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Abstract
Synaptic vesicle protein 2 (SV2) is expressed in neuroendocrine cells as three homologous isoforms, SV2A, SV2B and SV2C. Ca2+-dependent function in exocytosis has been attributed to SV2A and SV2B, without elucidation of the mechanism. The role of SV2C has not yet been addressed. Here we characterize the three SV2 isoforms and define their involvement in regulated insulin secretion. SV2A and SV2C are associated with insulin-containing granules and synaptic-like-microvesicles (SLM) in INS-1E insulinoma and primary beta-cells, whereas SV2B is only present on SLM. Neither overexpression nor isoform-specific silencing of SV2A or SV2C by RNA interference modifies depolarization-triggered cytosolic [Ca2+] rises or secretory granule [Ca2+], measured with a VAMP-2 aequorin chimera. This strongly argues against any Ca2+ transport function of SV2. Moreover, up- or downregulation of these isoforms has no influence on K+-induced insulin release suggesting that SV2 does not affect the Ca2+-dependent step(s) of exocytosis. By contrast, glucose-elicited secretion is inhibited during the sustained rather than the early phase, placing the [...]
SV2A and SV2C are not vesicular Ca\(^{2+}\) transporters but control glucose-evoked granule recruitment

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Summary

Synaptic vesicle protein 2 (SV2) is expressed in neuroendocrine cells as three homologous isoforms, SV2A, SV2B and SV2C. Ca\(^{2+}\)-dependent function in exocytosis has been attributed to SV2A and SV2B, without elucidation of the mechanism. The role of SV2C has not yet been addressed. Here we characterize the three SV2 isoforms and define their involvement in regulated insulin secretion. SV2A and SV2C are associated with insulin-containing granules and synaptic-like-microvesicles (SLM) in INS-1E insulinoma and primary β-cells, whereas SV2B is only present on SLM. Neither overexpression nor isoform-specific silencing of SV2A or SV2C by RNA interference modifies depolarization-triggered cytosolic [Ca\(^{2+}\)] rises or secretory granule [Ca\(^{2+}\)], measured with a VAMP-2 aequorin chimera. This strongly argues against any Ca\(^{2+}\) transport function of SV2. Moreover, up- or downregulation of these isoforms has no influence on K\(^+-\)-induced insulin release suggesting that SV2 does not affect the Ca\(^{2+}\)-dependent step(s) of exocytosis. By contrast, glucose-elicited secretion is inhibited during the sustained rather than the early phase, placing the action of SV2 on the recruitment of granules from the reserve pool to the plasma membrane. This conclusion is reinforced by capacitance measurements in glucose-stimulated SV2C-deficient cells. Like capacitance, evoked and basal hormone release are attenuated more by silencing of SV2C compared with SV2A. This indicates only partial redundancy and highlights a key role for SV2C in the secretory process.

Introduction

Pancreatic hormones released by the islets of Langerhans play a pivotal role in the regulation of nutrient homeostasis. Defective insulin secretion from the β-cells leads to diabetes mellitus (Wollheim and Maechler, 2002). The molecular details of β-cell exocytosis, which resembles neuroexocytosis, remain to be fully clarified. However, during the last few years several key components of the machinery permitting the targeting of secretory vesicles to the plasma membrane have been identified. In fact, SNARE proteins, the Ca\(^{2+}\) sensors synaptotagmins and Rab GTPases with their interacting partners, have been demonstrated to play a role in insulin release (Burgoyne and Morgan, 2003; Rorsman and Renstrom, 2003).

Synaptic vesicle protein 2 (SV2) is a highly glycosylated protein also involved in exocytosis, which is present in neurons and on secretory vesicles in endocrine cells including human islets and HIT-T15 insulin-secreting cells (Buckley and Kelly, 1985; Portela-Gomes et al., 2000). SV2 contains 12 potential transmembrane region homologous to bacterial and eukaryotic transporters with cytoplasmic N- and C-terminals (Bajjalieh et al., 1992; Feany et al., 1992). However, transport substrates of SV2 remain to be identified.

Vertebrates have three distinct genes encoding highly homologous proteins referred to as SV2A, -B and -C isoforms (Bajjalieh et al., 1992; Bajjalieh et al., 1993; Feany et al., 1992). SV2A and SV2C are more similar to each other than to SV2B (Janz et al., 1998). SV2A is expressed in all types of neurons; SV2B is nearly as prevalent, whereas SV2C is only present in a small subset (Bajjalieh et al., 1993; Janz and Sudhof, 1999). The three SV2 isoforms are also differentially distributed in the mouse retina (Wang et al., 2003). Regarding the neuroendocrine cells, SV2A is detected in the cell line PC12 whereas SV2B is associated with microvesicles of rat pinealocytes (Bajjalieh et al., 1994; Hayashi et al., 1998). In adrenal chromaffin cells, SV2A is mainly localized to granules whereas SV2C is enriched on microsomes (Janz and Sudhof, 1999). To date, the expression pattern and the intracellular distribution of the three SV2 isoforms in insulin-secreting cells have not been investigated.

Different hypotheses could explain SV2 function (Brose and Rosenmund, 1999). The glycosylated intravesicular domains of SV2 may trap soluble neurotransmitters to diminish the intravesicular osmotic pressure (Alvarez et al., 1993). Alternatively, SV2 could modulate exocytosis by interacting with synaptotagmin I (Schivell et al., 1996; Lazzell et al., 2004) or act as a scaffold protein that regulates vesicle shape (Janz et al., 1998).
A further study reported that SV2A and SV2A/SV2B double-knockout mice suffer from strong epileptic seizures and die postnatally, whereas mice lacking SV2B show no phenotype (Janz et al., 1999). The synaptic transmission was increased in double mutant hippocampal neurons and the effect was reversed by cytosolic Ca\(^{2+}\) buffering suggesting that presynaptic terminals accumulate abnormally high Ca\(^{2+}\) levels. Therefore, SV2A and -B may act by binding Ca\(^{2+}\) or, more likely, by pumping excess presynaptic Ca\(^{2+}\) into vesicles.

The epileptic seizures and the lethality of SV2A-deficient mice were reproduced in another investigation (Crowder et al., 1999). Nonetheless, a decrease in synaptic transmission was observed in hippocampal slices, indicating that SV2A positively regulates Ca\(^{2+}\)-triggered exocytosis perhaps by binding to synaptotagmin I. More recently, the same group analyzed chromaffin cells from SV2A and SV2A/SV2B double-knockout mice (Xu and Bajjalieh, 2001). In these cells, the Ca\(^{2+}\)-induced exocytotic burst, which defines the ready releasable pool (RPP) of secretory vesicles, was diminished. Moreover, loss of SV2A was associated with fewer SDS-resistant SNARE complexes in the brain. These findings indicate that SV2A controls the number of vesicles competent for fusion.

In this study, we investigate the presence and subcellular localization of the three SV2 isoforms in native β-cells and in the derived cell line INS-1E. We also examine the functional impact of SV2A and SV2C on Ca\(^{2+}\) levels and insulin exocytosis, comparing their overexpression to silencing by RNAi.

Materials and Methods

Materials

Rat SV2A, SV2B cDNAs and the monoclonal antibody against SV2A were a kind gift from S. Bajjalieh (University of Washington, Seattle, WA). Rat SV2C cDNA and rabbit polyclonal antibodies against SV2A, SV2B and SV2C were prepared as described previously (Janz et al., 1999). Recombinant adenovirus expressing cytosolic aequorin under the chicken actin promoter (AdCaActAq) was a kind gift from H. Ishihara (Tohoku University, Sendai, Japan). Chimeric cDNA encoding hemagglutinin-tagged Eaequorin fused to V AMP.Aq, generated as previously described (Mitchell et al., 2001), was supplied by P. Pinton (University of Ferrara, Italy). The monoclonal antibody against synaptotagmin IX was from BD Biosciences (Basel, Switzerland). Polyclonal antibodies against SV2A and SV2B were from Stressgen (Victoria, Canada). A rabbit polyclonal antibody directed against carboxypeptidase H was kindly provided by J. Parkinson (Sheffield Hallam University, Sheffield, UK). The polyclonal antibody against glucagon, somatostatin and pancreatic polypeptide were supplied by P. Herrera (University of Geneva, Switzerland).

Cell culture and transfection

Syrian baby hamster kidney (BHK) cells were cultured in RPMI 1640 medium with 10% FCS. The INS-1E clone from the insulin-secreting cell line INS-1 was cultured in RPMI 1640 medium and other additions as described (Merglen et al., 2004). Transient transfection of BHK and INS-1E cells was performed using the Lipofectamine 2000 reagent (Invitrogen, Groningen, Switzerland), according to the manufacturer’s instructions. In all the experiments, the DNA to Lipofectamine ratio was 1:0.5.

Rat pancreatic islets

Rat pancreatic islets were obtained by collagenase digestion and purified on a Ficoll gradient as described (Pralong et al., 1990). Islets were then homogenized and used for western blotting experiments as described below. Alternatively, the islets were dissociated by trypsin treatment, seeded on poly-ornithine-coated glass coverslips and maintained in culture 48 hours prior to the immunofluorescence experiments. In addition, islet cells were separated into two populations, non-β-cells and β-cells, by FACS and the latter were used for RT-PCR studies.

Homogenate preparation and immunoblot analysis

INS-1E cells, pancreatic islets and brain crude membranes were prepared as described (Iezzi et al., 2004). The homogenates were resolved by SDS-PAGE and transferred onto PVDF membrane. Immunoreactive bands were revealed by enhanced chemiluminescence (Pierce, Lausanne, Switzerland) using horseradish-peroxidase-coupled secondary antibodies.

Immunocytochemistry

INS-1E and primary pancreatic endocrine cells were fixed for 10 minutes in 4% paraformaldehyde in PBS and permeabilized for 1 hour in PBS containing 0.1% saponin and 0.5% BSA. The cells were incubated with primary antibodies overnight at 4°C and then exposed to FITC- or Rhodamine-conjugated secondary antibodies for 1 hour at room temperature. Samples were analyzed using a Zeiss laser confocal microscope (LSM 510, Zurich, Switzerland). Images were taken with a 60× objective.

Sucrose density gradient

Insulin-containing granules were separated as described (Iezzi et al., 1999). Briefly, INS-1E cells were homogenized in 5 mM HEPES pH 7.4, 1 mM EGTA, 10 μg/ml leupeptin and 2 μg/ml aprotinin. Cell debris and nuclei were eliminated by centrifuging the homogenate for 10 minutes at 3000 g. The supernatant was loaded on a continuous sucrose density gradient (0.45-2.0 M) and centrifuged for 18 hours at 110,000 g. The fractions were collected from the top of the gradient. The amount of insulin was monitored by radioimmunoassay (RIA).

Reverse-transcription and PCR amplification of SV2 cDNAs

Total RNA was extracted from rat brain, rat islet β-cells purified by FACs and INS-1E cells by the use of Trizol™ Reagent (Gibco Life Sciences, Basel, Switzerland). cDNA was synthesized for 45 minutes at 48°C followed by 2 minutes at 94°C using AMV reverse transcriptase (Promega Catalys, Walisellen, Switzerland). The primers used for reverse transcription and PCR amplification corresponded to nucleotides 675-697 of SV2A, 605-627 of SV2B and 516-536 of SV2C cDNA sequences. The PCR program involved 40 cycles of denaturation at 94°C for 30 seconds annealing at 60°C for 1 minute and elongation at 68°C for 2 minutes. PCR reactions were performed using Tfl DNA polymerase (Promega).

Adenovirus construction and infection

SV2A, SV2C and VAMP.Aq cDNAs were used for the adenovirus constructs. Recombinant adenoviruses encoding SV2A (AdCASV2A), SV2C (AdCASV2C) and the VAMP.Aq (AdCAVAMP.Aq) under the chicken actin promoter, were generated as previously described (Miyake et al., 1996). AdCALacZ, which expressed bacterial β-galactosidase, was used as a control adenovirus. INS-1E cells, spheroid clusters of INS-1E cells or isolated islets, were infected with adenoviruses for 90 minutes and further cultured for 24 hours (AdCASV2A, AdCASV2C and AdCAActAq) or 72 hours (AdCAVAMP.Aq) before experiments. INS-1E cells were infected with AdCASV2A and AdCASV2C at ~50 virus particles/cell, with AdCAActAq at ~20 virus particles/cell and with AdCAVAMP.Aq at ~10
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virus particles/cell; spheroids at $2 \times 10^5$ virus particles/spheroid (~50 virus particles/cell, assuming 4000 cells/spheroid) (Merglen et al., 2004) and islets at $3 \times 10^5$ virus particles/islet (~100 virus particles/cell, assuming 3000 cells/islet).

[Ca^{2+}] measurement with the expressed Ca^{2+}-sensitive photoprotein aequorin

For [Ca^{2+}] measurement, INS-1E cells were infected with AdCaAcAq adenovirus and cytosolic aequorin was reconstituted with 2.5 μM coelenterazine in INS-1E cell culture medium for ~2 hours. For [Ca^{2+}]SG measurement, INS-1E cells were infected with AdCA V AMP.Aq adenovirus. Cells were then Ca^{2+} depleted by incubation with 10 μM ionomycin, 10 μM monensin and 10 μM of the SERCA inhibitor cyclopiazonic acid (CPA), in Ca^{2+}-free Krebs-Ringer-Bicarbonate buffer (KRB; 135 mM NaCl, 3.6 mM KCl, 2 mM NaHCO3, 0.5 mM NaH 2PO4, 0.5 mM MgSO 4, 2.5 mM glucose and 10 mM HEPES, pH 7.4) supplemented with 1 mM EGTA for 5 minutes at 4°C. ER.aequorin was reconstituted in 0.1 mM EGTA, 5 μM coelenterazine-n for ~3 hours at 4°C.

For both [Ca^{2+}]c and [Ca^{2+}]SG measurement, the coverslips were placed in a chamber with a thermostat 5 mm from the photomultiplier apparatus (model EMI 9789; Thorn EMI, Hayes, UK) set at 37°C. Cells were perfused at a rate of 1 ml/minute in the appropriate buffer and permeabilized with 0.1 mM digitonin, 10 mM CaCl2 for calibration. Emitted light was collected every second with a photon-counting board (EMI 660). Insulin secretion was measured in the effluent collected every 20 seconds.

Insulin secretion

Attached cells

Prior to the experiment, infected INS-1E cells were maintained at 37°C for 2 hours in glucose-free culture medium. The cells were preincubated for 30 minutes in KRB buffer (135 mM NaCl, 3.6 mM KCl, 2 mM NaHCO3, 0.5 mM NaH 2PO4, 0.5 mM MgSO 4, 1.5 mM CaCl2, 10 mM HEPES, pH 7.4 and 0.1% BSA) containing 2.5 mM glucose and then incubated for 15 or 30 minutes in KRB supplemented with stimulators as indicated. Infected islets (10 islets/tube) were preincubated for 60 minutes in KRB containing 2.8 mM glucose and further incubated over a period of 15 or 30 minutes in KRB containing stimulators as specified.

Perifused cells

Spheroid clusters composed of INS-1E cells were prepared as described (Merglen et al., 2004). Briefly, ~500 infected spheroids were distributed per chamber and maintained at 37°C (Brandel, Gaithersburg, MD). The flow rate was set at 0.5 ml/minute and fractions were collected every minute after a 20 minute washing period at basal glucose concentration. Insulin contents were determined from acid-ethanol extraction. Insulin was measured by RIA.

Preparation of vectors for SVA and SV2C silencing

Mammalian expression vectors directing the synthesis of siRNAs targeted against SV2A and SV2C were prepared according to manufacturer’s guidelines (Ambion, Huntingdon, UK). cDNA fragments encoding a 19-nucleotide sequence and separated from its reverse 19-nucleotide complement by a short spacer were synthesized by Microsynth (Balgach, Switzerland). The SV2A silencers were generated using the sequences corresponding to nucleotides 128-150 (siRNA-1), 226-248 (siRNA-2) and 326-348 (siRNA-3) of rat SV2A cDNA. The SV2C silencers were constructed using the nucleotide sequences 301-321 (siRNA-1), 336-356 (siRNA-2) and 1705-1725 (siRNA-3) of Fig. 1.

Detection of SV2 isoforms in pancreatic endocrine cells. (A) PCR amplification of SV2 cDNAs from rat brain, INS-1E cells and rat islet β-cells purified by FACS. Control PCR reactions with no reverse transcriptase were performed for each isoform (–). The DNA size markers (bp) are also indicated. (B) Homogenates of rat brain (10 μg), rat islets and INS-1E cells (100 μg each) were separated by SDS-PAGE and subjected to immunoblotting with the monoclonal antibody against SV2 and with rabbit polyclonal antibodies against SV2A, SV2B or SV2C. Positions of molecular size markers are indicated in kDa. (C) Pancreatic endocrine cells were analyzed by confocal microscopy after double immunofluorescence with an antibody against glucagon (a), somatostatin (d) or pancreatic polypeptide (g) (revealed using FITC-coupled antibody) and with the anti-SV2 antibody (b, e and h) (detected using Rhodamine-coupled antibody). The merged images (c, f and i) were obtained after superposition of the green and red channels. Bars, 5 μm.
rat SV2C cDNA. The cDNA fragments were annealed and cloned in front of the U6-RNA promoter in the pSilencer™ 1.0-U6 siRNA Expression Vector (Ambion). The specificity of each sequence was verified by basic local alignment search tool (BLAST) search against the GenBank database.

hGH secretion
INS-1E cells were co-transfected with a plasmid encoding human growth hormone (hGH), and with plasmids containing the silencers. Four days later, the cells were maintained at 37°C for 2 hours in glucose-free culture medium before preincubation for 30 minutes in KRB buffer containing 2.5 mM glucose. The cells were then incubated for the indicated periods under basal condition or in the presence of stimulatory concentrations of glucose and KCl. Exocytosis from transfected cells was assessed by measuring the amount of hGH released into the medium by ELISA (Roche, Manheim, Germany).

Electrophysiology
INS-1E cells seeded on coverslips were co-transfected with GFP and the silencers. For patch-clamp capacitance recording, the coverslip was transferred to a temperature-controlled chamber and perfused with extracellular solution containing 120 mM NaCl, 20 mM tetraethyl ammonium, 3.6 mM KCl, 2.6 mM CaCl2, 2 mM NaHCO3, 0.5 mM NaH2PO4, 0.5 mM MgSO4, 15 mM glucose, 100 μM IBMX and 1 μM forskolin (pH 7.4 with NaOH). The pipette internal solution contained 125 mM CsCl, 10 mM sodium glutamate, 5 mM HEPES, 3 mM Mg-ATP, 1 mM MgCl2, 0.2 mM EGTA, 0.1 mM cAMP (pH 7.10 with CsOH). Capacitance measurements (EPC 9 amplifier; HEKA, Darmstadt, Germany) were performed after applying 1 kHz, 28 mV peak, to sinusoid stimulus from a DC holding potential of –70 mV (Olofsson et al., 2002). The sine+DC mode of the software lock-in extension of the PULSE software was used to calculate membrane capacitance (Cm), membrane conductance (Gm) and access resistance (Ra) from the current recordings.

Statistical analysis
Results are presented as mean±s.e. Differences between groups were analyzed by the Student’s t-test for unpaired data.

Results
Expression of SV2A, SV2B and SV2C in pancreatic endocrine cells
We first determined the presence of SV2A, -B and -C mRNAs in the insulin-secreting cells INS-1E, FACS-purified rat islet β-cells and rat brain. The latter was used as a positive control. RT-PCR with primers corresponding to the N-terminal region of SV2 isoforms, yielded products of the predicted size (SV2A, 190 bp; SV2B, 137 bp; SV2C, 144 bp) (Fig. 1A). Sequencing of the amplicons confirmed that INS-1E and β-cells express all three SV2 isoforms, SV2B being less abundant compared with expression in the brain.

Subsequently, we analyzed the expression of SV2 proteins. Homogenates of INS-1E cells, pancreatic islets and brain were resolved by SDS-PAGE and the proteins were detected with monospecific polyclonal antibodies directed against SV2A, -B or -C. Western blotting revealed that SV2 isoforms migrate at ~90-100 kDa and are present in all three homogenates (Fig. 1B). Similar results were obtained using commercial SV2A and -B antibodies (not shown). Each of the SV2 isoforms was less abundant in pancreatic islets compared with INS-1E cells. This observation was confirmed by immunoblotting performed with the general SV2 monoclonal antibody, recognizing all three isoforms (Fig. 1B). Note that SV2 proteins migrate as a heterogeneous set of bands reflecting their high degree of glycosylation (Buckley and Kelly, 1985).

To further examine the expression pattern of SV2, cultured primary pancreatic endocrine cells were immunostained with
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The monoclonal SV2 antibody and analyzed by confocal microscopy. SV2 is present in the glucagon, somatostatin and pancreatic polypeptide cells (Fig. 1C).

Intracellular distribution of SV2 isoforms in insulin-secreting cells

The subcellular localization of SV2 isoforms was determined by additional immunofluorescence in INS-1E and primary β-cells. Both cells possess two types of vesicle: insulin-containing secretory granules and GABA-containing synaptic-like microvesicles (SLM) (Reetz et al., 1991). Confocal microscopy analysis demonstrates that SV2A and SV2C mainly colocalize with secretory granules (Fig. 2); these isoforms are also associated with SLM (Fig. 3), which typically distributed to the perinuclear region (Reetz et al., 1991).

Neither of the tested anti-SV2B antibodies was appropriate for immunofluorescence experiments.

To further characterize the intracellular distribution of SV2, the two vesicle populations in INS-1E cells were separated on a continuous sucrose density gradient (Iezzi et al., 1999). Insulin granules were detected in the fractions 11-17 (Fig. 4, top panel), along with the secretory granule resident protein carboxypeptidase H, whereas synaptophysin, a marker of SLM was concentrated in fractions 7-10 (Fig. 4, lower panels). SV2A and SV2C were mainly found in fractions 7-17 consistent with an association with both insulin granules and SLM. By contrast, SV2B was only detected in fractions enriched in SLM. Western blotting performed with the general SV2 antibody confirmed these results. Remarkably, the lower abundance of SV2B RNA and protein in pancreatic cells compared with brain (see Fig. 1A,B) is in accordance with its unique localization on SLM. Subsequent experiments focused on SV2A and SV2C, the two isoforms being present on insulin granules.

Effect of overexpressed SV2A and SV2C on cytosolic and intravesicular [Ca²⁺]

To test whether SV2 has Ca²⁺-transporter function (Janz et al., 1999), we measured both cytosolic and secretory granule [Ca²⁺] in INS-1E cells overexpressing SV2A or -C. For this purpose, the cells were first infected with recombinant adenoviruses encoding SV2A (AdCASV2A) or SV2C (AdCASV2C) and analyzed by western blotting (supplementary material, Fig. S1A). The amounts of SV2A and -C proteins were increased in a dose-dependent manner and we selected a virus titer which did not provoke cell toxicity. Indeed, immunofluorescence showed that, analogous to endogenous proteins, exogenous SV2A and -C mainly associated with insulin granules (supplementary material, Fig. S1B); these isoforms were also detected in the nuclear-near area devoid of insulin, representing SLM (see also Fig. 3).

Next, for measurement of cytosolic [Ca²⁺] ([Ca²⁺]c), INS-1E cells were co-infected with adenoviruses encoding cytosolic aequorin (Merglen et al., 2004) and with either AdCASV2A or AdCASV2C (Fig. 5A). The SV2 isoforms were expressed at similar levels as demonstrated by western blotting performed with the monoclonal SV2 antibody (Fig. 5A, panel a). The cells were then stimulated with 30 mM KCl and [Ca²⁺]c rises were measured simultaneously with insulin release (Fig. 5A, panel b). The depolarizing agent transiently increased both [Ca²⁺]c and hormone secretion, and as expected the peak of insulin was delayed by ~90 seconds (Pralong et al., 1990). Overexpressed SV2A or -C did not significantly change either [Ca²⁺]c (Fig. 5A, panel c) or the insulin response (Fig. 5A, panel b). In addition, increasing the glucose concentration from 2.5 to 15.0
mM also raised [Ca\textsuperscript{2+}]\textsubscript{c}. Again SV2A or -C overexpression were without effect (see Fig. 5A, panel c, for averaged data). Similar results were obtained by monitoring [Ca\textsuperscript{2+}]\textsubscript{c} in rat pancreatic islets (not shown).

To dynamically monitor free Ca\textsuperscript{2+} concentration within secretory granules ([Ca\textsuperscript{2+]\textsubscript{SG}}), we expressed a chimeric cDNA encoding the vesicle-associated membrane protein VAMP-2 fused to aequorin (VAMP.Aq) (Mitchell et al., 2001). Since SLM only constitute 10% of the total secretory vesicle pool in INS-1 cells (MacDonald et al., 2005), this approach mainly measures [Ca\textsuperscript{2+}]\textsubscript{c} in the insulin granule population. Thus, INS-1E cells were co-infected with AdCAVAMP.Aq and with either SV2A or -C encoding adenoviruses (Fig. 5B). Both SV2 isoforms were expressed at comparable levels (Fig. 5B, panel a). To measure Ca\textsuperscript{2+} uptake into secretory granules, Ca\textsuperscript{2+} influx into cells was evoked by 30 mM KCl after Ca\textsuperscript{2+} depletion and reintroduction (Fig. 5B, panel b). KCl augmented [Ca\textsuperscript{2+]\textsubscript{SG}} from ~20 to 30 μM. Overexpressed SV2 proteins were without effect on [Ca\textsuperscript{2+]\textsubscript{SG}} after addition of CaCl\textsubscript{2} or KCl (Fig. 5B, panel c), and on insulin release (not shown).

Effect of SV2A and SV2C overexpression on insulin secretion

To investigate the role of SV2A and -C on exocytosis, we first overexpressed these proteins in INS-1E cells and evaluated their effect on static insulin release (Fig. 6A). As estimated by immunoblotting, both isoforms were expressed at similar amounts. We observed that in control cells, insulin secretion rose 2.4-fold and 5.0-fold in response to stimulatory concentrations of KCl and glucose, respectively. Overexpression of SV2A or -C neither affected basal nor K\textsuperscript{+}-induced exocytosis. By contrast, release triggered by glucose was significantly decreased by 36% for SV2A and 37% for SV2C. The overexpressed proteins did not change the cellular content of insulin (not shown).

Similar overexpression experiments were also performed in rat islets (supplementary material, Fig. S2). Comparable levels of SV2 production were first confirmed by western blotting. In control islets, 30 mM KCl and 16.7 mM glucose elicited insulin release by 1.8-fold and 3.8-fold, respectively. Both basal and KCl-evoked exocytosis remained unchanged after SV2 overexpression. By contrast, when hormone secretion was stimulated by glucose, there was strong inhibition reaching 66% and 69% for SV2A and -C, respectively. The potent attenuation was independent of the degree of overexpression as observed in four separate experiments.

We next assessed the impact of SV2A and -C overexpression on the dynamics of insulin secretion in perifused spheroid clusters of INS-1E cells (Merglen et al., 2004) (Fig. 6B). Immunoblotting revealed that both proteins were expressed at comparable amounts. When glucose was increased from 2.5 to 15.0 mM, there was a sixfold rise in insulin release with a first phase lasting for about 10 minutes, followed by a second sustained phase of 20 minutes. Both basal and first phase were not altered in spheroids expressing SV2A or -C. However, during the sustained phase, the secretory response was diminished by 42% for SV2A [area under the curve (AUC)=13.1±1.19 versus control value of 22.4±1.65] and by 46% for SV2C (6.4±0.87 versus 11.9±1.19). There was thus good agreement with the static incubations, as the averaged entire 30 minute release was reduced by 35% for SV2A (19.6±1.74 versus 29.9±2.51) and by 36% for SV2C (11.3±1.35 versus 17.7±1.79).

Selective decrease of SV2A and SV2C expression by RNAi

To further define their actions, we selectively reduced the endogenous level of SV2A and -C proteins by RNA interference (RNAi) (Fig. 7). Here we generated six plasmids capable of inducing the synthesis of small interfering RNAs (siRNAs) directed against the sequences of SV2A and SV2C. These siRNAs were first tested in BHK cells devoid of SV2. Western blotting on exogenous SV2A and -C confirm the highly specific antibodies (Fig. 7A). SV2A siRNA-2, -3 and SV2C siRNA-2, completely abolish the expression of the proteins whereas the other constructs strongly reduce it (Fig. 7B). Similar results were obtained after immunofluorescence studies performed on transfected BHK cells (not shown). Thus, for the following experiments we chose the SV2A siRNA-3 and SV2C siRNA-2 sequences.

To determine the silencing effect on the endogenous levels of SV2A and -C proteins, we infected INS-1E cells with a lentiviral vector encoding either SV2A or -C siRNA sequences (Merglen et al., 2004) (Fig. 7C). As estimated by immunoblotting, both isoforms were expressed at comparable amounts. When glucose was increased from 2.5 to 15.0 mM, there was a sixfold rise in insulin release with a first phase lasting for about 10 minutes, followed by a second sustained phase of 20 minutes. Both basal and first phase were not altered in spheroids expressing SV2A or -C. However, during the sustained phase, the secretory response was diminished by 42% for SV2A [area under the curve (AUC)=13.1±1.19 versus control value of 22.4±1.65] and by 46% for SV2C (6.4±0.87 versus 11.9±1.19). There was thus good agreement with the static incubations, as the averaged entire 30 minute release was reduced by 35% for SV2A (19.6±1.74 versus 29.9±2.51) and by 36% for SV2C (11.3±1.35 versus 17.7±1.79).

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To further define their actions, we selectively reduced the endogenous level of SV2A and -C proteins by RNA interference (RNAi) (Fig. 7). Here we generated six plasmids capable of inducing the synthesis of small interfering RNAs (siRNAs) directed against the sequences of SV2A and SV2C. These siRNAs were first tested in BHK cells devoid of SV2. Western blotting on exogenous SV2A and -C confirm the highly specific antibodies (Fig. 7A). SV2A siRNA-2, -3 and SV2C siRNA-2, completely abolish the expression of the proteins whereas the other constructs strongly reduce it (Fig. 7B). Similar results were obtained after immunofluorescence studies performed on transfected BHK cells (not shown). Thus, for the following experiments we chose the SV2A siRNA-3 and SV2C siRNA-2 sequences.

To determine the silencing effect on the endogenous levels of SV2A and -C proteins, we infected INS-1E cells with a lentiviral vector encoding either SV2A or -C siRNA sequences (Merglen et al., 2004) (Fig. 7C). As estimated by immunoblotting, both isoforms were expressed at comparable amounts. When glucose was increased from 2.5 to 15.0 mM, there was a sixfold rise in insulin release with a first phase lasting for about 10 minutes, followed by a second sustained phase of 20 minutes. Both basal and first phase were not altered in spheroids expressing SV2A or -C. However, during the sustained phase, the secretory response was diminished by 42% for SV2A [area under the curve (AUC)=13.1±1.19 versus control value of 22.4±1.65] and by 46% for SV2C (6.4±0.87 versus 11.9±1.19). There was thus good agreement with the static incubations, as the averaged entire 30 minute release was reduced by 35% for SV2A (19.6±1.74 versus 29.9±2.51) and by 36% for SV2C (11.3±1.35 versus 17.7±1.79).
proteins, INS-1E cells were co-transfected with the selected siRNAs and GFP. Homogenates of FACS-enriched GFP-expressing cells were analyzed by western blotting (Fig. 7C). SV2A siRNA-3 potently diminished the amount of SV2A (Fig. 7C, panel a) but did not affect SV2C (panel b). Similarly, SV2C siRNA-2 drastically decreased SV2C (panel c) without changing the level of SV2A (panel d). The effect of the chosen siRNAs was specific because the expression of synaptotagmin IX or other endogenous proteins (arrows) was not modified. Since we required suppression of both isoforms for subsequent secretion experiments, we demonstrated that combination of SV2A siRNA-3 and SV2C siRNA-2 strongly reduced the corresponding proteins (Fig. 7D). Neither the overall distribution of insulin granules nor the organization of the cortical actin cytoskeleton revealed by phalloidin staining was influenced by silencing of SV2 isoforms (not shown).

Effect of SV2A and SV2C suppression on \([\text{Ca}^{2+}]_c\) and \([\text{Ca}^{2+}]_{SG}\)

We next examined whether \([\text{Ca}^{2+}]_c\) and \([\text{Ca}^{2+}]_{SG}\) were altered in cells devoid of SV2A or -C (Fig. 8). For this purpose, INS-1E cells coexpressing GFP and the selected silencers were infected with AdCacAq (Fig. 8A) or AdCAVAMP.Aq (Fig. 8B) and subsequently separated by FACS. Perfusion of the cells with 30 mM KCl transiently raised \([\text{Ca}^{2+}]_c\) (Fig. 8A, panel i). This response remained unchanged after suppression of SV2A or SV2C (see Fig. 8A, panel ii, for averaged data). Regarding \(\text{Ca}^{2+}\) uptake into secretory granules, the depolarizing agent increased \([\text{Ca}^{2+}]_{SG}\) from ~20 to 30 \(\mu M\) (Fig. 8B, panel i). SV2 depletion did not modify \([\text{Ca}^{2+}]_{SG}\) after addition of CaCl\(_2\) or KCl to the perfusion medium (Fig. 8B, panel ii).

Impact of SV2A and SV2C silencing on hormone secretion and membrane capacitance

To study the effect of SV2A and -C silencing on INS-1E exocytosis, the siRNAs constructs were co-transfected with a plasmid encoding human growth hormone (hGH) (Fig. 9). Exogenous hGH is targeted to...
secretory granules and can be used to monitor secretion as an insulin substitute in the subpopulation of transfected cells (Iezzi et al., 2000). Under control conditions, hGH release increased 2.9-fold after KCl (Fig. 9A) and 7.3-fold after glucose stimulation (Fig. 9B). Loss of SV2C and SV2A/SV2C inhibited by 69% and 61% respectively basal secretion within 15 minutes (Fig. 9A), and by 51% and 50% within 30 minutes (Fig. 9B). By contrast, repression of SV2A had no effect. KCl-induced exocytosis was also unchanged in SV2A or SV2C deficient cells. However, the glucose-triggered release was diminished by 32%, 71% and 69% in the absence of SV2A, SV2C and SV2A/SV2C respectively. Remarkably, for SV2C and SV2A/SV2C the secretion was reduced to the level attained with KCl (only evoking early-phase release), suggesting preferential reduction of the sustained phase.

To analyze whether the two phases of insulin exocytosis were affected by the reduction of a given SV2 isoform, we measured hGH secreted during the first 10 minutes (early phase) and between 10 and 30 minutes (sustained phase) (Fig. 9C). Glucose elicited a 7.2- and 7.1-fold release during the first 10 and last 20 minutes, respectively. Again, basal secretion was significantly inhibited by loss of SV2C but not of SV2A. Silencing of SV2A and -C decreased the early phase by 25% and 41%, while strongly reducing the sustained phase by 39% and 84%, respectively. Thus, repression of SV2A and -C caused an impairment of both secretory phases, the defect being more marked on the sustained phase. In all these experiments, the silencers did not modify the cellular content of hGH (not shown).

To investigate the granule movement also at high temporal resolution we used patch-clamp capacitance recordings. Thus, we assessed the impact of SV2 depletion on membrane capacitance changes in conditions optimizing the recruitment of granules to the readily releasable pool (RRP) (Fig. 10). Ten trains of depolarizing current pulses were applied in glucose-stimulated SV2A or -C deficient cells (Fig. 10A). Without SV2C, the capacitance was reduced by 77% and 76% after the first and 10th pulse, respectively (see Fig. 10B for averaged data), indicating that the maximal Ca2+ influx was not capable of normalizing the refilling of RRP. However, only a tendency of inhibition was observed in the absence of SV2A. The silencers did not change the Ca2+ current density (Fig. 10C). These data are in agreement with those obtained for glucose-stimulated secretion (Fig. 9C) and further emphasize the role of SV2 in the translocation of granules to the plasma membrane.

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Fig. 6. Effect of SV2A and SV2C overexpression on insulin secretion in INS-1E cells. (A) Western blotting analysis of overexpressed SV2A and -C in INS-1E cells using the antibody against SV2 (top panel). INS-1E cells infected with the control AdCALacZ (LacZ), AdCASV2A (SV2A) or AdCASV2C (SV2C) adenoviruses, were incubated at basal 2.5 mM glucose (glc) and stimulated for 15 minutes with 30 mM KCl, or for 30 minutes with 15 mM glucose. Data are the mean±s.e. of five independent experiments. Asterisks indicate significant differences from levels in the LacZ control at P<0.05. (B) Western blotting of exogenous SV2A and -C in spheroid clusters of INS-1E cells with anti-SV2 antibody (left-hand panel). Spheroids of INS-1E cells infected with LacZ and SV2A, or SV2C encoding adenoviruses, were perifused with 2.5 mM glucose (glc) and stimulated at basal 2.5 mM glucose (glc) and stimulated for 15 minutes with 30 mM KCl, or for 30 minutes with 15 mM glucose. Data are the mean±s.e. of five independent experiments. Asterisks indicate a significant difference from levels in the LacZ control for corresponding time points at P<0.05.
The present work elucidates the role of SV2A, -B and -C in the regulation of insulin secretion. These isoforms are expressed in primary pancreatic endocrine cells and in the cell line INS-1E. In primary chromaffin cells SV2A was mostly recovered on granules, SV2C was enriched in the microsomal fraction containing synaptic-like-microvesicles (SLM), whereas SV2B was absent (Janz and Sudhof, 1999). By contrast, our results associate SV2A and -C with both insulin-containing granules and SLM, in INS-1E and native β-cells. The high sequence homology and the localization of these two isoforms in secretory granules point to their functional importance in hormone secretion. Like in the neuroendocrine pinealocytes (Hayashi et al., 1998), SV2B is only present on SLM in INS-1E cells.

SV2 was suggested to be a vesicular Ca\(^{2+}\) transporter clearing presynaptic [Ca\(^{2+}\)]\(_{\text{c}}\), although direct evidence pointing to Ca\(^{2+}\) handling was lacking (Janz et al., 1999). Here we measure cytosolic [Ca\(^{2+}\)]\(_{\text{c}}\), intravesicular [Ca\(^{2+}\)]\(_{\text{i}}\) and Ca\(^{2+}\) current in INS-1E cells. Neither overexpression nor silencing of SV2A and -C alter any of these parameters, strongly arguing against a role of SV2 in the final step(s) of the Ca\(^{2+}\)-dependent exocytosis. On the other hand, insulin release evoked by the fuel glucose is impaired
in both INS-1E cells and islets overexpressing the two isoforms.

Fuel-stimulated insulin secretion involves recruitment of granules from a reserve pool to the plasma membrane, docking, priming to achieve release competence, and formation of the readily and immediately releasable pools (IRP). Granules in

![Graph](image)

**Fig. 8.** Effect of SV2A and SV2C silencing on cytosolic and vesicular \([Ca^{2+}]\). (A) INS-1E cells were transiently co-transfected with GFP and an empty vector (Control), or with either the SV2A siRNA-3 (SV2A) or the SV2C siRNA-2 (SV2C). Three days later, the cells were infected with AdCAcAq, further cultured for 24 hours and separated by FACS for enrichment of transfected cells. (i) After cytosolic aequorin reconstitution, the cells were perifused with 2.5 mM glucose and stimulated with 30 mM KCl as indicated. (ii) Peak of \([Ca^{2+}]_c\). Data show the means±s.e. of four independent traces. (B) INS-1E cells were co-transfected as above. The day after, the cells were infected with AdCAVAMP.Aq, further cultured for 72 hours and separated by FACS. (i) The cells were perifused as detailed in Fig. 5B, panel b. (ii) Peak of \([Ca^{2+}]_{SG}\). Values represent the means±s.e. of four independent experiments.
the IRP will undergo exocytosis after Ca\textsuperscript{2+} influx (Straub and Sharp, 2002). Insulin release in response to glucose is biphasic with a first phase (early phase) peaking after about 5 minutes and a second phase (sustained phase) beginning after 10 minutes and lasting for hours (Wollheim and Sharp, 1981; Ishihara et al., 2003). The release of RRP granules (<5%) mainly accounts for first-phase secretion, and its end marks the depletion of this pool. The subsequent supply of new granules by mobilization/priming from a reserve pool (>95%) accounts for second-phase secretion (Rorsman and Renstrom, 2003). The latter is also referred to as the amplifying pathway and is observed with glucose but not with K\textsuperscript{+} stimulation (Henquin, 2000).

In perfused INS-1E spheroids overexpressing the SV2 isoforms, the secretory response is inhibited only during the second phase of insulin release. Thus, SV2 is likely to exert its function mainly on the translocation of granules from a reserve pool by interacting with proteins involved in this process. SV2 modulates the SNARE complex formation (Xu and Bajjalieh, 2001) and associates with synaptotagmin (Lazzell et al., 2004; Schivell et al., 1996); the latter in turn, undergoes Ca\textsuperscript{2+}-dependent binding to several components including syntaxin, SNAP-25, calmodulin, Na\textsuperscript{+}\textsuperscript{2+} and Ca\textsuperscript{2+} channels (Fukuda, 2003; Sudhof, 2004). Overexpression of SV2 may cause competition with these synaptotagmin partners thereby altering their regulatory activity. Similarly, overexpression of the t-SNARE-interacting protein complexin I, impaired insulin exocytosis by altering the formation of functional SNARE complexes (Abderrahmani et al., 2004). An excess of SV2 could also perturb secretion by decreasing the availability of synaptotagmin. Therefore, results based on overexpression should be complemented by suppression of the SV2 proteins to further elucidate their function.

We found that specific silencing of SV2A attenuates glucose-induced secretion, which is consistent with the diminished neurotransmitter release in SV2A-knockout mice.
Fig. 10. Effect of SV2A and SV2C depletion on membrane capacitance changes. INS-1E cells were transiently co-transfected with GFP and an empty vector (Control), or with either the SV2A siRNA-3 (siRNA SV2A) or the SV2C siRNA-2 (siRNA SV2C). Four days later, recordings were performed on GFP-expressing cells. (A) Single cell capacitance traces evoked by ten depolarizing pulses to 0 mV from a holding potential of –70 mV (left panels), and the corresponding Ca\(^{2+}\)-current traces of the first depolarization in the pulse train (right panels). The pulse train was started 60 seconds after establishing the whole-cell configuration (bottom). \(C_m\), membrane capacitance; ff, femtoFarad. (B) Capacitance changes normalized to the Ca\(^{2+}\)-current of the first pulse for every cell, after one and ten pulses. Values are the mean±s.e. of 8 to 12 cells. *P<0.05 vs. levels in the control. pA, picoAmpere; pF, picoFarad. (C) Ca\(^{2+}\)-current density measured from the first depolarization in the pulse train. The Ca\(^{2+}\)-current was evaluated 3 milliseconds after the onset of depolarization to avoid contamination of fast Na\(^+\) current.

(Crowder et al., 1999) and demonstrates that SV2 acts as a positive modulator of exocytosis. Likewise, the RRP of vesicles was impaired in SV2A-depleted chromaffin cells, despite increased expression of SV2C, suggesting that these isoforms do not perform entirely redundant functions (Xu and Bajjalieh, 2001). Here, INS-1E cells devoid of SV2A do not express more SV2C and vice-versa. In the secretion experiments, the small inhibition in SV2A-knockout cells could be compensated by SV2C whereas the strong reduction in SV2C-deficient cells could be only partially (or not at all) compensated by SV2A. Thus, SV2A and -C are not completely redundant, SV2C being functionally more relevant than SV2A. The lack of additive effect in the absence of both isoforms also speaks against an important role of SV2A.

In vitro SV2A and -B interaction with synaptotagmin I (Lazzell et al., 2004; Schivell et al., 1996) is modulated by SV2 phosphorylation (Pyle et al., 2000). In INS-1 cells, the insulin granule-associated synaptotagmin I, II, V or IX (Iezzi et al., 2004; Lang et al., 1997) could also bind SV2A or -C. Thus, isoform-specific interactions and/or phosphorylation changes,
might explain the extent to which secretion is inhibited after SV2 depletion. Like complexin I (Abderrahmani et al., 2004), the similar effects of up- and downregulation of SV2 isoforms, indicate that correct endogenous levels are required for appropriate protein stoichiometry.

In addition to stimulated conditions, silencing of SV2C also inhibits basal secretion. This may either correspond to diminished transport of insulin vesicles from the Golgi apparatus to the cell periphery or to impaired exocytosis. INS-1 cells express synaptotagmin I and IX participating not only in exocytosis (Iezzi et al., 2004; Lang et al., 1997) but also in endocytosis (Haberman et al., 2003; Jarousse et al., 2001). Accordingly, SV2C may be involved in synaptotagmin endocytosis, as suggested for retinal photoreceptor synapses of SV2B-knockout mice after reduced expression of synaptotagmin I (Lazzell et al., 2004). Since SV2A and -B interact with synaptotagmin I in a Ca\(^{2+}\)-independent manner (Lazzell et al., 2004; Schivell et al., 1996), during basal release, a Ca\(^{2+}\)-independent binding of synaptotagmins to SV2C would be operative. Interestingly, if SV2 were a vesicular Ca\(^{2+}\) transporter, increased rather decreased basal secretion would be expected.

Suppression of SV2A and -C led to an inhibition in the sustained phase of glucose-induced exocytosis. The early phase is also diminished but the effect is much weaker. Consistent with these data, capacitance recordings reveal that loss of SV2C strongly impairs glucose-triggered granule recruitment to the RRP, whereas there is only a tendency for SV2A. This is in accordance with the decreased pool of fusion-competent vesicles in SV2A-deficient chromaffin cells (Xu and Bajjalieh, 2001). Of note, the unaltered K\(^+\)-evoked hGH secretion seems at variance with the capacitance results. However, during K\(^+\)-depolarization, vesicular translocation does not occur (Olofsson et al., 2002). Taken together, these findings demonstrate that SV2 is mostly involved in the glucose-dependent progression of granules to the plasma membrane.

In addition to synaptotagmin isoforms, SNARE proteins as well as different Rab GTPases with their effectors and regulatory proteins have been implicated in \(\beta\)-cell exocytosis (Burgoyne and Morgan, 2003; Lang, 1999; Rorsman and Renstrom, 2003). Dynamic association of granules with the actin cytoskeleton is also required for the final step of glucose-dependent insulin secretion (Thurmond et al., 2003). Moreover, glucose but not high K\(^+\) stimulates the mobilization of insulin granules to the plasma membrane, probably because of ATP generation (Varadi et al., 2002). Thus, it is possible that SV2 regulates the reorganization of the actin cytoskeleton to recruit granules, modulates the binding with synaptotagmins or controls the formation of the release machinery.

In conclusion, we show that SV2A, -B and -C are expressed with distinct locations in insulin-secreting cells. The secretory-granule-associated SV2A and SV2C are not Ca\(^{2+}\) transporters, but regulate the vesicular recruitment to the plasma membrane, without affecting the final step of exocytosis. Finally, SV2C is functionally more important than SV2A, which emphasizes a novel role for this isoform in endocrine secretion.

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