Evidence that Electrostatic Interactions between Vesicle-associated Membrane Protein 2 and Acidic Phospholipids May Modulate the Fusion of Transport Vesicles with the Plasma Membrane

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Submitted April 8, 2009; Revised September 21, 2009; Accepted September 29, 2009
Monitoring Editor: Thomas F.J. Martin

The juxtamembrane domain of vesicle-associated membrane protein (VAMP) 2 (also known as synaptobrevin2) contains a conserved cluster of basic/hydrophobic residues that may play an important role in membrane fusion. Our measurements on peptides corresponding to this domain determine the electrostatic and hydrophobic energies by which this domain of VAMP2 could bind to the adjacent lipid bilayer in an insulin granule or other transport vesicle. Mutation of residues within the juxtamembrane domain that reduce the VAMP2 net positive charge, and thus its interaction with membranes, inhibits secretion of insulin granules in β cells. Increasing salt concentration in permeabilized cells, which reduces electrostatic interactions, also results in an inhibition of insulin secretion. Similarly, amphipathic weak bases (e.g., sphingosine) that reverse the negative electrostatic surface potential of a bilayer reverse membrane binding of the positively charged juxtamembrane domain of a reconstituted VAMP2 protein and inhibit membrane fusion. We propose a model in which the positively charged VAMP and syntaxin juxtamembrane regions facilitate fusion by bridging the negatively charged vesicle and plasma membrane leaflets.

INTRODUCTION

Several investigators have recently reviewed the process by which transport vesicles fuse with the plasma membrane (Chernomordik and Kozlov, 2008; Jackson and Chapman, 2008; Jahn, 2008; Malsam *et al.*, 2008; Rizo and Rosenmund, 2008; Wickner and Schekman, 2008). There is substantial evidence that the fusion reaction is initiated by interactions between soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins that assemble into a four-helix bundle between the vesicle and plasma membranes (Jahn and Scheller, 2006). However, the exact molecular events driving both the close approximation of these two phospholipid bilayers and their subsequent fusion remain elusive, as discussed in the reviews indicated above. In this study, we address the problem of bringing the two bilayers together.

Specifically, because the bilayer components of both the plasma membrane and transport vesicles contain acidic lipids and bear a net negative charge, a mechanism should be present that can overcome their electrostatic repulsion, which is significant when the distance between the bilayers is <2 -Debye lengths (~ 2 nm). One popular model is that the

zippering of the SNARE coiled-coil domains from the N to C termini pulls the membranes together with sufficient force to induce membrane fusion (Fasshauer *et al.*, 1997a,b; Hanson *et al.*, 1997; Sutton *et al.*, 1998). However, recent evidence suggests the zippering process may not generate sufficient energy to drive fusion (Li *et al.*, 2007; Wiederhold and Fasshauer, 2009).

Several investigators have noted that the carboxy-terminal juxtamembrane domains (JMDs) of both the plasma membrane SNARE protein syntaxin and transport vesicle SNARE protein vesicle-associated membrane protein (VAMP) typically contain an excess of positively charged residues, which may interact with acidic lipids (Sutton *et al.*, 1998; Montal, 1999; Kweon *et al.*, 2003). More recently, Stuenkel and coworkers provided strong evidence that the basic JMD cluster in syntaxin was important for exocytosis (Lam *et al.*, 2008). In this report, we examined the role of the basic JMD cluster in VAMP2 (also known as synaptobrevin2). We first determined the electrostatic interaction of the JMD of VAMP2 with phospholipid membranes using both peptides corresponding to this JMD region and reconstituted VAMP2 protein. We then studied the potential role of the VAMP2 JMD in fusion using the well-established cell biological properties of insulin granule secretion in β cells and glucose transporter (GLUT) 4 translocation/fusion in adipocytes. Our data suggest that the electrostatic interactions between acidic phospholipids and the SNARE protein JMD contribute to the membrane fusion process.

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E09-04-0284>) on October 7, 2009.

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MATERIALS AND METHODS

Materials

Insulin, dexamethasone, isobutyl-1-methylxanthine, bovine serum albumin, donkey serum, potassium glutamate, 1,4-piperazinediethanesulfonic acid (PIPES), EGTA, digitonin, *N*-(4-aminobutyl)-1-naphthalenesulfonamide hydrochloride (W12), *N*-(4-aminobutyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W13), sphingosine, and the protease and phosphatase inhibitor cocktails were purchased from Sigma-Aldrich (St. Louis, MO). All lipids, palmitoylcholinephosphatidylcholine (PC), dioleoylphosphatidylserine (PS), and phosphatidylinositol-4,5-bisphosphate (PIP₂) and palmitoylcholinephosphatidylcholine (PA) were purchased from Avanti Polar Lipids (Alabaster, AL). The c-myc antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-mouse immunoglobulin G Alexa-Fluor secondary antibody was purchased from Invitrogen (Carlsbad, CA), and horseradish peroxidase-conjugated secondary antibodies were purchased from Pierce Chemical (Rockford, IL). The human growth hormone (hGH) enzyme-linked immunosorbent (ELISA) assay was purchased from Roche Applied Science (Indianapolis, IN), and the insulin ELISA kit was obtained from Mercodia (Winston Salem, NC). Lipofectamine 2000 was obtained from Invitrogen.

Plasmids

The exofacial myc-tagged GLUT4-enhanced green fluorescent protein (eGFP) pcDNA reporter was constructed as described previously (Watson *et al.*, 2004b). The full-length human VAMP2 coding sequence was directly cloned from pCMV-Sport6-VAMP2 cDNA (I.M.A.G.E clone; American Type Culture Collection, Manassas, VA) by polymerase chain reaction (PCR) using Taq Platinum high fidelity (Invitrogen) and placed into the pGFP-C2 vector. Mutation of the KR residues at position 85/86 to ED was prepared by overlapping PCR. The full-length human VAMP2 coding sequence was directly cloned from pCMV-Sport6-VAMP2 cDNA by PCR (Pfx high fidelity polymerase; Invitrogen) into Topo-D pET200 by using the forward primer CACCGGATCCATGCTCTGCTACCGCTGCCACGG and the reverse primer GGCGCGCTTAAGTGCTGAAGTAACTATG to generate a His₆-VAMP2 bacterial expression plasmid.

Large Unilamellar Vesicles and Peptide Preparations

Sucrose-loaded large unilamellar vesicles (LUVs) were used for centrifugation experiments. As described previously (Wang *et al.*, 2002), solutions of PC, PS, and PIP₂ dissolved in 1–2 ml of chloroform were added to a 50-ml round-bottomed flask that was then immersed into a 30°C water bath and attached to a rotary evaporator to rapidly remove the solvent. Adding a solution containing 176 mM sucrose, 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.0, produces a solution of multilamellar vesicles, which were frozen and thawed five times. We formed 100-nm-diameter LUVs by extruding the solution through polycarbonate filters and then resuspending the sucrose-loaded vesicles in 100 mM KCl, 10 mM MOPS, pH 7.0; centrifuging; and then resuspending again. Approximately 1 µg of synthetic VAMP2(79–95) peptide containing an additional N-terminal cysteine (American Peptide, Sunnyvale, CA) was labeled with [³H]N-ethylmaleimide (NEM) and purified in acetonitrile by high-performance liquid chromatography for use in the binding experiments.

Peptide Binding Analysis

The binding of ³H-labeled VAMP2(79–95) and other peptides to sucrose-loaded PC/PS, PC/PIP₂, and PC/PS/PIP₂ LUVs was measured as described previously (Wang *et al.*, 2002). In summary, LUVs were mixed with trace concentrations (~10 nM) of radiolabeled peptide and the mixture centrifuged at 100,000 × *g* for 1 h. The radioactivity of the supernatant (free peptide) and pellet (peptide bound to sucrose-loaded vesicles) was compared and used to calculate the percentage of bound peptide. The molar partition coefficient *K* is given by the following equation:

$$\frac{[P_m]}{[P_{total}]} = \frac{K[L]}{(1 + K[L])}$$

where [P_m] is the molar concentration of peptide partitioned onto the membrane; [P_{total}] is the total concentration of peptide in solution, and [L] is the accessible lipid concentration, which is one half the total lipid concentration (we add radioactive peptide to preformed vesicles). We applied the equation to the experimental data to obtain a value for *K*. Note that *K* is the reciprocal of the accessible lipid concentration required to bind 50% of the peptide. The derivation of the equation makes no assumption about the mechanism by which the peptide binds to the membrane.

Proteoliposome Reconstitution of VAMP2

The different VAMP2 proteoliposomes were reconstituted and purified as described in Vicogne *et al.* (2006). In brief, the His₆-VAMP2-wild type (WT) and the His₆-VAMP2-KR/ED expression vectors were transformed in B121 Star (DE3) *Escherichia coli* (Invitrogen). The recombinant VAMP2-WT and

VAMP2-KR/ED proteins were expressed and purified Co²⁺-nitrilotriacetic acid affinity chromatography. Protein purity was assessed by NuPAGE gel (Invitrogen) and quantified by protein assay (BCA protein assay kit; Pierce Chemical). The VAMP2-WT and VAMP2-KR/ED proteins were reconstituted by mixing octyl-β-D-glucopyranoside-soluble proteins with various detergent-solubilized phospholipids mixtures as indicated in the figure legends. After rapid dilution, the proteoliposomes were purified by dialysis and sucrose gradient flotation. VAMP2 protein copy numbers were calculated based upon the vesicle volume and number (determined by dynamic light scattering) and the amount of VAMP2 protein (moles) present in the sample (Vicogne *et al.*, 2006). VAMP2-WT, VAMP2-WW/AA, and VAMP2-KR/ED copy number were adjusted to obtain ~300 copies per vesicle giving a protein:lipid ratio of ~1:50.

Tryptophan Fluorescence

The full-length VAMP2-WT protein (~1 µM) was reconstituted into LUVs containing either PC or 3:1 PC/PS (~50 µM total lipid) as described above. The tryptophan fluorescence signal was normalized by subtraction of the signal from a VAMP2-WW/AA mutant protein reconstituted into vesicles with the same lipid composition. For the peptide measurements, 100 nm of LUVs (100 µM accessible lipid) were prepared in buffer (100 mM KCl and 10 mM MOPS, pH 7.0) and mixed with 1 µM VAMP2(79–95) peptide. Fluorescence emission spectra (excitation, 274 nm; emission, 300–370 nm) were obtained using an SLM Aminco Bowman Series 2 spectrofluorometer (SLM Aminco Instruments, Rochester, NY) at 24°C. Sphingosine was added directly to the cuvette. A standard Gaussian deconvolution was performed using SigmaPlot (Systat Software, San Jose, CA), with maximal predicted peaks at 335 and 355 nm.

Cell Culture and Transient Transfection

Murine 3T3L1 preadipocytes were purchased from the American Type Tissue repository (<http://www.atcc.org/Home/tabid/57/Default.aspx>). Cells were cultured in DMEM supplemented with 25 mM glucose and 10% calf serum at 37°C with 8% carbon dioxide. Cells were differentiated into adipocytes with the addition of 1 µg/ml insulin, 1 µM dexamethasone, and 0.5 mM isobutyl-1-methylxanthine. Adipocytes were used in experiments 6–9 d after the initiation of differentiation. Cells were electroporated using the Gene Pulser II (Bio-Rad Laboratories, Hercules, CA) with settings of 0.16 kV and 960 microfarads. For all experiments, 50 µg of DNA was used for electroporation. After electroporation, cells were plated on glass coverslips and allowed to recover in complete medium.

Min6B1 (transformed mouse β) cells were grown at 37°C in 5% CO₂ in DMEM supplemented with 10% fetal bovine serum containing penicillin-streptomycin and 70 µM 2-mercaptoethanol. For hGH secretory assays, the cells were transfected with 2 µg of hGH cDNA plus either 6 µg VAMP2 cDNA or empty vector by using Lipofectamine 2000 according to the manufacturer's instructions.

β-Cell Secretion

Min6B1 cells were incubated in KRBH buffer (4.7 mM KCl, 125 mM NaCl, 5 mM NaHCO₃, 1.2 mM MgSO₄, 1 mM CaCl₂, 25 mM HEPES, pH 7.4, 1.2 mM KH₂PO₄, and 0.1% bovine serum albumin) containing 2.8 mM glucose for 2 h. Cells were then treated with vehicle (buffer solution), 100 µM sphingosine, or 100 µM W13 for 20 min followed by stimulation with 30 mM KCl for 10 min. Insulin release into the medium and total amount of insulin were then determined by an insulin ELISA assay. Secretion was expressed as percentage of the amount of insulin released into the medium relative to the total cellular insulin levels. In cells cotransfected with the VAMP2 cDNAs and hGH for 48 h, KCl-stimulated secretion was determined using a hGH ELISA assay.

The digitonin permeabilization experiments were performed as described by Holz and Senter (1986). In brief, cells were permeabilized in a solution containing 139 mM potassium glutamate, 20 mM PIPES, 5 mM EGTA, 2 mM Mg²⁺, and 20 µM digitonin, pH 6.6, for 6 min. Directly after permeabilization, the solution was changed to solutions containing 20 mM PIPES, 5 mM EGTA, and 2 mM Mg²⁺ with or without 10 µM free Ca²⁺ and the indicated concentrations of potassium glutamate. After 15 min of stimulation, Ca²⁺-dependent secretion was determined using an insulin ELISA assay.

Immunofluorescence and Image Analysis

Differentiated 3T3L1 adipocytes transfected with the exofacially tagged myc-GLUT4-green fluorescent protein (GFP) cDNA with or without Myr-Akt and were grown on cover slips and serum-deprived in DMEM for 2 h before each experiment. When relevant, cells were treated for 20 min or 60 min with the weak bases sphingosine (100 µM) or W13 (100 µM). The cells were then fixed for 10 min at room temperature using 4% paraformaldehyde. Cells were then blocked using 1% bovine serum albumin solution with 5% donkey serum for 1 h at room temperature. Coverslips were then incubated in primary antibody solution for 1 h at 37°C. After incubation, the coverslips were washed three times with phosphate-buffered saline (PBS) solution, followed by incubation in Alexa 594 anti-mouse secondary antibody for 1 h at 37°C. Coverslips were then again washed three times with PBS and mounted with VECTASHIELD medium (Vector Laboratories, Burlingame, CA) and evaluated by laser scanning confocal microscopy. The ratio of exofacial myc signal to total cellular

Figure 1. The VAMP2 JMD binds to acidic phospholipids through electrostatic interactions. (A) Amino acid sequence alignment of the human VAMP2 JMD residues (77-96) with the human VAMP1 and VAMP3 proteins. Conserved basic residues (+) are in blue, acidic residues (−) are in red, and hydrophobic residues (Φ) are in green. The mutation of the human VAMP2 used in this study is underlined. (B) A peptide corresponding to the VAMP2 JMD, VAMP2(79-95), was labeled with [³H]NEM and incubated with sucrose-loaded LUVs containing the indicated mole ratios of PC/PS. After separation by centrifugation the percentage of bound peptide was determined directly from the radioactivity in the supernatant and the pellet. The molar partition coefficient, K , was determined by plotting the percentage of bound peptide versus concentration of lipid accessible to the peptide ($1/2$ total lipid concentration because the membrane-impermeable peptide is added to the preformed vesicles). The curves illustrate the best fit of the equation in *Materials and Methods* to the data; note K is the reciprocal of the lipid concentration that binds 50% of the peptide. (C) The value of K obtained from Figure 1B was plotted as a function of the mole % PS in the PC/PS LUVs. Note the binding of the positively charged (6 basic residues) peptide increases exponentially with the mole fraction of acidic lipid PS in the membrane, as predicted theoretically for electrostatic interactions. (D) The binding of the [³H]NEM-labeled VAMP2(79-95) to PC/PIP₂ (99:1) or PC/PS/PIP₂ (74:25:1) vesicles was determined as described above. Data for 3:1 PC/PS vesicles reproduced from B to facilitate comparison.

GFP signal was quantified as a measure of GLUT4 fusion as described previously (Jiang *et al.*, 2002; Williams and Pessin, 2008). GLUT4 translocation was quantified as the ratio of GFP signal localized to within $0.5 \mu m$ of the cell surface to total GFP cellular GFP signal. Images of 15 representative cells per condition were processed using LSM510 software (Carl Zeiss, Thornwood, NY) and the results represent the mean $\pm$ SD from 3 independent experiments.

RESULTS

The VAMP2 JMD Binds to Negatively Charged Phospholipid Bilayer Membranes

The mammalian VAMP protein isoforms involved in plasma membrane fusion (VAMP1–3) all have JMD with four basic residues (K Φ KRK, Φ hydrophobic) followed by tandem hydrophobic Trp residues (WW) flanked by an additional two basic residues (KN Φ K) (Figure 1A). This core sequence of basic amino acid residues with two invariant Trp is conserved in most of the VAMP2 orthologues found in amphibians, fish, flies, worms and yeast (Supplemental Figure 1S). This level of conservation suggests that the JMD may play an important role in VAMP protein function. It is well established that clusters of basic amino acids within proteins can associate with acidic phospholipid head groups through direct electrostatic interactions (McLaughlin and Murray, 2005; Heo *et al.*, 2006), as hypothesized previously for the

basic clusters on syntaxin and VAMP (Sutton *et al.*, 1998). Recent studies on the JMD of syntaxin1 have suggested that the conserved basic cluster is important for synaptic membrane fusion in PC-12 cells (Lam *et al.*, 2008).

To determine how strongly the VAMP2 JMD interacts with membranes containing acidic phospholipids, we first examined the binding of the VAMP2 JMD peptide in vitro. An ³H-labeled peptide corresponding to the VAMP2(79-95) region was incubated with sucrose-loaded 100-nm LUVs composed of mixtures of phosphatidylcholine (PC: net charge = 0), phosphatidylserine (PS: net charge = −1), and PIP₂ (net charge = −4). The membrane-bound and free peptide were separated by ultracentrifugation; the percentage of peptide bound to the vesicles is shown in Figure 1B as a function of lipid concentration. The peptide binding increased as more negatively charged PS was incorporated into the vesicles (the molar partition coefficient, K , is the reciprocal of the lipid concentration that binds 50% of the peptide, see the equation in *Materials and Methods*). Figure 1C shows the expected exponential dependence of VAMP2(79-95) peptide binding, K , on the mole fraction of acidic lipid in the membrane (Arbuzova *et al.*, 2000; Rusu *et al.*, 2004; McLaughlin and Murray, 2005; McLaughlin *et al.*, 2005; Nomikos *et al.*, 2007). The increase in binding to a 3:1 PC/PS membrane produced by PS corresponds to an electrostatic energy of ~ 3

Figure 2. The VAMP2 JMD tandem Trp residues penetrate inside the low dielectric acyl chain region of the lipid bilayer. (A) The VAMP2(79-95) peptide (1 μ M) in an aqueous environment (reconstitution buffer) was excited at 274 nm and the emission spectra scanned from 310 to 370 nm (dotted line). The VAMP2 (79-95) peptide was incubated with 100 μ M (accessible lipid concentration = 1/2 total) PC (dashed line) or 3:1 PC/PS LUVs (solid line), and Trp fluorescence was determined as described in *Materials and Methods*. The blue shift and enhanced fluorescence observed when the peptide binds to the PC/PS vesicles suggest the Trp residues insert into the low dielectric interior of the bilayer. (B) Fluorescence of $\sim$ 1 μ M full-length VAMP2 WT protein reconstituted into PC or 3:1 PC/PS LUVs ($\sim$ 50 μ M lipid) were scanned from 310 to 370 nm (excitation, 274 nm). Maximal peak of the Trp signal was normalized to 100 arbitrary units to illustrate the slightly greater blue shift observed with PC/PS versus PC membranes.

kcal/mol, whereas the binding of the VAMP2(79-95) peptide to PC LUVs is too weak to be measured directly (diamonds in Figure 1B), but the extrapolated value from Figure 1C gave $K \sim 30 \text{ M}^{-1}$ (energy, $\sim 2 \text{ kcal/mol}$). This affinity for PC (neutral) LUVs reflects the binding of the hydrophobic residues in the JMD, whereas K increased from $\sim 10^3$ to 10^5 M^{-1} as the mole fraction of PS increased from 17 to 33% (Figure 1, B and C).

Two experiments confirm this increase is due to nonspecific electrostatics rather than a specific chemical interaction with PS. First, the binding is independent of the chemical nature of the monovalent acidic lipid; replacing PS with PG did not change the affinity (data not shown). Second, electrostatic theory predicts that increasing the salt concentration in the bathing solution should decrease significantly the molar partition coefficient. This was confirmed experimentally; an increase in KCl concentration decreased the binding of the JMD peptide (Supplemental Figure 2S). Because the cytoplasmic leaflet of a plasma membrane typically contains 1–3% PIP_2 , which has a charge of -4 at pH 7.0 (McLaughlin *et al.*, 2002), we next examined the ability of the VAMP2 JMD peptide to bind PC/PS/ PIP_2 74:25:1 LUVs (Figure 1D). The addition of 1% PIP_2 significantly enhanced the binding of the VAMP2 JMD peptide to the PC/PS membranes.

The VAMP2 JMD Trp Residues Penetrate into the Phospholipid Bilayer

Electron spin resonance (ESR) and other spectroscopic studies have suggested that, in reconstituted vesicles, the VAMP2 JMD tandem Trp residues are inserted into the lipid bilayer (Kweon *et al.*, 2003; Bowen and Brunger, 2006). To confirm and extend these measurements, we took advantage of the known property that Trp fluorescence is sensitive to the dielectric constant of its microenvironment. As observed for other basic peptides containing Trp residues, excitation of Trp residues at 274 nm results in a peak of fluorescence emission at $\sim 350 \text{ nm}$ in an aqueous environment. However, in the low dielectric interior of a bilayer the emission peak is blue shifted to $\sim 335 \text{ nm}$ and the emission is significantly enhanced, typically two- to threefold (see Supplemental Figure 3S for results with a peptide corresponding to a basic/hydrophobic membrane-binding cluster of the scaffolding protein Gravin).

We therefore determined Trp fluorescence of the VAMP2(79-95) peptide in aqueous buffer, and bound to 3:1 PC/PS LUVs (Figure 2A). As expected, Trp fluorescence in both aqueous buffer and in the presence of PC LUVs (to which the peptide

does not bind significantly, as shown in Figure 1B) was essentially identical, with fluorescence maximum peak at $\sim 350 \text{ nm}$. However, in the presence of 3:1 PC/PS LUVs (to which the peptide binds as shown in Figure 1, B and C), the Trp fluorescence was blue shifted to $\sim 330 \text{ nm}$ and enhanced ~ 2.5 -fold in magnitude (Figure 2A). These data suggest that when the VAMP2 JMD peptide binds to a membrane, the Trp residues penetrate into the hydrophobic interior of the lipid bilayer.

To examine whether the JMD of the full-length VAMP2 protein (VAMP2-WT) also binds to phospholipid bilayers, we reconstituted the VAMP2-WT protein into PC or 3:1 PC/PS LUVs. Fortunately, the two JMD Trp are the only Trp residues present in VAMP2. The VAMP2-WT protein reconstituted into either PC or PC/PS vesicles displayed a significant blue shift in normalized Trp fluorescence (from ~ 350 to $< 340 \text{ nm}$) (Figure 2B). The JMD of the reconstituted protein binds to PC because it experiences an ~ 1000 -fold higher effective lipid concentration than the simple JMD peptide shown in Figure 2A. The blue shift for the PC/PS vesicles (3:1) was slightly greater than for PC, which may reflect a deeper penetration of Trp residues into the hydrophobic membrane interior. Both spectra (PC/PS and PC reconstituted VAMP2-WT) in Figure 2B were normalized to more clearly demonstrate the wavelength shifts. These findings are consistent with previous ESR and other spectroscopic measurements that suggest the JMD of reconstituted VAMP binds to the phospholipid membrane. These data are also consistent with the interpretation that VAMP2 JMD in transport vesicles is bound to the cytoplasmic leaflet of the vesicles through both electrostatic and hydrophobic interactions.

We next reasoned that if the binding of the JMD to the phospholipid leaflet is driven mainly by nonselective electrostatic (3 kcal/mol) and hydrophobic (2 kcal/mol) interactions, reversing the negative charge on the bilayer membrane should repel the positively charged JMD from the surface and thus move the Trp residues from a low dielectric membrane interior to the higher dielectric aqueous phase. To test this prediction, the full-length VAMP2 protein was reconstituted into 3:1 PC/PS vesicles and incubated with increasing concentrations of sphingosine, an amphipathic weak base that can reverse the charge on a PC/PS vesicle when present at free concentrations higher than 2 μM (McLaughlin *et al.*, 2005). Sphingosine does not display any fluorescence between 300 and 400 nm when excited at 274 nm and therefore will not interfere with the determination of

Figure 3. Reversing the net negative charge on a phospholipid vesicle reverses the membrane binding of the positively charged JMD of reconstituted VAMP2. The full-length VAMP2-WT protein ($\sim 1 \mu\text{M}$) was reconstituted into 3:1 PC/PS LUVs and incubated with the indicated concentrations of the amphipathic weak base sphingosine. The lipid concentration of the reconstituted LUVs, after purification, was $\sim 50 \mu\text{M}$, which corresponds to $\sim 15 \mu\text{M}$ PS. (A) The samples were excited at 274 nm and emission spectra scanned from 310 to 370 nm. Trp fluorescence was determined by subtraction of fluorescence from full-length VAMP2-WW/AA reconstituted into LUVs. (B and C) The Trp fluorescence emission spectra obtained in panel A were deconvoluted into two components representing the hydrophobic (B) emission spectra (335 nm maximal emission peak) and the hydrophilic (C) emission spectra (355 nm maximal emission peak). (D) Percentage of the Trp inserted in the low dielectric acyl chain region of the membrane was determined by calculating the mass contribution (half width of the maximum peak method) of the 335 and 355 nm maximal peak of the deconvoluted spectra.

Trp fluorescence. As expected, increasing concentrations of sphingosine not only decreased the Trp fluorescence signal at 335 nm but also resulted in a red shift (Figure 3A). Deconvolution of these emission spectra illustrates a sphingosine-dependent reduction at 335 nm with a concomitant increase at 355 nm (Figure 3, B and C). The phospholipid concentration in these proteoliposomes was estimated to be $\sim 50\text{--}60 \mu\text{M}$, which corresponds to $\sim 15 \mu\text{M}$ PS. Thus, the addition of $10 \mu\text{M}$ sphingosine should reduce and $20 \mu\text{M}$ sphingosine should reverse the charge on these vesicles. Consistent with these calculations $20 \mu\text{M}$ sphingosine produced a large decrease ($\sim 70\%$) in the 335 nm peak (Figure 3B) with a concomitant increase in the 355 nm peak (Figure 3C). Figure 3D summarizes the sphingosine concentration dependent desorption of the VAMP Trp residues out of the reconstituted hydrophobic membrane environment.

Mutation of the VAMP2 JMD Basic Cluster Inhibits Insulin Granule Secretion

If electrostatic binding of the VAMP2 JMD to membranes is involved in the fusion mechanism, then functional consequences of perturbing either the number of basic charges in this region or the electrostatic surface potential of the membrane should be observable in living cells. Thus, we decreased the net positive charge of the VAMP2 JMD by generating a VAMP2-KR/ED mutant in which lysine K85 and arginine R86 were replaced with glutamic and aspartic acids residues, respectively, thereby reducing the net charge of the JMD basic region from +6 to +2. We next examined how this mutation affected insulin secretion from pancreatic β -cells.

In this system, calcium-triggered fusion can be initiated by the addition of KCl to induce membrane depolarization, resulting in opening of the voltage-dependent calcium channels, influx of extracellular calcium and fusion

of the readily releasable pool of predocked insulin granules (Daniel *et al.*, 1999). Under these conditions, the membrane depolarization of pancreatic β -cells primarily results in the fusion of predocked insulin granules at the plasma membrane (Michael *et al.*, 2006).

We therefore cotransfected Min6B1 cultured β -cells with either VAMP2-WT or VAMP2-KR/ED and with the hGH cDNA as a reporter for insulin secretion (Iezzi *et al.*, 2000; Cheviet *et al.*, 2004). KCl-induced depolarization resulted in an approximate threefold increase in hGH release in empty vector and VAMP2-WT cotransfected Min6B1 cells (Figure 4A). In contrast, expression of the VAMP2-KR/ED mutant inhibited KCl-stimulated hGH secretion, indicating that this mutant functions in a dominant-interfering manner. To assess whether the VAMP2-KR/ED mutant displayed the same intracellular localization as VAMP2-WT, we cotransfected the Min6B1 cells with both constructs and examined their subcellular distribution by confocal fluorescent microscopy (Figure 4B). The VAMP2-WT and VAMP2-KR/ED fluorescent signals were strongly overlapped in both a single plane or when assessed by three-dimensional (z-axis) reconstruction (Supplemental Figure 4). These data suggest that both VAMP2-WT and VAMP2-KR/ED are expressed and traffic properly in Min6B1 cells.

To further investigate how the net charge of the VAMP2 JMD affects insulin secretion, we generated a series of alanine substitution mutations that successively reduce the net positive charge of this region from +6 to +4, +3, and +2 (Figure 5). A greater reduction in positive charge correlated with a greater degree of inhibition of insulin secretion. Moreover, the specific position of the mutations was without effect. For example, the KR/AA and KNLK/ANLA mutants both contain four positive charges and display the same degree of inhibition. Similarly, the KLKRK/ALAAA and the KR/ED mutants both contain two net positive charges and result in the same degree of inhibition.

Figure 4. Decreasing the positive charge of the VAMP2 JMD results in inhibition of calcium-dependent fusion of insulin granules. Min6B1 cells were cotransfected with 6 μ g of the VAMP2-WT or VAMP2-KR/ED mutant expression plasmids and with 2 μ g of the hGH expression plasmid. (A) Twenty-four hours later, the cells were either left untreated (open bars) or stimulated (filled bars) with 30 mM KCl for 10 min. The cell supernatants and total cell extracts were then assayed for hGH and the release of hGH was determined as the percentage of total hGH content. These data are the average $\pm$ SD for three independent experiments, * $p < 0.05$. (B) The cells were fixed and subjected to confocal microscopy. White bar, 5 μ m (c). This is a representative image showing the colocalization of the coexpressed VAMP2-WT and VAMP2-KR/ED mutant.

Insulin Secretion Is Salt Dependent

As shown in Supplemental Figure 2S, increasing the concentration of salt screens the electrostatic interactions and inhibits

Figure 5. Insulin secretion is dependent upon the net positive charge of the VAMP2 JMD but is independent of the specific amino acid residues that are mutated. Min6B1 cells were cotransfected with 2 μ g of the hGH expression plasmid plus either 6 μ g of empty vector, VAMP2-WT (+6), VAMP2-KR/AA (+4), VAMP2-KNLK/ANLA (+4), VAMP2-KRK/AAA (+3), VAMP2-KLKRK/ALAAA (+2), or VAMP2-KR/ED (+2) mutants that have net JMD charges as indicated in the brackets and in the figure. Twenty-four hours later, the cells were either left untreated (open bars) or stimulated (closed bars) with 30 mM KCl for 10 min. The cell supernatants and total cell extracts were then assayed for the release of hGH as the percentage of total cellular hGH content. These data are the average $\pm$ SD from three independent experiments, * $p < 0.05$.

Figure 6. Increased intracellular salt concentrations, which screen electrostatic interactions inhibit insulin secretion. Min6B1 cells were treated briefly with a low concentration of the detergent digitonin to permeabilize the plasma membrane. The cells were then incubated with a buffer containing the indicated concentrations of potassium glutamate. The cells were then treated with or without 10 μ M free Ca²⁺ in solutions containing the indicated concentrations of potassium glutamate. After 15 min of stimulation, Ca²⁺-dependent secretion was determined using an insulin ELISA assay and insulin release was reported as the percentage of total cellular insulin content. These data are the average $\pm$ SD from three independent experiments.

the binding of the JMD peptide to membranes containing acidic phospholipids. To determine whether insulin release *in vivo* depends on salt concentration, we permeabilized Min6B1 cells with digitonin and incubated the cells with increasing concentrations of potassium glutamate as described previously for chromaffin granule secretion (Holz and Senter, 1986). The cells were then stimulated with 10 μ M calcium to initiate the fusion of predocked insulin granules. As shown in Figure 6, an increase in salt concentration produced a decrease in the calcium-stimulated insulin secretion but had no significant effect on the basal level of insulin release.

Amphipathic Weak Bases Inhibit Insulin Granules Secretion and GLUT4 Vesicle Fusion

If reducing the net positive charge of the JMD inhibited insulin secretion by reducing the membrane binding of this region, it follows, as a corollary, that reducing the net negative charge on the biological membranes should also reduce insulin granule fusion. The amphipathic weak bases sphingosine and W13 are known to neutralize the electrostatic surface potential of a phospholipid membrane of similar composition to the cytoplasmic leaflet of a plasma membrane (Sengupta *et al.*, 2007). Because the plasma membrane is permeable to the neutral form of the weak bases, these amphipathic reagents can transverse the bilayer where they combine with a proton from the cytoplasm and then adsorb to the cytoplasmic leaflets of both vesicle and plasma membranes. In this case, unlike transfection, we could directly measure insulin secretion, as the amphipathic weak bases will partition into all of the cells.

Incubation of Min6B1 cells with sphingosine and W13 markedly inhibited KCl-stimulated insulin secretion (Figure 7A). In contrast to W13, the structurally related analogue W12 binds less strongly to membranes, is less able to neutralize the membrane electrostatic surface potential, and was without any significant effect at the concentrations used (Figure 7A). The relative dose response of sphingosine, W13 and W12 on KCl-stimulated insulin secretion is shown in Figure 7B and is consistent with their effect on the surface (ζ) potential of acidic phospholipid vesicles (Sengupta *et al.*, 2007).

Figure 7. Weak bases that neutralize the electrostatic surface potential of phospholipid membranes inhibit calcium-dependent fusion of insulin granules. (A) Min6B1 cells were treated with vehicle, 100 μ M sphingosine, 100 μ M W13, or 100 μ M W12 for 20 min followed by stimulation with 30 mM KCl for 10 min. The cell supernatants were then collected and assayed for the presence of released insulin. These data are the average $\pm$ SD for two to five independent experiments, * $p < 0.05$. (B) Min6B1 cells were treated with vehicle or the indicated concentrations of W12, W13, or sphingosine for 20 min followed by stimulation with 30 mM KCl for 10 min. The supernatants and total cell extracts were then collected and insulin release was determined as the percentage of total cellular insulin content. These data are the average $\pm$ SD for two independent experiments each performed in duplicate.

In adipocytes, the GLUT4 protein is localized to small transport vesicles (referred to as insulin responsive vesicles) that continually cycle from the cell interior to the plasma membrane with a very low extent of fusion with the plasma membrane. However, after insulin-stimulated activation of the Akt kinase the rate of fusion is markedly increased (Lizunov *et al.*, 2005; Bai *et al.*, 2007; Huang *et al.*, 2007). Fusion of these vesicles with the plasma membrane requires the plasma membrane-localized syntaxin4/SNAP23 proteins, the transport vesicle VAMP2, and regulatory SM protein Munc18c (Bryant *et al.*, 2002; Watson *et al.*, 2004a; Ishiki and Klip, 2005). Unlike β -cells that have predocked insulin granules, GLUT4 transport vesicles first undergo a regulated recruitment (trafficking) to the plasma membrane from intracellular sites before docking and subsequent fusion. The trafficking of GLUT4 vesicles to the plasma membrane is dependent upon the formation of phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) to induce the phosphorylation and activation of the serine/threonine kinase Akt (Bryant *et al.*, 2002; Watson *et al.*, 2004a; Huang and Czech, 2007).

To avoid any potential effects of the amphipathic bases on PIP₃ formation/function, we cotransfected 3T3L1 adipocytes with a myc-GLUT4-GFP reporter construct and a constitutively active Akt mutant (Myr-Akt) that provides the necessary downstream signal for GLUT4 translocation in the absence of insulin or phosphatidylinositol 3-kinase activity (Kohn *et al.*, 1996). When we expressed the GLUT4 reporter alone, we observed no significant exofacial exposure of the myc epitope; that is, no fusion with the plasma membrane occurred (Figure 8Ab). In contrast, coexpression with constitutively active Myr-Akt resulted in a marked increase in exofacial myc epitope labeling compared with myc-GLUT4-GFP expressing cells in the absence of Myr-Akt (Figure 8A, compare b with d). Addition of the amphipathic bases sphingosine or W13 for 20 min partially inhibited exofacial

Figure 8. Weak bases that neutralize the electrostatic surface potential of membranes inhibit GLUT4 vesicle fusion with the plasma membrane. Fully differentiated 3T3L1 adipocytes were cotransfected with the myc-GLUT4-GFP reporter construct and the constitutively active Myr-Akt construct as described in *Materials and Methods*. Twenty-four hours later, cells were treated with vehicle, 100 μ M sphingosine, or 100 μ M W13 for 20 or 60 min. Cells were then fixed and subjected to confocal microscopy for the localization of the myc-GLUT4-GFP reporter. (A) Representative images from each treatment condition. White bar, 10 μ m (t). (B) The percentage of GLUT4 vesicle fusion was calculated by a single-cell measurement of the ratio of exposed exofacial myc label (Alexa 594) to total GLUT4 (GFP label). These data represent the analysis of 15 cells/experiment from three independent experiments, * $p < 0.05$ compared with vehicle controls. (C) The percentage of GLUT4 translocation was calculated by a single-cell measurement of the ratio of plasma membrane GFP label to total GFP label. These data represent the analysis of 15 cells/experiment from three independent experiments, * $p < 0.05$ compared with vehicle controls. Error bars represent SD.

myc epitope exposure (Figure 8A, compare h and l with d) without significantly affecting GLUT4 translocation to the plasma membrane; the latter was detected by the presence of

a plasma membrane rim of the GFP signal (Figure 8A, compare g and k with c). Incubation with the amphipathic bases for 60 min resulted in a dramatic decrease in GLUT4 fusion (Figure 8A, compare p and t with d), again without any change in the translocation of GLUT4 to the plasma membrane (Figure 8A, compare o and s with c). Quantification of the amount of GLUT4 reporter fusion and plasma membrane translocation are presented in Figure 8, B and C, respectively. The data in Figure 8B demonstrate that treatment with the amphipathic bases for 20 min inhibited GLUT4 fusion by 35%, whereas treatment for 60 min resulted in 80% inhibition. The data in Figure 8C show plasma membrane translocation is unaffected by sphingosine and W13. Together, these *in vivo* data on GLUT4 trafficking in adipocytes and calcium-stimulated β -cell insulin secretion support the hypothesis that membrane electrostatic interactions play an important role in VAMP2-dependent vesicle fusion with the plasma membrane.

DISCUSSION

Although it is well established that the assembly of vesicle and plasma membrane SNARE proteins is required for membrane fusion, the molecular mechanism of fusion remains an enigma (Jahn and Scheller, 2006). As a prelude to fusion, the SNARE (and/or other) proteins must overcome the electrostatic repulsion between the negatively charged cytoplasmic leaflets of the plasma membrane and transport vesicle, but the details of this process are also unclear. Some clues as to how the positively charged JMDs of the SNAREs might cause a close apposition of the negatively charged bilayer leaflets emerge from our studies with VAMP2 and recent work on syntaxin (Lam *et al.*, 2008).

Our data demonstrate that a peptide corresponding to the VAMP2 JMD binds phospholipid vesicles with an energy

that increases linearly with the mole fraction of monovalent acidic lipid (PS) in the vesicle (Figure 1C). Previous results show that the membrane binding energy of basic peptides also increases linearly with the number of basic residues. For example, peptides with three, five, and seven lysine residues bind to 2:1 PC/PS membranes with energies of 3, 5, and 7 kcal/mol, respectively (Ben-Tal *et al.*, 1996). The available evidence suggests the membrane binding of the VAMP2 JMD is primarily electrostatic in nature: the binding depends on the concentration of salt (Supplemental Figure 2S) and is independent of the chemical nature of the monovalent acidic lipid (PS vs. PG). The data reported in Figures 2 and 3 support previous measurements that strongly suggest the tandem Trp in the VAMP JMD insert within the hydrophobic bilayer interior (Kweon *et al.*, 2003; Bowen and Brunger, 2006). In addition, our data suggest that the membrane binding of the Trp residues in a reconstituted VAMP2 vesicle can be reversed: this occurs if the electrostatic attraction of the positively charged JMD region for the negatively charged membrane is transformed into an electrostatic repulsion by reversing the charge of the membrane with sphingosine (Figure 3).

Does membrane binding of the VAMP JMD affect membrane fusion in cells? If we hypothesize that it does, then both decreasing the net positive charge of the JMD and decreasing the net negative charge of the membrane should decrease membrane binding and thus decrease fusion. Consistent with this supposition, we have observed that decreasing the net positive charge of the VAMP2 JMD by mutation of the VAMP2 protein, independently of the amino acid position of the positive charges inhibited membrane fusion. Similarly, decreasing electrostatic interactions by increasing salt concentrations inhibits the binding of the VAMP2 JMD to acidic phospholipids *in vitro* and also inhibits insulin granule secretion *in vivo*. Moreover, decreasing the net neg-

Figure 9. Cartoon of how the SNARE JMDs may interact with the cytoplasmic leaflets of the plasma and vesicle membranes. Top, the positively charged JMD of VAMP2 is strongly attracted by the negative electrostatic potential produced by acidic phospholipids in the GLUT4 cargo vesicles; the two hydrophobic Trp residues penetrate into the bilayer. Similarly, the positively charged JMD of syntaxin 4 is strongly attracted by the negative electrostatic potential produced by the acidic phospholipids in the plasma membrane. Bottom, after the assembly of the SNARE complex, the opposing membranes come in proximity and the two phospholipid leaflets share the two basic clusters, which glue the bilayers together through nonspecific electrostatic interactions. Only a single JMD for VAMP2 and syntaxin are shown for clarity. See text for details.

active charge on the vesicle (and plasma) membrane by addition of amphipathic weak bases inhibited membrane fusion. This was true for both depolarization-induced insulin secretion in β -cells and GLUT4 plasma membrane fusion in adipocytes. Importantly, the inhibition of GLUT4 plasma membrane fusion occurred with a normal extent of GLUT4 translocation from the intracellular vesicle storage sites to the cytosolic face of the plasma membrane. The time course of the effects of sphingosine/W13 observed in Figure 7B are consistent with a near complete inhibition of GLUT4 exocytosis but with a normal rate of plasma membrane endocytosis (Williams and Pessin, 2008). Although it is formally possible that each *in vivo* manipulation we used to alter electrostatic interactions (JMD mutants, salt concentration and amphipathic bases) could have other effects, our results and data on the syntaxin JMD mutants (Lam *et al.*, 2008) are consistent with the hypothesis that membrane electrostatic interactions may play an important role in bringing the two membranes together before fusion.

The inner leaflet of the plasma membrane is enriched in acidic phospholipids such as PS (20–30%) and PIP₂ (1–3%) and the sites of vesicle docking have been suggested to contain higher levels of PIP₂ (Verkley *et al.*, 1973; James *et al.*, 2008), which is required for calcium-mediated fusion of vesicles with the plasma membrane (Hay *et al.*, 1995; Holz *et al.*, 2000; James *et al.*, 2008). The basic JMD of syntaxin, which is required for membrane fusion (Lam *et al.*, 2008), should bind to the inner leaflet of the plasma membrane (Figure 9, top). Although transport vesicles contain no appreciable PIP₂ and only 5–10% PS (Matsuoka *et al.*, 1998; Yeung *et al.*, 2007), the available data (e.g., Figure 2B) suggest that the JMD of VAMP2 should bind to the vesicle membrane with the two Trp residues inserted into the hydrocarbon interior (Figure 9, top). A recent report has suggested that sphingosine present in synaptic vesicles activates VAMP to form the SNARE complex by causing desorption of the VAMP JMD from the vesicles (Darios *et al.*, 2009). Our data provide evidence that sphingosine can desorb the VAMP JMD from reconstituted vesicles, but it remains to be determined whether synaptic vesicles contain the very high (>20 mol %) fraction of sphingosine required to produce this electrostatic effect in reconstituted vesicles.

We propose the following working model (Figure 9). The syntaxin and VAMP2 JMD are shown as monomers for clarity, although recent studies have suggested that the SNARE proteins may be present as oligomers during the fusion process (Lu *et al.*, 2008). As shown in the top of Figure 9, and described in more detail in a previous review (Jahn and Scheller, 2006), the assembly of the SNARE proteins brings the negatively charged bilayers together to some degree. Without the basic clusters in the JMDs, however, the mutual electrostatic repulsion of these two membranes should keep them >1 nm apart (Debye length, $1/\kappa \sim 1$ nm). We speculate, as shown in the bottom of Figure 9, that the two phospholipid leaflets share the two basic clusters, which “glue” the bilayers together through nonspecific electrostatic interactions. Specifically, we propose that the local positive potential produced by the membrane-bound VAMP JMD attracts the negatively charged inner leaflet of the plasma membrane; similarly the membrane-bound syntaxin JMD attracts the vesicle membrane. The local positive potential produced by a membrane-bound cluster of basic residues is illustrated elsewhere (McLaughlin and Murray, 2005).

In support of our proposal, we note it is well established that basic peptides, including one corresponding to the VAMP2 JMD (data not shown), cause aggregation of negatively charged lipid vesicles by this simple electrostatic

mechanism. Furthermore, multivalent acidic phospholipids (e.g., PIP₂) will facilitate the postulated juxtaposition of the two basic clusters on the syntaxin and VAMP2 JMD by reducing their electrostatic repulsion (Figure 9, bottom). Specifically, FRET experiments with membrane-bound peptides corresponding to the syntaxin JMD (data not shown) demonstrate that the local positive potential attracts and concentrates PIP₂ as described in detail previously (McLaughlin and Murray, 2005). The sandwich composed of negative vesicle membrane, positive JMD, and negative plasma membrane should be held together very strongly, particularly when several sets of JMD act together. Although these hypothetical events shown in Figure 9 allow for SNARE-dependent zippering to closely approximate the two bilayers, the working model makes no specific predictions about molecular pathways by which the phospholipids themselves physically interchange between the two bilayers, and which additional proteins (e.g., Munc18, CAPS, and synaptotagmin) might facilitate this interchange and thus initiate fusion. In this regard, two recent reports discuss a specific mechanism by which membrane insertion of hydrophobic residues at the tips of the C2 domains of synaptotagmin could induce membrane buckling and thus lower the activation barrier for fusion (Lynch *et al.*, 2008; Paddock *et al.*, 2008).

Note added in Proof. Following online Publication, it was brought to our attention that we had omitted the following three references, which constitute the initial reports of interactions between VAMP and phospholipids and their role in vesicle fusion.

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ACKNOWLEDGMENTS

We thank Dr. Ronald Holz for helpful discussions during the course of this study.

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