.*, 2011; Rauch *et al.*, 2012). While the effects of both incretin and adrengergic receptor signaling are thought be cAMP-dependent, the mechanism(s) that underlie disparate regulation of glucagon secretion by GLP-1 and adrenaline are incompletely understood. One report suggest that α-cell action potential (AP) firing and exocytosis may be differentially regulated by the magnitude of the cAMP response conferred by differing GLP-1 and adrenergic receptor expression levels in α-cells (De Marinis *et al.*, 2010).

Insulin secretion is regulated by post-translational SUMOylation, whereby a small ubiquitin-like modifier (SUMO) peptide is covalently attached to protein targets (Yeh, 2009). In β-cells, SUMOylation controls ion channel activity, exocytosis, metabolic sensing by glucokinase, and gene transcription (Manning Fox *et al.*, 2012; Aukrust *et al.*, 2013). Additionally, SUMOylation may modulate responsiveness to circulating hormones. GLP-1 receptor signaling in β-cells is down-

regulated by SUMOylation (Rajan *et al.*, 2012). Also, the stimulus-dependent re-internalization of β-adrenergic receptors may require SUMOylation of arrestins (Wyatt *et al.*, 2011). The role of SUMOylation in α-cell function has not been investigated.

We therefore sought to examine the role for SUMOylation in α-cell function and glucagon secretion, and whether this modifies the responses to GLP-1 and adrenergic receptor activation. We demonstrate that SUMO1 increases α-cell AP firing, likely due to an up-regulation of L-type Ca^{2+} channels, and enhances α-cell exocytosis. While over-expressed SUMO1 also prevented the inhibition of Na^+ currents and exocytosis by the GLP-1 receptor agonist exendin 4, it did not prevent the exendin 4-dependent suppression of glucagon secretion. Rather, the ability of SUMO1 to enhance α-cell exocytosis depended on the presence of cAMP within the patch-clamp pipette. Indeed, SUMO1 was able to enhance the stimulatory action of forskolin and adrenaline on α-cell exocytosis, and potentiated adrenaline-induced glucagon secretion from intact islets. Thus, SUMOylation exerts an important control of the distal machinery governing glucagon exocytosis in a cAMP-dependent manner. The ability of SUMOylation to enhance α-cell exocytosis and glucagon secretion is in contrast to its direct inhibitory action on insulin secretion.

Materials and Methods

Cells and cell culture

Mouse islets from male C57BL6 mice (Charles River, Montreal, QC, Canada) were isolated by standard collagenase digestion followed by hand-picking and then cultured overnight in RPMI (Gibco 11875; Life Technologies, Carlsbad, CA, USA) with 10% FBS (vol./vol.) and 100 U/ml penicillin/streptomycin at 37°C and 5% CO_2. Human islets from the Clinical Islet Laboratory at the University of Alberta and the Alberta Diabetes Institute IsletCore were cultured for 1-3 days in DMEM (Gibco 11885; Life Technologies) with 10% FBS and 100 U/ml penicillin/streptomycin at 37°C and 5% CO_2. Islets were dispersed into single cells by incubation for 10 minutes at 37°C in a Hank's-based enzyme-free cell dissociation buffer (Life Technologies), followed by mechanical dispersion with a flame-polished glass pipette. All studies were approved by the Animal Care and Use Committee and the Human Research Ethics Board at the University of Alberta.

Constructs, adenoviruses, and recombinant peptides

Recombinant adenoviruses expressing green fluorescent protein (GFP) alone (Ad-GFP) or together with SUMO1 (Ad-SUMO1), and expressing an shRNA sequence targeted against SENP1 (Ad-shSENP1) or a scrambled control (Ad-Scrambled), have been characterized (Dai *et al.*, 2009; 2011). Ad-SUMO1 was constructed by cloning the human SUMO1 sequence into the pAdtrackCMV plasmid for adenoviral generation. Sequences for shRNA constructs were as follows: Ad-shSENP1 (GATCCCGCCGAAAGACCTCAAGTGGATTTTCAAGAGAAATCCACT-TGAGGTCTTTCGGTTTTTTCCAAA); Ad-Scrambled (GATCCCGATTGCGCCAGATTGAAGA-TTCAAGAGATCTTCAATCTGGCGCAATCTTTTTTCCAAA). All constructs co-expressed GFP to allow identification of infected cells prior to patch-clamp recordings and to estimate infection efficiency. Recombinant human SUMO1 and glutathione S-transferase (GST) proteins were from Sigma-Aldrich Canada (Oakville, Canada), and infused into cells at 4 μg/ml.

Glucagon secretion assay

Glucagon secretion was assessed by perifusion using a BioRep PERI4 system with batches of 85 mouse islets 36-48 hours following infection with either Ad-GFP or Ad-SUMO1. Islets were perifused at 50 μl/minute with KRBH solution containing (in mM): 140 NaCl, 3.6 KCl, 2.6 $CaCl_2$, 0.5 NaH_2PO_4, 0.5 $MgSO_4$•$7H_2O$, 5 HEPES, 2 $NaHCO_3$, and glucose as indicated at a pH of 7.45 with NaOH. Islets were pre-incubated at 3 mM glucose for 1 hour prior to fraction collection at 2 minute intervals. Samples were assayed using the Mouse/Rat Glucagon MULTI-ARRAY Assay System from Meso Scale Discovery (Rockville, MD, USA).

Patch-clamp electrophysiology

The standard whole-cell technique with the sine+DC lock-in function of an EPC10 amplifier and Patchmaster software (HEKA Electronics, Lambrecht/Pfalz, Germany) was used in experiments performed at 32°C to 35°C. Patch pipettes, pulled from borosilicate glass and coated with Sylgard, had a resistance of 3 to 4 MΩ when filled with pipette solution; liquid junction potentials were corrected as appropriate. For depolarization-stimulated capacitance measurements and voltage-gated Na^+ and Ca^{2+} current by standard whole-cell patch clamp, the pipette solution contained (in mM): 125 Cs-glutamate, 10 CsCl, 10 NaCl, 1 $MgCl_2$, 0.05 EGTA, 5 HEPES, 0.1 cAMP and 3 MgATP (pH 7.15 with CsOH). The extracellular bath contained (in mM): 118 NaCl, 20 tetraethylammonium chloride, 5.6

KCl, 1.2 $MgCl_2$, 2.6 $CaCl_2$, 1 glucose and 5 HEPES (pH 7.4 with NaOH). Some experiments were performed with perforated patch pipettes pulled from thick walled borosilicate glass tubes, with resistances between 8 and 10 MΩ when filled with (in mM) 76 K_2SO_4, 10 KCl, 10 NaCl, 1 $MgCl_2$ and 5 HEPES (pH 7.25 with KOH), and back-filled with 0.24 mg/ml amphotericin B. Extracellular solution for these experiments was (in mM): 140 NaCl, 3.6 KCl, 1.5 $CaCl_2$, 0.5 $MgSO_4$, 10 HEPES, 0.5 NaH_2PO_4, 5 $NaHCO_3$, 1 glucose (pH 7.3 with NaOH). VDCC activity was also measured by substituting Ba^{2+} for Ca^{2+}. The pipette solution for this was (in mM): 140 Cs-glutamate, 1 $MgCl_2$, 20 tetraethylammonium chloride, 5 EGTA, 20 HEPES and 3 MgATP (pH 7.3 with CsOH). The extracellular bath contained (in mM): 20 $BaCl_2$, 100 NaCl, 5 CsCl, 1 $MgCl_2$, 1 glucose, 10 HEPES, and 0.5 µM tetrodotoxin (pH 7.35 with CsOH). For Kv currents, the intracellular solution contained (in mM): 140 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 EGTA and 3 MgATP (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 1 glucose (pH 7.3 with NaOH).

Blockers of L-type Ca^{2+} channels (isradipine, 5 μM) and N-type (and perhaps P/Q-type) Ca^{2+} channels (ω-conotoxin, 100 nM), the GLP-1 receptor agonist exendin 4 (10 nM; Sigma-Aldrich Canada), or adrenaline (10 μM; Sigma-Aldrich Canada) were added to the bath as indicated. Measurements were performed on separate groups of cells (from the same human or mouse islet isolation). Cells were infected 4-6 hours after dispersion (as above), and then for an additional 36 hours (for over-expression) or 72 hours (for shRNA experiments) before patch-clamp experiments. Measurements were normalized to initial cell size and expressed as femtofarad (fF) per picofarad (pF) and picoampere (pA) per pF. In all experiments, mouse and human α-cells were positively identified by immunostaining for glucagon (guinea pig anti-glucagon, 1:5,000; Linco, St Charles, MO, USA), and appropriate secondary antibodies (Alexa Fluor 594 goat anti-guinea pig 1:200; Invitrogen, Eugene, OR, USA).

Data Analysis

Analysis was performed using Fit-Master software (HEKA Electronik) and SigmaPlot 11 (Systat Software, Point Richmond, CA). Groups are compared by multiple comparison ANOVA followed by Bonferroni or Fisher's LSD (AUC) post-test, or by the unpaired Student's t test. Data are presented as means ± standard errors, and $P<0.05$ is considered significant.

Results

SUMO1 regulates α-cell action potentials through distinct effects on K^+ and Ca^{2+} currents

SUMO1 inhibits β-cell Kv currents, increasing the duration of action potentials (APs) and reducing firing frequency (Dai *et al.*, 2009). We expressed SUMO1 in mouse α-cells, identified by positive glucagon immunostaining, and examined AP firing in the presence of a hyperpolarizing -2 pA current injection (Figure 1a, b) since we found previously that this facilitates α-cell AP firing (Spigelman *et al.*, 2010). Ad-SUMO1 (n=11) increased mouse α-cell AP half-width ($P<0.05$), firing frequency ($P<0.01$), decreased the threshold for AP firing ($P<0.01$), and increased AP amplitudes ($P<0.001$) compared with cells infected with the control Ad-GFP (n=11-15; Figure 1a-g). The AP peak potentials were not increased by Ad-SUMO1, suggesting that the elevated AP amplitudes are due almost entirely to a lower firing threshold.

We assessed the effect of SUMO1 on voltage-gated Na^+, K^+, and Ca^{2+} channels in mouse α-cells (Figure 1h-j). Voltage-dependent Na^+ currents, elicited by a series of short (10 ms) depolarizations from -70 mV to voltages between -60 and +60 mV, were unaffected by SUMO1 (n=26). Kv currents were slightly, but significantly, increased in α-cells infected with Ad-SUMO1 (n=20; $P<0.05$) compared with Ad-GFP (n=15). This may contribute to the observed increase in AP firing frequency. Conversely, voltage-dependent Ba^{2+} currents (a measure of Ca^{2+} channel activity) elicited by a 500 ms step-wise depolarization from -70 to 0 mV were significantly (n=8, $P<0.01$) increased when SUMO1 was up-regulated. This occurred despite a hyperpolarizing shift in VDCC voltage-dependent inactivation (from V_{50}=-34.6+/-3.9 to -48.5+/-2.5 mV, n=14,13, $P<0.01$) that cannot explain the up-regulated Ba^{2+} current. Thus, the increased AP amplitude and duration upon up-regulation of SUMO1 is consistent with an increased Ca^{2+} current.

SUMOylation enhances α-cell exocytosis

Given that SUMO1 increases α-cell Ca^{2+} currents, we examined whether this translates into an elevated α-cell exocytotic response. Increasing SUMO1 in mouse α-cells enhanced exocytosis triggered by a series of membrane depolarizations (n=43-50, $P<0.001$; Figure 2a,b). The deSUMOylating enzyme SENP1 cleaves SUMO1 from it's target proteins (Yeh, 2009). Expression of a SENP1-targeted shRNA adenoviral construct (Ad-shSENP1; n=29) resulted in an increased exocytotic response in mouse α-cells compared with a scrambled control (Ad-Scrambled; n=22,

P<0.01), similar to the effect of SUMO1. The SUMO1-dependent increase in exocytosis was also observed in human α-cells infected with Ad-SUMO1 (n=32-33, P<0.01, Figure 2e,f). The direct intracellular infusion of recombinant SUMO1 peptide had no effect on α-cell exocytosis or Ca^{2+} currents (not shown). Finally, we confirmed that SUMO1 also increases Ca^{2+} current in human α-cells (n=29, P<0.05; Figure 2g,h). This increase in Ca^{2+} current could be prevented by the L-type channel blocker isradipine (5 μM, n=11; Figure 2g,h).

SUMO1-enhanced α-cell exocytosis is dependent on L-type Ca^{2+} channels

To determine the nature of α-cell exocytosis following up-regulation of SUMO1, we examined the effect of the VDCC inhibitors isradipine and ω-conotixin. These block L- and N-type channels, respectively, although it has been suggested that ω-conotoxin may additionally block P/Q-type channels (Rorsman *et al.*, 2012). The exocytotic response in mouse α-cells under control conditions (Ad-GFP) was suppressed by 100 nM ω-conotoxin (by 74%, n=6; Figure 3a, *left*). We find however that ω-conotoxin is ineffective at suppressing exocytosis when SUMO1 is up-regulated (n=13; Figure 3a, *right*). Conversely, while isradipine (5 μM) had little effect on exocytosis in control α-cells (n=9, Figure 3b,c), it prevented the up-regulation of α-cell exocytosis by SUMO1 (n=17, P<0.01; Figure 3b,d). We also examined the effect of ω-conotoxin and isradipine on exocytosis in response to a 500 ms depolarization in mouse α-cells following SENP1 knockdown (Figure 3e,f). Again, exocytosis was inhibited by ω-conotoxin only under the control condition (Ad-Scrambled; n=15-24, P<0.01), while isradipine was effective at inhibiting exocytosis in cells infected with Ad-shSENP1 (n=12-15, P<0.05; Figure 3e,f). Finally, to determine whether increased Ca^{2+} entry *per se* is responsible for the SUMO1-dependent increase in α-cell exocytosis, we monitored capacitance upon infusion of 200 nM free Ca^{2+} (Figure 3g,h). Under these conditions, Ca^{2+}-induced exocytosis was similar between α-cells infected with Ad-GFP (n=8) and Ad-SUMO1 (n=15).

SUMO1 prevents the suppression of α-cell Na^{+} currents and exocytosis by exendin 4

Since SUMO1 negatively regulates the trafficking and activity of the GLP-1 receptor in β-cells (Rajan *et al.*, 2012), we examined whether SUMO1 could modulate the cellular response(s) to the GLP-1 receptor agonist exendin 4 in α-cells. In our hands, the effects of exendin 4 on α-cell action

potentials are consistent with an inhibition of voltage-gated Na^+ currents, leading to decreased AP height and peak potential (not shown). Indeed, the rapidly inactivating Na^+ currents in mouse α-cells, elicited by short (10 ms) depolarizations from -70 mV to voltages between -60 and +60 mV, were significantly (n=8, P<0.01) blunted by exendin 4 (10 nM; Figure 4a,b). This results from a hyperpolarizing shift in voltage-dependent inactivation of the Na^+ current from a half-inactivation (V_{50}) of -64.6+/-1.5 (n=10) to -82.8+/-1.6 mV (n=10, p<0.001, Figure 4c,d). While SUMO1 did not affect α-cell Na^+ current amplitude (n=26) or inactivation time constant at 0 mV (τ=4.3+/-0.7 versus 3.5+/-0.7 ms, n=14, 9), consistent with the findings in Figure 1, infection of α-cells with Ad-SUMO1 prevented the exendin 4-induced Na^+ current inhibition (n=8, Figure 4a,b; τ=6.0+/-1.8 ms) and leftward-shift in voltage-dependent inactivation (Figure 4c,d).

Activation of the GLP-1 receptor inhibits α-cell exocytosis (De Marinis *et al.*, 2010). Indeed, exendin 4 (10 nM) suppressed exocytosis elicited by membrane depolarization in mouse α-cells infected with Ad-GFP (Figure 5). This inhibitory effect was observable when intracellular cAMP levels were clamped at 0.1 (n=7-11, P<0.05) or 1 mM (n=8-11, P<0.01). Consistent with the above results we find that SUMO1 alone (n=14) was sufficient to enhance the α-cell exocytosis beyond that observed in control cells (n=23, P<0.05). Following up-regulation of SUMO1, exendin 4 (10 nM) was no longer able to suppress α-cell exocytosis (n=6-7; Figure 5). Together, our data suggests that the ability of SUMO1 to prevent the effects of exendin 4 on Na^+ currents and exocytotic responsiveness is not due to impairment of cAMP responses (which were clamped in these experiments), but rather to an as-yet-unappreciated signaling mechanism.

The ability of SUMO1 to enhance α-cell exocytosis is cAMP-dependent

To further explore the interaction between SUMO1 and cAMP in α-cell exocytotic responses, we monitored α-cell capacitance without including cAMP in our patch pipette (Figure 6). In the absence of cAMP, the exocytotic response of α-cells infected with Ad-GFP or Ad-SUMO1 were similar (n=19-29). This contrasts with the robust increase in exocytosis seen in α-cells infected with Ad-SUMO1 at 0.1 or 1 mM cAMP (Figure 5). Indeed, up-regulation of SUMO1 significantly increased the α-cell exocytotic response to cAMP-raising agents forskolin (10 μM; n=25-29, P<0.01) and

adrenaline (10 μM; n=21-22, P<0.01; Figure 6). Cells were pre-treated with these agents in the bath solution, immediately prior to and during the patch-clamp experiment.

SUMO1 enhances glucagon secretion stimulated by adrenaline

To assess the effect of SUMO1 on glucagon secretion *per se*, we performed perifusion analysis of mouse islets infected with either Ad-GFP or Ad-SUMO1. Interestingly, up-regulation of SUMO1 generally had little effect on glucagon secretion at either 1 or 6 mM glucose (Figure 7) and had no effect on islet glucagon content (not shown). In one set of experiments (Figure 7b) the slight increase in glucagon release with Ad-SUMO1 was significant (n=5, P<0.05) at only one time point. Also, the glucagon response to 20 mM KCl was modestly increased (AUC in Figure 7a; n=4, P<0.05). This is consistent with the lack of effect of SUMO1 on α-cell exocytosis in the absence of elevated cAMP. Somewhat surprisingly, exendin 4 (10 nM) remained effective in inhibiting glucagon secretion at 1 mM glucose in islets infected with Ad-SUMO1 (n=5, Figure 7b). Finally, consistent with the cAMP-dependence of the effect of SUMO1 on α-cell exocytosis, glucagon secretion in response to 10 μM adrenaline was significantly elevated in islets infected with Ad-SUMO1 compared with Ad-GFP (n=5, P<0.01) and remained elevated following removal of adrenaline (P<0.001), although this latter effect may in part be related to the slow perifusion rate (50 μl/minute) used in these experiments.

Discussion

The importance of glucagon secretion in the physiologic control of glucose and the pathophysiology of diabetes is receiving renewed interest (Cryer, 2012). We however know surprisingly little about the cellular machinery that determines α-cell function, and how this is regulated by metabolic and hormonal inputs. Significant parallels can be drawn between β-cell and α-cell function; in fact they express much of the same downstream secretory machinery that includes similar compliments of ion channels and exocytotic proteins (Andersson *et al.*, 2011; Rorsman *et al.*, 2012). It is thus no surprise that the final events of glucagon secretion, including AP firing and Ca^{2+}-dependent exocytosis, are quite similar to that of insulin secretion. The control of this machinery by both internal and external cues however differs strikingly between β- and α-cells. For example, insulin and glucagon secretion are regulated in opposite manners by intracellular PAS kinase (da Silva

Xavier *et al.*, 2004; Fontés *et al.*, 2009; da Silva Xavier *et al.*, 2011), adrenergic signals (Dunning & Taborsky, 1991; Straub & Sharp, 2012), and GLP-1 receptor signaling (Gromada & Rorsman, 2004).

SUMO1 increases AP amplitudes and frequency in α-cells. This likely results from a combination of increases in Kv and L-type Ca^{2+} currents. Effects of SUMOylation on ion channels (Rajan *et al.*, 2005; Benson *et al.*, 2007; Dai *et al.*, 2009; Plant *et al.*, 2010; 2011), and Ca^{2+} handling (Feligioni *et al.*, 2009; Kho *et al.*, 2011) have been demonstrated, and a recent abstract suggests the SUMO-dependent inhibition of P/Q-type Ca^{2+} channels (Davila *et al.*, 2010), however a SUMO1-dependent up-regulation of L-type Ca^{2+} current has not been demonstrated previously. This up-regulated Ca^{2+} current cannot be explained the changes in voltage-dependent inactivation or by acute effects of SUMO1 on the channels (since dialysis had no effect). Thus, given the known roles for SUMOylation in gene transcription (Yeh, 2009), SUMOylation in α-cells may increase Ca^{2+} currents primarily by up-regulating L-type Ca^{2+} channel density.

We wondered if the SUMO1-dependent increase in L-type Ca^{2+} current results in enhanced depolarization-induced exocytosis. Indeed, we find that SUMO1 (or knockdown of SENP1) enhances depolarization-induced exocytosis in α-cells. This is mediated entirely by the up-regulated Ca^{2+} current, as evidenced by the inability of SUMO1 affect exocytotic responses to direct intracellular Ca^{2+} dialysis. This is in stark contrast to the effects of SUMOylation in β-cells, where exocytosis is inhibited directly by SUMOylation with no effect on VDCC activity or intracellular Ca^{2+} responses (Dai *et al.*, 2011). Furthermore, a key role for the L-type Ca^{2+} channel in the up-regulated α-cell exocytosis is supported by the sensitivity of this response to block by isradipine. Our observation that SUMO1 promotes a shift from ω-conotoxin- to isradipine-sensitive exocytosis in α-cells is reminiscent of recent work demonstrating a similar response of glucagon secretion to stimulation with adrenaline (De Marinis *et al.*, 2010), where cAMP was proposed to underlie the shift from non-L-type to L-type channel dependent glucagon secretion.

Inhibition of the non-L-type Ca^{2+} channels that are coupled to glucagon exocytosis under basal conditions are proposed to underlie the suppression of glucagon secretion by GLP-1 (De Marinis *et al.*, 2010). These Ca^{2+} currents, likely mediated by N- or P/Q-type channels, are generally quite small in α-cells (Rorsman *et al.*, 2012). As noted above we do find that a large proportion of α-cell exocytosis under control conditions is blocked by ω-conotoxin, however in our hands exendin 4 remains able to inhibit α-cell exocytotic responses in the presence of 1 mM intracellular cAMP. This

argues against 'low cAMP' as the mediator, and is consistent with the suggestion that additional cAMP-independent signals contribute to the inhibition of glucagon secretion by GLP-1 (Tian *et al.*, 2011). Nonetheless, we cannot exclude a small change in Ca^{2+} current, or perhaps an uncoupling of glucagon granules from the non-L-type VDCCs, contributing to the suppression of glucagon secretion by exendin 4.

We also now provide evidence for an exendin-4-dependent inhibition of α-cell Na_v channels, resulting from a hyperpolarizing shift in the voltage-dependence of inactivation. This likely contributes to reduced AP height and would serve to reduce the overall activation of high-voltage-activated N- or P/Q-type Ca^{2+} channels on which glucagon secretion depends under basal conditions. While in apparent contrast to the suggestion of transient α-cell hyperpolarization by GLP-1 leading to increased in AP amplitude in two of six α-cells examined from intact mouse islets (De Marinis *et al.*, 2010), our experiments examined APs elicited by current injection (to allow us to study the AP characteristics, rather than glucose-dependence *per se*) following a longer exposure to exendin 4 (10-30 minutes) in isolated α-cells. As such, the methodological differences may account for this, and our results are consistent with a decreased AP firing frequency reported in the steady-state following GLP-1 treatment and the lack of effect of GLP-1 on AP duration in that study.

Based upon the ability of SUMOylation to down-regulate GLP-1 receptor signaling in β-cells (Rajan *et al.*, 2012), we investigated whether SUMO1 would prevent the α-cell responses to the GLP-1 receptor agonist exendin 4. Indeed, up-regulation of SUMO1 prevented the inhibition of α-cell Na^+ currents and exocytosis by GLP-1 receptor activation. While this may appear consistent with an inhibition of GLP-1 receptor signaling, our finding that SUMO1 fails to prevent exendin 4-mediated suppression of glucagon secretion suggests that GLP-1 receptor signaling is preserved in the intact islets. This discrepancy can be explained by a key experimental difference between the single-cell patch-clamp and whole islet secretion studies: our initial patch-clamp studies were performed with a high level of intracellular cAMP, which is likely much lower (if increased at all) following GLP-1 receptor activation in α-cells of the intact islet (De Marinis *et al.*, 2010; Tian *et al.*, 2011). Thus SUMO1 is unlikely to block GLP-1 receptor signaling directly in α-cells, but may have a more direct cAMP-dependent effect to increase glucagon secretion.

We find that the facilitation of α-cell exocytosis by SUMO1 is indeed cAMP-dependent. This likely explains the inability of Ad-SUMO1 to enhance glucagon secretion stimulated by low glucose or

exendin 4, since GLP-1 receptor activation produces only a small rise in α-cell cAMP, if any (De Marinis *et al.*, 2010; Tian *et al.*, 2011). On the other hand, signaling of β- and α_1-adrenergic receptors is generally considered to produce a robust increase in α-cell cAMP (Schuit & Pipeleers, 1986; Vieira *et al.*, 2004; Tian *et al.*, 2011). Stimulation of single α-cells with adrenaline in our hands facilitated depolarization-induced α-cell exocytosis to a degree similar to that of forskolin or direct infusion of cAMP, and enabled a further SUMO1-dependent increase in exocytosis. Thus while up-regulation of SUMO1 results in increased α-cell L-type Ca^{2+} channel currents and enhanced excitability, a rise in cAMP and shift to L-type-dependent glucagon exocytosis is required to realize an enhancement of glucagon secretion such as that observed upon stimulation with adrenaline (Figure 8).

Thus, we now demonstrate that SUMO1 regulates glucagon secretion in a manner opposite to its control of insulin secretion. SUMO1 enhances glucagon secretion by increasing L-type Ca^{2+} currents in α-cells, increasing exocytosis under conditions of elevated cAMP where glucagon granules become more dependent on Ca^{2+} entry through L-type channels. The result is that glucagon secretion in response to adrenergic stimulation is enhanced by SUMO1. Interestingly, while our data suggest that GLP-1 receptor inhibition of α-cell Na_v channels and exocytosis is lost following SUMO1 up-regulation, this is insufficient to prevent exendin 4-mediated suppression of glucagon secretion where α-cell cAMP levels may be low. Further understanding the interaction of the SUMOylation and cAMP pathways may reveal novel approaches to control insulin and glucagon secretion in diabetes. The demonstration that SUMOylation regulates key components of the α-cell secretory mechanism in a manner opposite that in the β-cell provides a novel mechanism underlying the differential regulation of glucagon and insulin secretion.

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Figure 1. *SUMO1 increases α-cell excitability and up-regulates voltage-dependent Ca^{2+} currents.* We measured APs from mouse α-cells in the perforated patch condition, positively identified by glucagon immunostaining, with 0 and -2 pA current injection. **(*A*)** Representative APs from three different α-cells transduced with Ad-GFP. **(*B*)** Representative APs from three different α-cells transduced with Ad-SUMO1. Up-regulation of SUMO1 increased AP half-width **(*C*)** and firing frequency **(*D*)**, decreased the firing threshold **(*E*)**, and increased AP amplitudes without affecting peak potential **(*F*,*G*)**. We then measured voltage-dependent Na^+ **(*H*)**, K^+ **(*I*)** and Ba^{2+} **(*J*)** currents from mouse α-cells infected with Ad-GFP (open circles) or Ad-SUMO1 (black circles), positively identified by glucagon immunostaining, in the whole-cell configuration. *P<0.05, **P<0.01, and ***p<0.001 comparing the Ad-SUMO1 with Ad-GFP.

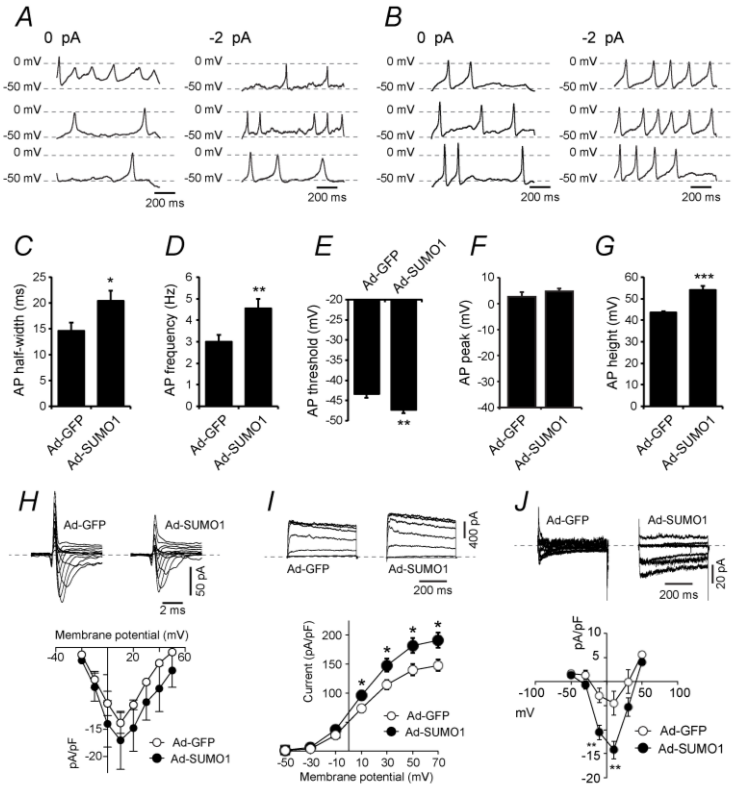

Figure 2. *SUMOylation increases the α-cell exocytotic response to membrane depolarization, and up-regulates L-type Ca^{2+} currents.* We measured exocytosis as increases in membrane capacitance in mouse and human α-cells, positively identified by glucagon immunostaining, during a series of ten membrane depolarizations from -70 to 0 mV. Representative **(*A*)** and quantified **(*B*)** responses of mouse α-cells infected with Ad-GFP (open circles) or Ad-SUMO1 (black circles). Representative **(*C*)** and quantified **(*D*)** responses of mouse α-cells infected with Ad-shScramble (open squares) or Ad-shSENP1 (black squares). Representative **(*E*)** and quantified **(*F*)** responses of human α-cells infected with Ad-GFP (open circles) or Ad-SUMO1 (black circles). Representative **(*G*)** and quantified **(*H*)** Ca^{2+} currents from human α-cells infected by Ad-GFP or Ad-SUMO1, elicited by a single depolarization from -70 to 0 mV, in the absence or presence of 5 μM isradipine. *P<0.05, **P<0.01, and ***P<0.001 compared with the control.

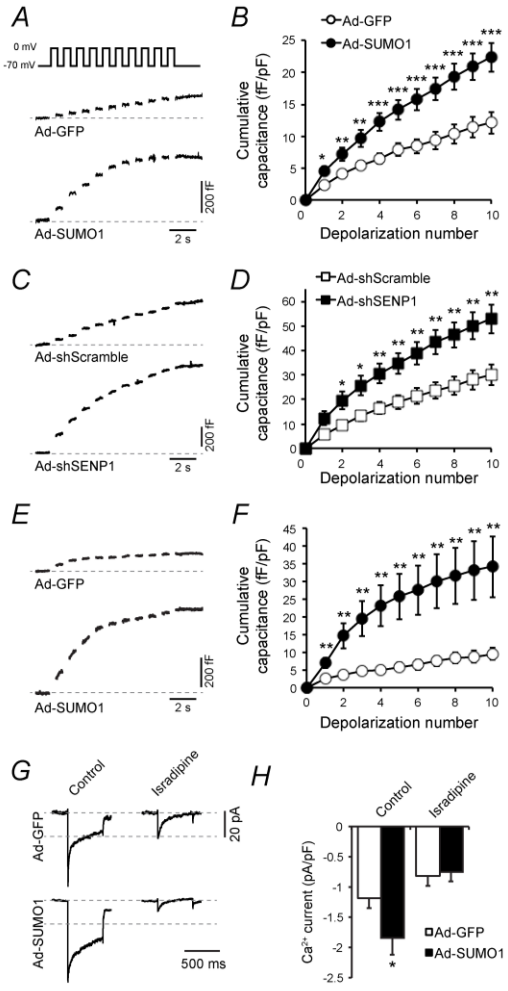

Figure 3. *SUMOylation shifts the dependence of exocytosis from non-L-type to L-type VDCCs.* We measured exocytosis as increases in membrane capacitance in mouse α-cells, identified by glucagon immunostaining, in response to membrane depolarization. **(*A*)** Representative capacitance responses of α-cells infected with Ad-GFP or Ad-SUMO1 under control conditions (black traces) or in the presence of 100 nM ω-conotoxin (blue traces). **(*B*)** Representative capacitance responses of α-cells infected with Ad-GFP or Ad-SUMO1 under control conditions (black traces) or in the presence of 5 μM isradipine (red traces). **(*C*)** The cumulative capacitance response from α-cells expressing Ad-GFP in the absence (open circles) and presence (open squares) of 5 μM isradipine. **(*D*)** The cumulative capacitance response from α-cells expressing Ad-SUMO1 in the absence (black circles) and presence (black squares) of 5 μM isradipine. **(*E*)** Representative capacitance traces in response to a single depolarization of α-cells infected with Ad-Scrambled or Ad-shSENP1 in the absence of VDCC inhibitor (black traces), or in the presence of 100 nM ω-conotoxin (blue traces) or 5 μM isradipine (red traces). **(*F*)** Average capacitance responses to the single depolarization in cells infected with Ad-Scrambled (open bars) or Ad-shSENP1 (black bars) in the absence or presence of 100 nM ω-conotoxin and 5 μM isradipine. **(*G*)** Continuous recording of membrane capacitance during infusion of 200 nM free Ca^{2+} following establishment of whole-cell access (arrows). **(*H*)** The percentage increase in membrane capacitance (C_m) over 200 seconds. *P<0.05, **P<0.01, and ***P<0.001 compared with the respective control, or as indicated.

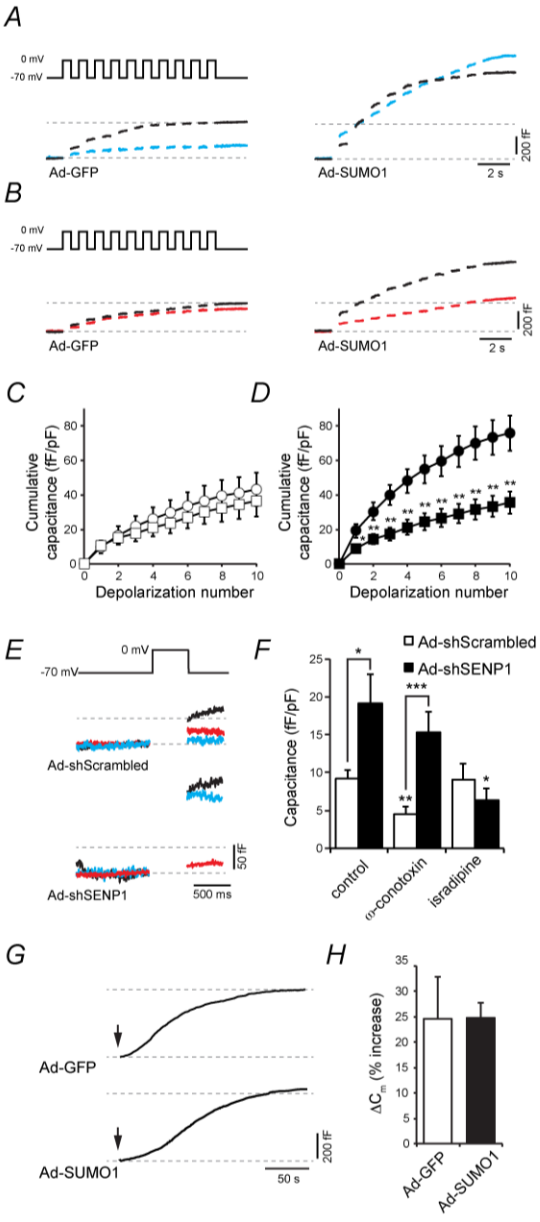

Figure 4. *SUMO1 prevents GLP-1 receptor-dependent inhibition of α-cell Na^+ currents.* We measured voltage-dependent Na^+ currents in mouse α-cells, positively identified by glucagon immunostaining, in the whole-cell configuration. **(*A*)** Representative responses to a series of membrane depolarizations from a holding potential of -70 mV in α-cells infected with Ad-GFP and exposed to 0 or 10 nM exendin 4 during the experiment, and in α-cells infected with Ad-SUMO1 then treated with 0 or 10 nM exendin 4. **(*B*)** Quantification of peak Na^+ currents in cells infected with Ad-GFP (open bars) or Ad-SUMO1 (black bars). **(*C*)** Exendin 4 (10 nM) inhibits α-cell Na^+ currents by causing leftward shift of voltage-dependent inactivation (open squares). This is prevented by up-regulation of SUMO1 (black squares). **(*D*)** Half-maximal current inactivation (V_{50}) is shown for cells infected with Ad-GFP (open bars) or Ad-SUMO1 (black bars). *P<0.01 and ***P<0.001 compared with the control.

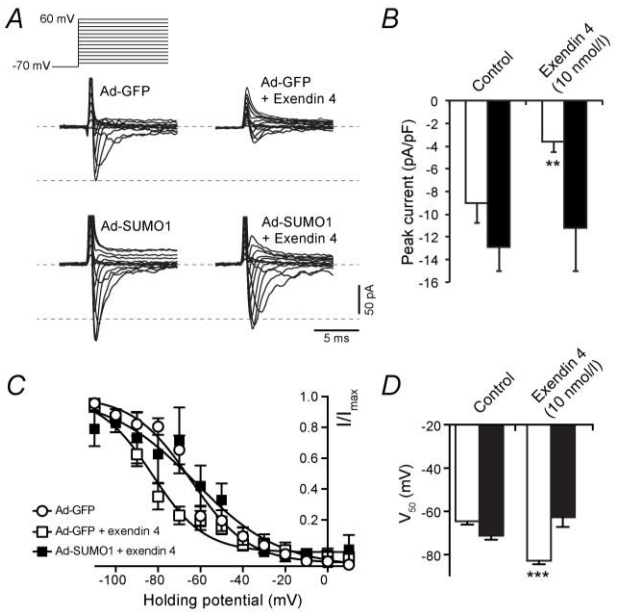

Figure 5. *SUMO1 enhances α-cell exocytosis and prevents GLP-1 receptor-mediated inhibition.* We measured exocytosis as increases in membrane capacitance in mouse α-cells, positively identified by glucagon immunostaining, in response to membrane depolarization. **(*A*)** Representative capacitance responses of mouse α-cells infected with Ad-GFP (black traces) or Ad-SUMO1 (red traces). **(*B*)** The cumulative capacitance responses from mouse α-cells expressing Ad-GFP (open bars) and Ad-SUMO1 (black bars). Treatment with exendin 4 in the bath solution suppressed the exocytotic response, regardless of the presence of 0.1 or 1 mM cAMP in the patch-pipette, but could not suppress the elevated responses observed in α-cells infected with Ad-SUMO1. *P<0.05, **P<0.01 and ***P<0.001 compared with the respective control, or as indicated.

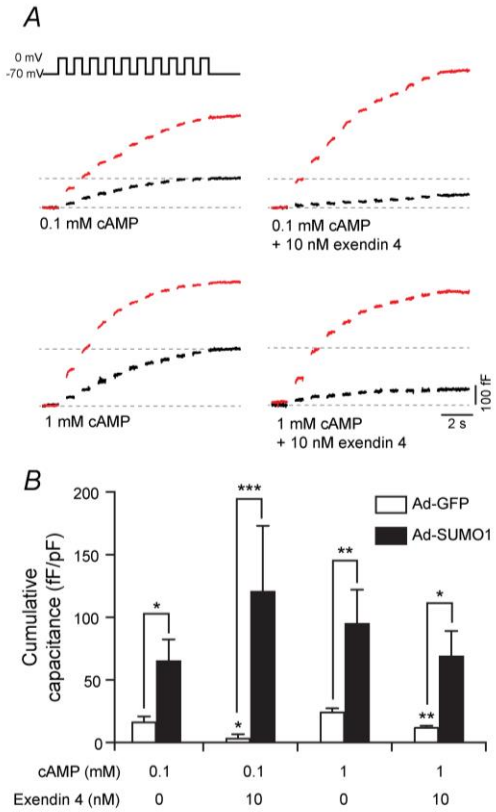

Figure 6. *SUMO1 increases α-cell exocytosis in a cAMP-dependent manner.* We measured exocytosis as increases in membrane capacitance in mouse α-cells, identified by glucagon immunostaining, in response to membrane depolarization in the whole-cell configuration. **(*A*)** Representative capacitance responses of α-cells infected with Ad-GFP (black traces) or Ad-SUMO1 (red traces) without cAMP included in the patch-pipette and following treatment with adrenaline or forskolin as indicated. **(*B*)** The cumulative capacitance response of α-cells infected with Ad-GFP (open bars) or Ad-SUMO1 (black bars). Ad-SUMO1 was unable to increase α-cell exocytosis in the absence of cAMP, but this response was restored following treatment with forskolin or adrenaline. *P<0.05, **P<0.01, and ***P<0.001 compared with the respective control, or as indicated.

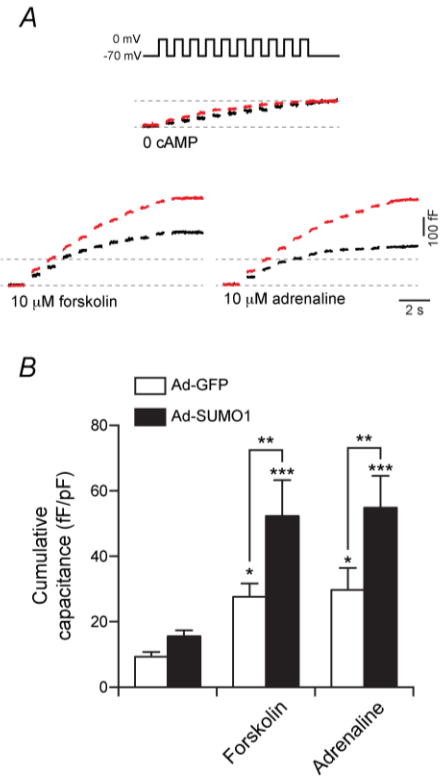

Figure 7. *SUMO1 increases adrenaline-stimulated glucagon secretion from intact islets.* We measured glucagon secretion from mouse islets infected with Ad-GFP (open circles/bars) or Ad-SUMO1 (black circles/bars) during perifusion. **(*A*)** Glucagon secretion in response 1 mM glucose followed by 20 mM KCl. Area under the curve (AUC) for the various conditions are shown at right. **(*B*)** Glucagon secretion in response 1 mM glucose followed by 10 nM exendin 4. AUC for the various conditions are shown at right. **(*C*)** Glucagon secretion in response 1 mM glucose followed by 10 μM adrenaline. AUC for the various conditions are shown at right. *P<0.05, **P<0.01, and ***P<0.001 compared with the Ad-GFP control.

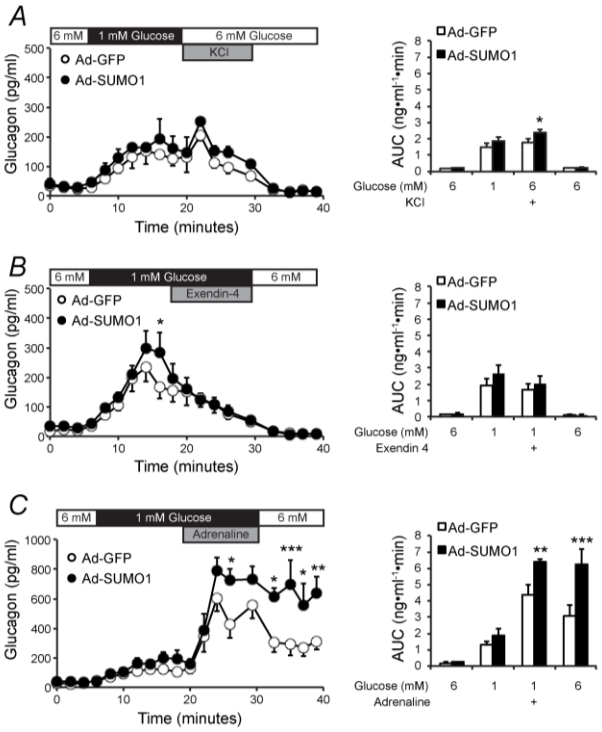

Figure 8. *Schematic hypothesis for the interaction between SUMOylation and cAMP on glucagon secretion.* ***(A)*** In the absence of elevated cAMP or SUMO1, glucagon exocytosis is triggered by Ca^{2+} entry through non-L-type Ca^{2+} channels. ***(B)*** In response to elevated cAMP, such as that stimulated by adrenaline, glucagon granules become more coupled to L-type channels. However, the majority of glucagon exocytosis remains mediated by the non-L-type Ca^{2+} channels. ***(C)*** Increased SUMOylation enhances L-type Ca^{2+} channel currents in α-cells, but in the absence of the increased coupling of glucagon granules to these channels conferred by elevated cAMP, this does not result in increased glucagon exocytosis. ***(D)*** Only when cAMP is elevated, and the coupling of glucagon granules to the L-type Ca^{2+} channels is increased, does SUMOylation increase glucagon exocytosis as a result of increased L-type Ca^{2+} current.