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Extracellular CADM1 Interactions Influence Insulin Secretion by Rat and Human Islet Beta-Cells and Promote Clustering of Syntaxin-1

Running head: Role of CADM1 in insulin secretion

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ABSTRACT

Contact between β-cells is necessary for their normal function. Identification of the proteins mediating the effects of β-cell-to-β-cell contact is a necessary step towards gaining a full understanding of the determinants of β-cell function and insulin secretion. The secretory machinery of the β-cells is nearly identical to that of central nervous system (CNS) synapses, and we hypothesize that the trans-cellular protein interactions that drive maturation of the two secretory machineries upon contact of one cell (or neural process) with another are also highly similar. Two such trans-cellular interactions, important for both synaptic and β-cell function, have been identified: EphA/ephrin-A and neuroligin/neurexin. Here we test the role of another synaptic-cleft protein, CADM1, in insulinoma cells and in rat and human islet β-cells. We find that CADM1 is a predominant CADM isoform in β-cells. In INS-1 cells and primary β-cells, CADM1 constrains insulin secretion, and its expression decreases after prolonged glucose stimulation. Using a coculture model, we find that CADM1 also influences insulin secretion in a trans-cellular manner. We ask whether extracellular CADM1 interactions exert their influence via the same mechanisms by which they influence neurotransmitter exocytosis. Our results suggest that, as in the CNS, CADM1 interactions drive exocytic site assembly and promote actin network formation. These results support the broader hypothesis that the effects of cell-cell contact on β-cell maturation and function are mediated by the same extracellular protein interactions that drive the formation of the presynaptic exocytic machinery. These interactions may be therapeutic targets for reversing β-cell dysfunction in diabetes.

KEYWORDS: CADM1, SynCam, pancreatic islet, insulin secretion

INTRODUCTION

β-cells require contact with other β-cells to mature and function normally (12, 19, 27). This contact gives rise to trans-cellular protein interactions that drive the maturation and help regulate the function of the insulin secretory machinery (19, 27, 31, 40, 50). Consistent with an essential role of interactions between β-cells, insulin exocytic complexes assemble under the plasma membrane at sites of β-cell-to-β-cell contact (16). Identifying the trans-cellular protein interactions that mediate the effects of β-cell-to-β-cell contact and help guide assembly and functioning of the insulin secretory machinery is crucial for understanding how contact between β-cells promotes functional maturation and helps to control insulin secretion.

β-cells and neurons are very much alike—with similar patterns of protein expression and shared developmental pathways—and likely derive from a common evolutionary ancestral cell type in the primitive central nervous system (CNS) (1, 2, 41, 58). The insulin secretory machinery, in particular, bears a striking resemblance to the synaptic machinery for neurotransmitter release, and the width of the interstitial space between β-cells approximates that of the synaptic cleft (1, 2, 29, 48). Synapse formation (synaptogenesis) is triggered by direct interactions between proteins on the surfaces of contacting neural processes (13, 47). Given the parallels between the synaptic and β-cell exocytic machinery, the cell-surface proteins mediating the effects of contact between β-cells may be the same as those that guide synaptogenesis (50). We previously described one such synaptogenic protein interaction, neuroligin-neurexin, that influences β-cell function; another, EphA-ephrin-A, was described elsewhere (31, 40, 50).

Like members of the neuroligin/neurexin and Eph/ephrin protein families, members of the CADM (cell adhesion molecule) protein family are synaptogenic: trans-cellular interactions between CADM proteins on contacting neural processes trigger pre- and post-synaptic differentiation (7). CADMs are their own extracellular binding partners: interactions are either homophilic or heterophilic with other CADM isoforms (14). We previously found that CADM1 (also referred to as SynCAM1, Necl2, TSLC1 and IGSF4) is expressed in islet α- and β-cells (48). Subsequently, CADM1 was found to be a key target of the microRNA *miR-375*

(51, 52). This is the most abundant β-cell microRNA and participates in the regulation of islet function, including insulin and glucagon secretion, and α- and β-cell proliferation (42, 51, 52). Regulation of CADM1 expression by *miR-375* underscores the potential importance of the protein in β-cell development and function.

In α-cells, CADM1 helps constrain glucagon secretion (23). Enhanced insulin secretion in CADM1 global knockout mice suggests that CADM1 similarly inhibits insulin exocytosis (38). Alternatively, the increased secretion in this mouse model could reflect an effect of CADM1 deficiency on the CNS or some other tissue. The subplasmalemmal insulin secretory machinery includes a set of proteins that constitute a mechanism for halting insulin secretion just prior to insulin release (26, 40, 63). Determination that CADM1 inhibited insulin exocytosis would implicate it in this regulatory mechanism.

Here we investigated the role of CADM in β-cell function. We found that CADM1 is the predominant CADM isoform in human islets and, along with CADM4, one of two predominant isoforms in INS-1 cells and rat islets. We show that insulin secretion varies inversely with CADM1 expression. Further, we show that β-cell expression of CADM1 decreases after glucose stimulation and that CADM1 binds essential components of the β-cell secretory machinery. Asking whether—as in the synapse—trans-cellular interactions contribute to the effect of CADM1 on exocytic function, we found that trans-cellular CADM1 interactions do, indeed, influence insulin secretion, and we provide evidence that, as in the synapse, they do so through effects on assembly of the secretory machinery and the cortical actin network. These results bring to three the number of synaptic-cleft, synaptogenic protein interactions known to also help determine insulin secretion via extracellular interactions. They provide further evidence that parallel sets of trans-cellular protein interactions organize the synaptic neurotransmitter secretory machinery and the submembrane β-cell insulin secretory apparatus.

RESEARCH DESIGN AND METHODS

Antibody and plasmid reagents. Antibodies used were: rabbit anti-CADM1 and mouse anti-GADPH, anti-FLAG, anti-syntaxin-1 and anti-CASK (all from Sigma, St. Louis, MO); mouse anti-synaptophysin and anti-Munc18 (BD, Franklin Lakes, NJ); rabbit anti-EPB41L3/DAL-1 (ThermoFisher, Waltham, MA); IRDye 680-conjugated anti-mouse IgG and IRDye 800CW-conjugated anti-rabbit IgG (LI-COR); and Alexa Fluor 488-anti-rabbit and 594-anti-mouse IgG (Life Technologies, Carlsbad, CA). The expression construct for FLAG-tagged CASPR1 was generously provided by Davide Comoletti (Robert Wood Johnson Medical School). The expression plasmid encoding FLAG-tagged CADM1 was generated by adding a FLAG-tag to full-length CADM1 cDNA (kindly provided by Thomas Biederer, Tufts University) and insertion into pcDNA4 (Life Technologies).

Cell Culture and Transfection. INS-1 cells were cultured in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol and penicillin-streptomycin. Islets were cultured in the same medium without 2-mercaptoethanol or sodium pyruvate. COS-7 cells were cultured in DMEM containing 10% FBS, 2 mM L-glutamine and penicillin-streptomycin. COS cells were also cocultured with INS-1 cells or islet cells in a 1:1 mixture of the RPMI- and DMEM-based media. For dissociation, islets were incubated overnight and then washed with Hanks Buffered Saline Solution (HBSS) without calcium or magnesium. Islets were then treated with 0.01% trypsin solution in HBSS for 3 min at 37°C followed by mechanical disruption using a P200 pipette. Cocultures were seeded with cells from approximately 50 islets (rat) or islet equivalents (human) per well. Details regarding this coculture method are available in video and print (61). Cells were maintained in a humidified 37°C incubator with 5% CO_2. Transfections took place in 24-well plates using DNA constructs or siRNA duplexes mixed with Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocols. RNA interference experiments used pooled siRNAs and a non-

targeting control siRNA pool (Dharmacon, Lafayette, CO). Knockdown was quantified by qPCR analysis. COS cells were transfected at 100% confluency. INS-1 cells were transfected at 30% confluency and were harvested 24 or 72 h after transfection with plasmid or siRNA, respectively.

Immunoblotting. Protein extracts were prepared by lysing cells in RIPA buffer (150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, 2 mM EDTA, 1 mM phenylmethanesulfonylfluoride, and protease inhibitor cocktail (Sigma)). Protein was quantified using the DC Protein Assay (Biorad, Irvine, CA). Proteins (20 µg per lane) were electrophoresed in 4-12% Bis-Tris NuPAGE gels with an IR protein ladder (LI-COR) and then transferred to PVDF membranes. Membranes were blocked with 5% milk in PBS and probed with primary antibodies in Odyssey Blocking Buffer (LI-COR) overnight followed by IRDye-conjugated secondary antibodies in 5% milk in PBS with 0.1% Tween-20. Membranes were imaged and band density quantified using an Odyssey Infrared Imaging System (LI-COR).

Immunoprecipitation. Cells were lysed in 150 mM NaCl, 1% Nonidet P-40, 50 mM Tris (pH 8), 1 mM phenylmethanesulfonylfluoride, and a protease inhibitor cocktail (Sigma). Lysates were precleared using protein G-Sepharose beads then incubated overnight at 4°C with 5 µg of anti-CADM1 or purified rabbit nonspecific IgG. Next, incubation with protein G-Sepharose beads at 4°C for 2 h was followed by thorough washing with PBS. Samples were denatured in LDS sample buffer (Life Technologies) and dithiothreitol prior to Western blotting.

Islets. Islets were isolated from adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) as previously described (49) with adherence to UC Irvine guidelines for the use and care of laboratory animals and under an IACUC-approved protocol. Human islets were provided by the Integrated Islet Distribution Program (coordinated at the City of Hope, Duarte, CA; sponsor: NIDDK).

Real-time Quantitative PCR (qPCR). Total RNA was isolated using GenElute mammalian RNA kit (Sigma) and then reverse-transcribed. Brain RNA was obtained from Clontech Laboratories. qPCR was performed using PerfeCTa SYBR Green FastMix (Quanta BioSciences, Gaithersburg, MD) on an ABI 7500 Fast Real-Time qPCR system. Samples were analyzed in duplicate alongside no-RT and no-template controls; values were normalized to 18S RNA. Primers were designed using Primer3 software and are shown in Table 1 (56). Analysis of qPCR results to yield relative change in message levels was by calculation of $2^{-\Delta\Delta CT}$ (36).

TABLE 1. *qRT-PCR primer sequences*

Gene	Forward primer	Reverse primer
hCADM1	GGTGATGGGCAGAATCTGTT	ACCAGGACTGTGATGGTGGT
hCADM2	ATCCAGAAACGCAGGTGTTC	CGCCGCTAAGTACACATTGA
hCADM3	GTGCTCAAGTGCCAAGTGAA	GGCTGTGTCTTTTTCCCGTA
hCADM4	GGTTCCTATCTGACCCACGA	CCTCACTTCTGGCCCTTACA
rCADM1	GAAGGACAGCAGGTTTCAGC	GCCTTTGAGTTCCTTGTTCC
rCADM2	GACCGTAGCGATGATGGAGT	CAGGTTCTGGCAGTGGTTTT
rCADM3	GGACCGCCAAGTCCCTCGTC	ATTCGCGTCTGGTCCCCGTG
rCADM4	GTCATCTGTGAAGCGCAGAA	AGCACATGTCAGCACCAGAG

qRT-PCR, quantitative real time-PCR; h, human; r, rat

Flow cytometry. FACS analysis was kindly overseen by Alberto Hayek (UC San Diego) and carried out by Orion BioSolutions (Vista, CA) using a chicken anti-CADM1 monoclonal antibody (CM004-3, MBL International, Woburn, MA) as previously described (28). Briefly, dissociated islet cells were fixed in 2% paraformaldehyde, washed in ice-cold PBS and permeabilized in 0.05% Triton X-100. Cells were labeled with antibodies to CADM1, proinsulin (Abcam) and amylase (Sigma) or

non-immune chicken IgY (MBL International). Antibody-labeled cells were stained with fluorescein isothiocyanate (FITC)- or R-phycoerythrin (PE)-labeled secondary antibodies. Cells were washed and resuspended in PBS and were analyzed using a BD FacsScan instrument and CellQuest software (Cytometri Research LLC).

Insulin Secretion and Glucose Stimulation. INS-1 cells were preincubated with 2.75 mM glucose in Krebs-Ringer bicarbonate buffer (KRB) for 1 h and next incubated for 1 h in fresh KRB containing either 2.75 mM glucose alone (basal conditions) or 16.7 mM glucose with 0.1 mM IBMX (stimulating conditions). IBMX was added along with glucose to potentiate glucose-stimulated insulin secretion, which is otherwise reduced substantially below physiologic (in vivo) levels in insulinoma cells such as INS-1 cells and dissociated primary β-cells. Use of IBMX in this manner has been described previously and is fairly common in tissue culture studies of β-cell function, e.g. see refs (9, 20, 54, 59). For potassium-stimulation studies, 30 mM KCl instead of glucose was used. When indicated, latrunculin-B (10 μM, Adipogen, San Diego, CA) was added to the media. After 1 h, media was collected and cell lysates prepared by 30 min incubation in RIPA buffer at 4°C. Insulin was measured by RIA (Millipore, Billerica, MA). Secreted insulin was normalized to total insulin content determined from cell lysates.

Syntaxin Clustering. Quantitative immunofluorescence analysis of syntaxin-1 clustering in INS-1 cells was carried out in cocultures exactly as described before (50), except for the use of transfected COS-7 cells in place of HEK293 cells (see Fig. 10 for an explanatory diagram). COS-7 cells were pre-transfected to express FLAG-tagged CADM1 or FLAG-tagged CASPR2. The latter is an unrelated, neuronal, non-synaptogenic transmembrane protein (57). After a 24 h coculture, cells were washed with PBS, fixed with 4% paraformaldehyde for 1 h, and then washed with 1% BSA in PBS containing 0.1% Tween 20 (PBST). Cells were stained for 1 h with anti-FLAG primary antibody (1:500 dilution) to label transfected COS-7 cells and with anti-syntaxin-1A antibody (1:100). After washing with 1% BSA in PBST, cells were incubated with 1:200 Alexa Fluor-488 anti-rabbit IgG and 1:500 Alexa Fluor-594 anti-mouse IgG. To quantify clustering of syntaxin-1 using immunofluorescence, imaging software was employed for pixel-by-pixel determination of the signal intensity of syntaxin-positive pixels. Images of 8 random, non-overlapping regions within each culture well were captured using Zeiss LSM 700 Confocal Microscope (UCI Optical Biology Core Facility) and analyzed using Zeiss Zen Digital Imaging software as previously described (50).

Statistical Analysis. Data are presented as mean ± SEM. Differences between quantitative data sets was analyzed by two-tailed Student's t-test. Linear regression from syntaxin clustering data and slope analysis by F-test were performed using GraphPad Prism 5 software. $P < 0.05$ was considered statistically significant.

RESULTS

CADM expression in human and rat islets. There are four CADM protein family members; all are expressed and functional in the brain (7, 14). We previously determined that CADM1 is expressed on the surface of α- and β-cells in rat and human islets (48). Because islet expression of the other three isoforms was not previously characterized, we used qPCR to analyze transcript levels in rat and human islets and in INS-1 β-cells, comparing levels to those in the brain. CADM1 was the predominant transcript in INS-1 cells, with levels closest to those in rat brain, whereas CADM2 was not detectable (Fig. 1A). All four transcripts were detected in rat islets (Fig. 1A). Here, CADM4 levels were closest to those in brain. In human islets, CADM1 transcript levels were comparable to those in brain while levels of the other three transcripts were substantially lower (Fig. 1B).

We also analyzed CADM expression data yielded by previous transcriptome-wide microarray and RNA sequencing (RNA-seq) studies. These results, unlike our qPCR results, are not normalized to brain expression, and they

TABLE 2. CADM isoform gene expression

		CADM1	CADM2	CADM3	CADM4	Ref.
Integrated microarray results	Human islets	+++	+	+	+	(32)
	Human β-cells	+++	NM	++	++	
	Rat islets	+++	NM	-	+++	
	Rat β-cells	+++	NM	-	+++	
	INS-1 cells	+++	NM	+	+++	
RNA-seq	Human α -cells	23.89	0.01	0.00	4.04	(8)
	Human β-cells	66.10	0.04	0.03	1.00*	
	Rat islets	7.17	0.28	0.67	1.00*	(24)

Top, Transcript levels yielded from analysis and integration of data from 27 microarray studies; cutoff values as defined in the Beta Cell Gene Atlas: -, no expression; + low expression; ++, moderate expression; +++, enriched expression. NM=not measured. (For details regarding the Atlas, see ref. (32)). **Bottom,** mRNA expression data (RNA-seq) from highly purified human islet α- and β-cells and from whole rat islets. Average FKPM (fragments per kilobase of exon per million reads; normalized as described in the references) were obtained from datasets deposited in NCBI GEO (8, 24). To help interpret relative isoform abundance, expression levels are shown normalized to human β-cell or rat islet CADM4 values (indicated by *).

therefore provide further insights into CADM isoform expression. The Beta Cell Gene Atlas, a compilation of integrated gene expression data calculated from 27 microarray studies (32), confirms that CADM1 transcript levels are enriched in human islets and β-cells (Table 2). In rat islets, purified rat β-cells and INS-1 cells, there is predominant expression of CADM1 and CADM4 (CADM2 data not available). In addition to providing further evidence of the predominance of CADM1 expression, RNA-seq results (Table 2) indicate that CADM1 is the most abundant CADM isoform in rat islets and in both human α- and β-cells. CADM4 mRNA is relatively more abundant in rat islet cells than in human β-cells.

Normalization of islet CADM1 transcript levels to levels in the brain in our qPCR study (Fig. 1A) might have masked the relative abundance in rat islets evident in Table 2. Together, the results in Fig. 1 and Table 2 indicate that CADM1 is the predominant islet isoform and that CADM4 is also enriched in islet cells.

We used FACS analysis to confirm human β-cell expression of CADM1 protein and, since human β-cells are heterogeneous, to ask whether there is a population of β-cells lacking CADM1 expression (4). Human β-cells were uniformly positive for CADM1 expression (Fig. 1C, middle panel). A proinsulin-negative population of cells was also CADM1-positive (Fig. 1C, middle panel, right lower quadrant) while amylase-positive cells were CADM1 negative (panel on right). These results are consistent with prior immunostaining studies showing CADM1 expression in β-cells and other islet endocrine cell types but not in exocrine tissue (30).

CADM1 constrains insulin secretion in INS-1 β-cells. We next asked whether alterations in

CADM1 expression levels would affect insulin secretion. As noted earlier, increased insulin secretion in whole-body CADM1 knockout mice suggests an inhibitory effect (38). Although it seems paradoxical that a component of the membrane secretory apparatus would function to inhibit secretion, granuphilin, tomosyn-2 and a number of other constituents of the submembrane insulin secretory machinery have such an effect, most likely because they participate in a late-stage regulatory mechanism that constrains secretion (17, 26, 40, 63). Our results indicate that CADM1 behaves like these other proteins: its overexpression in INS-1 cells resulted in decreased insulin secretion at basal and stimulating glucose levels (Fig. 2A). Conversely, siRNA-mediated CADM1 knockdown—yielding a mean 91% reduction in CADM1 transcript levels—increased glucose-stimulated insulin secretion (Fig. 2B, C).

Because CADM1 inhibits insulin secretion, its expression may fall in response to glucose stimulation. Such is the case with tomosyn-2 and neurexin: expression of both declines in response to glucose (5, 40). We found that, likewise, CADM1 transcript and protein levels also fall at stimulating glucose concentrations (Fig 2D-F).

CADM1 constrains insulin secretion in primary rat β-cells. We next tested the effect of CADM1 overexpression and knockdown in primary rat islet β cells (Fig 3). The results paralleled that in INS-1 β-cells: overexpression decreased glucose-stimulated insulin secretion (Fig. 3A) while knockdown increased secretion (Fig. 3B). As in α-cells, then, CADM1 in islet β-cells appears to function as an inhibitor of hormone release (23).

Time-course of decrease in CADM1 protein levels. We next asked whether the decrease in CADM1 expression at elevated glucose concentrations (Fig. 2D-F) could be part of the acute response of the β-cell to increased ambient glycemia or a longer-term adaptation. We analyzed CADM1 protein content in INS-1 cells at different times after exposure to a raised, stimulating glucose concentration (Fig. 4). The time-course reveals that CADM1 protein levels did not immediately decline: there was a lag of at least 6 h. Subsequently, levels reach ~50% (48% ± 8%) of starting levels by 24 h (Fig. 4; corresponding change in CADM1 transcript shown in Fig. 2D).

Effect of trans-cellular CADM1 interactions on insulin secretion. In the CNS, CADM molecules on the surface of one neural process bind across the synaptic cleft to CADM molecules on the surface of an apposed process to drive synaptogenesis (13, 47). Such interactions are trans-cellular, occurring between proteins situated on the surfaces of neighboring cells, and can be studied in vitro—as has been previously shown—by coculture with transfected HEK293 or COS-7 cells (13, 35, 50).

We cocultured β-cells in contact with COS-7 cells pre-transfected with CADM1 or, as a control, with empty vector. Coculture of INS-1 cells with COS-7 cells expressing CADM1 increased both glucose-stimulated insulin secretion (Fig. 5A) and potassium-stimulated insulin secretion (Fig 5B), indicative of an effect on insulin secretion downstream of glucose sensing. CADM1 also acted in a trans-cellular manner to influence insulin secretion by rat and human islet β-cells; however, here the result was a decrease rather than increase in insulin secretion (Fig. 5C, D).

Association of CADM1 with the subplasmalemmal insulin secretory apparatus. The short, cytoplasmic, carboxyl-terminal tails of the CADM family members contain motifs for binding to synaptic scaffolding molecules with PDZ type II domains and to members of the protein 4.1 family (6). The latter function as subplasmalemmal hubs that help anchor and organize the submembrane actin cytoskeleton and bind a number of membrane-associated proteins, including regulators of cytoskeleton formation and proteins that drive clustering of exocytic proteins (21). CADM1 promotes assembly of the

presynaptic neurotransmitter secretory apparatus through binding to CASK, a PDZ-domain-containing scaffolding protein (25, 45). We previously found that CASK is expressed in β-cells and, as in neurons, interacts with neurexin (40).

To help determine whether CADM1 affects insulin secretion through direct interactions with the secretory machinery and, in β-cells, whether CADM1 similarly associates with CASK, we immunoprecipitated CADM1 from INS-1 cell lysates. Fig. 6 shows that CADM1 co-precipitated with CASK as well as Munc18 and syntaxin-1A, two additional constituents of the submembrane insulin secretory assembly. Thus, in both β-cells and neurons, CADM1 interacts with constituents of the submembrane protein assemblies that mediate regulated insulin or neurotransmitter secretion (25, 45).

CADM1—as has been demonstrated in a variety of cell types—plays an essential role in cytoskeletal organization and remodeling (10, 11, 39, 44). It helps anchor F-actin to subplasmalemmal sites and binds proteins that regulate actin cytoskeletal dynamics (11, 39). Consistent with CADM1 having parallel function in β-cells, CADM1 co-immunoprecipitated DAL-1 (Fig. 6), a protein 4.1 family member also known as EPB41L3 and protein 4.1B and shown previously to interact with CADM1 in other cell types (10, 44).

Trans-cellular CADM1 interactions enhance syntaxin-1 clustering. In studies of synapse formation, analysis of the punctate immunofluorescent staining of pre- or post-synaptic components of the neurotransmitter signaling machinery is used to follow synaptic maturation. Assembly of the pre-synaptic exocytic protein complexes is accompanied by the clustering of syntaxin-1 or synapsin at discrete sites, and this clustering is signaled by the resultant increased intensity of punctate staining (3, 13, 46). A defining property of synaptogenic proteins such as CADM1 is the induction of such clustering (13, 47).

As in neurons, membrane-associated SNARE and other exocytic proteins cluster during maturation of the islet β-cell secretory machinery (33, 50). We previously found that neuroligin-2 increases syntaxin-1 clustering in β-cells (50). Using the same approach, we asked whether trans-cellular CADM1 interactions would do the same.

A schematic of the "artificial synapse formation assay" used to determine whether proteins drive secretory machinery assembly, adapted as previously described to β-cells, is included in Fig. 10 (50). INS-1 cells were seeded onto pre-transfected COS-7 cells and cocultured overnight. The intensity of INS-1 cell syntaxin-1 puncta in different regions was determined, as was the efficiency of COS-7-cell transfection in the same regions. Image analysis showed that the intensity of syntaxin-1 puncta in INS-1 cells increased in proportion to the level of CADM1 expression in the underlying COS-7 cells (Fig. 7). This suggests that trans-cellular CADM1 interactions drive syntaxin-1 clustering.

Latrunculin counteracts trans-cellular CADM1 inhibition of insulin secretion. CADM1 promotes F-actin assembly and helps anchor the cortical actin network to the plasma membrane. As a result, exocytic sites that assemble around the CADM1 cytoplasmic domain are also sites of actin filament nucleation and of membrane-tethering of the cytoskeleton (11, 39, 60). Because the actin network helps regulate insulin granule trafficking, we asked whether enhancement of local actin network formation might be an additional mechanism through which CADM1 influences insulin secretion.

The effects of the cortical actin network on glucose-stimulated insulin secretion vary between cell types. In primary β-cells, the actin mesh impedes insulin granule trafficking, and F-actin depolymerization contributes to increased glucose-stimulated insulin secretion (22, 53). This is in contrast to poorly granulated β-cell lines such as INS-1, where the actin network does not hinder granule trafficking and its depolymerization does not significantly increase insulin secretion (18, 34, 55). These divergent effects of actin mesh on secretion could help explain why trans-cellular CADM1 interactions

decrease insulin secretion by primary β-cells while increasing secretion by INS-1 cells.

We used latrunculin, an inhibitor of actin polymerization, to test the role of F-actin in CADM1-mediated changes in insulin secretion. In cocultures with INS-1 cells, latrunculin did not change the stimulatory effect of trans-cellular CADM1 interactions on insulin secretion (Fig. 8A). In primary rat β-cells, on the other hand, latrunculin markedly attenuated the inhibitory effect on insulin exocytosis of extracellular CADM1 interactions (Fig. 8B). This suggests that trans-cellular, CADM1-mediated inhibition of insulin secretion in primary β-cells is brought about, at least in part, by effects on the actin network.

Latrunculin increased insulin secretion in rat β-cells co-cultured with control COS-7 cells by ~100% (Fig. 8C, right side, white column). This effect was augmented to ~175% by co-culture with CADM1-transfected COS-7 cells (Fig. 8C, right, black column). Inhibition of actin polymerization, in other words, caused a much greater increase in insulin secretion in primary β-cells co-cultured with CADM1-expressing COS-7 cells than with control cells COS-7 cells. Taken together, these data indicate that trans-cellular CADM1 interactions did indeed influence insulin secretion by primary β-cells in part through effects on the actin cytoskeleton.

DISCUSSION

The submembrane protein complexes that mediate insulin exocytosis in the pancreatic islets and neurotransmitter exocytosis in the brain are nearly identical, so it is natural to wonder whether the mechanisms guiding their formation are also the same (1, 2, 41, 58). In the CNS, assembly of these submembrane protein complexes is guided by "synaptogenic" proteins that interact across the nascent synaptic cleft. There is evidence to suggest that similar trans-cellular interactions help direct the formation of the insulin exocytic machinery. This evidence includes the dependence of β-cell function and maturation on contact between β-cells as well as the tendency for exocytic complexes to form beneath the β-cell plasma membrane at sites where such cell-to-cell contact occurs (12, 16, 27).

Our results show that the synaptogenic protein CADM1 interacts with the submembrane secretory machinery in β-cells and constrains insulin secretion. After an at least 6 h lag, CADM1 protein levels fall following glucose stimulation. Our coculture experiments reveal that trans-cellular CADM1 interactions also influence insulin secretion. CADM1 therefore provides the third example of a synaptogenic, extracellular protein interaction that modulates insulin secretion. The two previously-identified examples involve the proteins neuroligin/neurexin and EphA/Ephrin-A (31, 40).

Extracellular CADM1 interactions are either homophilic or heterophilic with CADM2 (7, 14). We have found that CADM1 is the predominant islet β-cell isoform transcript. CADM4 expression tends also to be enriched in islet cells. In contrast to brain, expression of CADM2 and CADM3 is markedly lower.

Synaptogenic protein interactions are defined by their ability to trigger the formation of pre- or post-synaptic sites ("hemi-synapses") in coculture experiments closely akin to those employed here (13, 47). In neuronal coculture assays—and in the β-cell coculture assays that we adapted from the neuronal system—maturation of submembrane secretory complexes is assessed by using immunofluorescence to analyze punctae of syntaxin-1 or other membrane-associated SNARE proteins. Punctate staining intensity, which increases as exocytic sites assemble, is enhanced by trans-cellular CADM1 interactions in both neural processes and, as now revealed here, in β-cells (3, 7, 13, 46).

Transcellular CADM1 interactions likely promote assembly of the submembrane β-cell secretory complexes—sometimes referred to as "excitosomes"—at sites of β-cell-β-cell contact (37, 43). CADM1 functions in this regard similarly to neurexin, which is also expressed in both β-cells and brain (40). Both proteins recruit

CASK and other exocytic scaffolding proteins to the submembrane exocytic assemblies via a cytoplasmic PDZ-binding motif. Both also co-immunoprecipitate with the key t-SNARE syntaxin-1 and also with constituents of the secretory machinery, such as Munc-18, that enable a late-stage mechanism constraining insulin secretion (40, 62). Consistent with participation in this constraining mechanism, decreased expression of either CADM1 or neurexin increases insulin secretion whereas overexpression of either has the opposite effect (40). CADM1 expression, like that of neurexin, decreases in response to raised glucose levels (40). This glucose effect on CADM1 expression could serve to enhance insulin secretion when ambient glucose levels are persistently elevated.

Coculture experiments allowed the effects of transcellular CADM interactions to be observed without directly altering β-cell CADM1 protein expression. β-cell gene expression and secretory mechanisms were not manipulated, and the observed effects on insulin secretion and sytaxin-1A clustering reflected the endogenous β-cell response to CADM1 expression by neighboring cells. With INS-1 cells, contact with CADM1-expressing COS-7 cells promoted, in addition to syntaxin-1 clustering, increased glucose-stimulated insulin secretion. Consistent with an effect downstream of glucose-sensing, potassium-stimulated insulin secretion was also increased. With rat and human primary β-cells, as is discussed below, glucose-stimulated insulin secretion decreased in CADM1 coculture experiments.

Overexpression and gene silencing experiments yielded the same result in INS-1 cells and primary β-cells: insulin secretion increased as CADM1 expression decreased. In contrast, insulin secretion by INS-1 cells and primary β-cells (rat and human) responded differently in the coculture experiments with CADM1-expressing COS-7 cells. Several explanations for this divergence can be envisioned. First, granuphilin, Munc-18 and other constituents of the submembrane secretory apparatus impart on the exocytic machinery an ability to constrain insulin secretion (17, 26, 40, 63). This built-in inhibitory mechanism provides a potential brake on insulin release: a final control point where insulin release can be checked just prior to membrane fusion (26). Our results suggest that, like neurexin, CADM1 both participates in this inhibitory mechanism and also interacts with and helps drive assembly of the rest of the secretory assembly (40). Because INS-1 cells differ from primary β-cells in important ways, such as having far fewer granules and responding less robustly to glucose, it seems likely that the complex interplay between the pro-secretory and secretion-constraining activities of CADM1 in the coculture experiments could favor the former in INS-1 cells and the latter in primary β-cells (17, 26, 40, 63). As another explanation for the divergent insulin secretion responses, CADM1 is an anchor point for the cortical actin network and promotes its formation at exocytic sites that assemble around the CADM1 cytoplasmic domain (11, 39, 60). The actin network impedes insulin exocytosis in primary β-cells, and, in these cells, we found that pharmacological actin depolymerization rescued insulin secretion from inhibition by CADM1-expressing COS cells. In contrast to primary β-cells, insulin secretion from INS-1 cells is subject to, at most, only minimal inhibition by actin. It is likely that CADM1-influenced F-actin assembly at exocytic sites inhibited insulin secretion by primary β-cells while having no or minimal impact on secretion by INS-1 cells.

The findings reported here, together with our prior findings regarding neurexin and neuroligin, are consistent with the overall hypothesis that β-cell excitosome assembly—in parallel to formation of the closely related presynaptic active sites of secretion—occurs around the intracellular domains of neurexin, CADM1, and perhaps other presynaptic, synaptogenic proteins (Fig. 9) (48, 50). Four such synaptogenic, transmembrane proteins have been found to interact with the insulin secretory machinery: neurexin-1α and -2β, ephrin-A (not shown in Fig 9) and now CADM1 (31, 40). Neurexin-neuroligin and CADM1 interactions across the synaptic cleft induce “lateral” clustering—meaning lateral movement of these proteins through the plasma membrane leading to accumulation at discrete sites—which in turn

triggers synaptogenesis (15, 47). The model in Fig. 9 posits that, in β-cells, similar interactions drive formation of the secretory microdomains.

CADM1 helps organize, anchor and promote formation of the cortical actin network. Interaction with F-actin-binding proteins and inducers of actin filament formation, including DAL-1 (EPB41L3), has been demonstrated here and elsewhere (11, 39, 60). This aspect of CADM1 function is also incorporated in the model (Fig. 9). The model is largely derived from findings in the neurobiology field and provides a framework for further investigations.

In conclusion, our results suggest that, like neurexin-neuroligin and EphA-ephrin-A, CADM1 molecules engage in trans-cellular interactions between β-cells paralleling identical interactions in the central nervous system. As occurs during maturation of the presynaptic machinery for neurotransmitter exocytosis, extracellular CADM1 interactions drive clustering of syntaxin-1, and CADM1 associates with CASK and other components of the submembrane insulin secretory machinery. As in other cell types, CADM1 interacts with the actin-binding protein DAL-1 and by inference with the cortical actin network. CADM1 has a constraining effect on insulin secretion analogous to that of neurexin, granuphilin, and other components of the insulin exocytic machinery, and its expression decreases after glucose stimulation. These results support the idea that functional maturation of the β-cell insulin secretory machinery is guided by a set of the same trans-cellular interactions that trigger the formation and drive the maturation of presynaptic exocytic sites in the CNS. The importance of such interactions for normal β-cell maturation and insulin secretion is underscored by the dependence of β-cell function on contact with other β-cells.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors

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AUTHOR CONTRIBUTIONS

C.Z., T.A.C., M.R.M. and S.D.C. designed the study. C.Z., T.A.C., M.R.M., D.D. and E.J.P. carried out the key experiments. N.W.C. assisted with gene knockdown experiments and data analysis and helped write and edit the manuscript. C.Z. and S.D.C. analyzed data and wrote the manuscript.

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FIGURE CAPTIONS

Figure 1. CADM expression in rat and human β-cells. *A*, mRNAs from INS-1 cells and from rat brain and islets were reverse-transcribed and the expression of each CADM isoform quantified by qPCR. The results are presented as β-cell expression as percent of brain expression, mean +/- SEM. *B*, mRNAs from human brain and islets were analyzed as in *A*. *C*, FACS analysis of dissociated human islet cells. Antibodies to proinsulin were used to tag β-cells. Antibodies to amylase were used to tag pancreatic exocrine cells remaining in the islet preparations. The left panel depicts results with a non-immune control IgY and the remaining two panels show results with anti-CADM1 IgY antibody. The populations of cells tagged by the anti-CADM1 antibody appear to the right of the vertical line in the right two panels. The populations of cells tagged by the anti-proinsulin antibody appear above the horizontal line in the left two panels. (qPCR, n=3 individual preparations assayed in duplicate).

Figure 2. Effect of CADM1 expression level on insulin secretion; glucose-sensitive expression of CADM1. *A*, INS-1 cells were transfected with a CADM1 expression plasmid (black columns) or mock-transfected with empty vector (white columns) and incubated for 48 h followed by 1 h incubation in either 2.75 mM (low) or 16.7 mM (high) glucose with 0.1 mM IBMX. Insulin secretion is shown normalized to total cellular insulin content. *B*, INS-1 cells were transfected for 72 h with pools of either non-targeting, scrambled (white columns) or CADM1 (black columns) siRNAs and then insulin secretion analyzed as in *A*. *C*, RNA was isolated from siRNA-treated INS-1 cells and degree of CADM1 knockdown determined by qPCR. Data are shown normalized to control values obtained using scrambled (SCR) siRNA. *D*, INS-1 cells were incubated for 18 hours in 2.75 mM (low) or 16.7 mM (high) glucose. RNA was isolated from cells and CADM1 transcript levels measured by qPCR. Data are shown as expression level normalized to that in low (2.75 mM) glucose samples. *E*, INS-1 cells were incubated in 2.75 mM or 16.7 mM glucose as in *D* and then CADM1 protein levels analyzed by immunoblot analysis of cell lysates. GAPDH protein was probed as a loading control. *F*, bands from *E* were quantitated. GAPDH-normalized CADM1 levels were normalized to control levels. All data are represented as mean +/- SEM from 6 samples and representative of 3 experiments; qPCR and insulin RIA samples were assayed in duplicate; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

Figure 3. Effect of altering CADM1 expression on insulin secretion by primary rat islet cells. *A,* Dispersed rat islets were transfected with a CADM1-expressing plasmid or mock-transfected (Mock) with empty vector then incubated for 48 h. Insulin secretion was then measured during a 1-h incubation in 16.7 mM glucose with 0.1 mM IBMX. *B*, Dispersed rat islets were transfected for 72 hours with pool of either scrambled (Scr) or CADM1 siRNA. Insulin secretion was measured over a 1-h incubation in 16.7 mM glucose with 0.1 mM IBMX. Total insulin secreted is shown normalized to total cellular insulin content. *C,* To verify effectiveness of CADM1 siRNA, CADM1 mRNA was measured by RT-qPCR. CADM1 mRNA level after treatment with CADM1 siRNA is shown relative to level after treatment with control (scrambled) siRNA. Data are represented as a mean +/- SEM of 3 experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

Figure 4. Kinetics of glucose-induced downregulation of CADM1 protein. INS-1 cells were incubated overnight in 5 mM glucose followed by exposure (at time t=0) to 16.7 mM glucose. *A,* Cell lysates at various time points were collected and equal amounts of protein immunoblotted for CADM1 and a loading control, GAPDH. The immunoblot shown is representative of four independent experiments. *B*, Time points repeated at least 3 times over the course of the separate time-course experiments were quantitated by infrared fluorescent imaging of the immunoblots using a LiCor Odyssey imaging system. Data are shown as CADM1 expression levels normalized to GAPDH and relative to the 0-time point (mean normalized CADM1 band intensity at time 0 is 1). Columns are mean +/- SEM of 3 experiments. ***, $P < 0.005$

Figure 5. Insulin secretion by β-cells cocultured with COS-7 cells expressing CADM1. *A-B,* INS-1 cells were cocultured (CCx) with COS-7 cells pre-transfected with CADM1 expression vector (black columns) or mock transfected with empty (control) vector (white columns; mock). After 24 h, the cocultures were incubated for 1 h in (*A*) 2.75 mM (low) glucose or in 16.7 mM (high) glucose with 0.1 mM IBMX or (*B*) in 2.74 mM glucose supplemented with either 0 (-) or 30 mM (+) KCl. Insulin secreted during this last hour is shown as percent of cellular insulin content. *C*, Islets were isolated from male Sprague Dawley rats and dispersed. Islets were then cocultured with COS-7 cells as in *A* for 24 h followed by 1 h incubation in low or in high glucose with 0.1 mM IBMX. *D*, Human islets were dispersed and then cocultured as in *A* for 24 hours followed by 1 h incubation in low or high glucose with 0.1 mM IBMX. Insulin secreted was normalized to total cellular insulin content. All data are represented as mean +/- SEM from 6 samples assayed in duplicate and representative of 3 experiments; *, $P < 0.05$; ***, $P < 0.005$.

Figure 6. CADM1 interacts with components of the insulin exocytic assembly and the actin-binding protein DAL-1. Immunoprecipitates were prepared from INS-1 cell lysates using an anti-CADM1 antibody or non-immune rabbit IgG (control). Immunoprecipitated proteins were analyzed by western blotting, probing for the proteins indicated to the right of each row. CASK, Munc18 and syntaxin-1 are constituents of the subplasmalemmal insulin secretory machinery. The F-actin binding protein DAL-1, also known as EPB41L3 and protein 4.1B, is known to bind to CADM1 in other cell types. Synaptophysin, unlike the other proteins, is a vesicle-associated protein, not a component of the submembrane secretory apparatus, and no corresponding band was detected—even after adjusting the fluorescent imaging system for maximal sensitivity—during immunoblot analysis of precipitated proteins. (Results representative of three separate experiments).

Figure 7. Coculture with CADM1-expressing COS-7 cells promotes clustering of syntaxin-1. INS-1 cells were cocultured with COS-7 cells expressing FLAG-tagged CADM1 (FLAG-CADM1) or, as a negative control, with FLAG-tagged CASPR2 (FLAG-CASPR2). After 24 h, cells were fixed and stained for syntaxin-1 and for the FLAG epitope. Stained cocultures were imaged in small, non-overlapping fields and the following were determined for each field: a) percentage of field area staining positive for FLAG and b) the average intensity of the syntaxin puncta. These values were normalized to their respective maximum values and plotted for the FLAG-CASPR2 (A) and the FLAG-CADM1 (B) cocultures. There was no correlation between the intensity of the syntaxin puncta in INS-1 cells (Y axis) and the level of FLAG-CASPR2 expression by the cocultured COS-7 cells (X axis; panel A). In contrast, with FLAG-CADM1-expressing COS-7 cells, the average immunofluorescent intensity of the syntaxin puncta in INS-1 cells increased in proportion to the FLAG-CADM1 expression level (the percentage of the underlying area staining for CADM1); $P<0.01$. Data from three separate experiments are shown together here; the same relationship between transfection efficiency and intensity of syntaxin-1 punctae is also present when the three experiments are analyzed separately.

Figure 8. Effect of latrunculin on CADM1-induced changes in insulin secretion. *A-B,* INS-1 cells (*A*) and cells from dissociated rat islets (*B*) were cocultured (CCx) with COS-7 cells expressing CADM1 (black columns, CCx-CADM1) or with mock-transfected COS-7 cells (white columns, CCx-mock). After 24 h, cells were incubated for 30 min in high (16.7 mM) glucose with 0.1 mM IBMX with or without latrunculin. Insulin secretion (as % of cellular content) is shown. *C*, The percent increase in insulin secretion caused by latrunculin in control cocultures (mock-transfected, white columns) and CADM1 cocultures (black columns) is shown. The left two columns and the right two columns of *C* show the latrunculin-induced percent increase in insulin secretion by INS-1 cells and by rat islet cells, respectively. Note that latrunculin caused a greater percent increase in insulin secretion by rat islet β cells in CADM1 cocultures than by rat islet β cells in control cocultures (compare the rightmost two columns in *C*). What appears to be a slight latrunculin-induced increase in insulin secretion by INS-1 cells (left two columns) was not statistically significant. All data are represented as mean +/- SEM from 6 samples and representative of 3 experiments; insulin RIA samples were assayed in duplicate; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

Figure 9. Proposed model of a site of cell-cell contact between β-cells. Trans-cellular CADM1-CADM1 and neuroligin (NL)-neurexin (NRX) binding interactions are depicted in the extracellular space. Also shown is the interaction of CADM1 and neurexin-1α and -2β (NRX 1a/2b) with submembrane exocytic proteins such as syntaxin-1 and with other constituents of the exocytic complex, such as Munc18, that can constrain secretion. The membrane domain in red (through which neurexin passes) and the underlying exocytic proteins represent an exocytic microdomain (excitosome). CADM1, like neurexin, binds the scaffolding protein CASK and, both directly and indirectly, other components of the submembrane insulin exocytic machinery. Granuphilin, a component the secretory protein complex that acts to constrain insulin secretion, is also associated—either directly or indirectly—with CADM1 and neurexin. CADM1 also binds proteins (EPB41L3/DAL-1 and FARP) that regulate the assembly of and help anchor the cortical actin network. Neuroligin-2 passes through an as-yet unidentified membrane domain (yellow) and binds the postsynaptic scaffolding protein gephyrin.

Figure 10. Method of analysis of syntaxin-1A clustering. COS-7 cells in culture dishes (top left; illustration not to scale) were transfected with FLAG-tagged CADM1 or with the non-synaptogenic, transmembrane protein CASPR2 (control), also FLAG epitope-tagged. INS-1 cells were cultured for 24 h on the COS-7 cells. Eight randomly-selected regions were analyzed per dish in blinded fashion. If expression of the FLAG-tagged protein by COS-7 cells increases the intensity of syntaxin-1A punctae in contacting INS-1 cells, then mean syntaxin-1A intensity in each region will vary as a function of the proportion the region stained positively with an anti-FLAG antibody. Details regarding co-culture and use of this assay to analyze neuroligin-2 have been previously published (50, 61). The assay is adapted from the artificial synapse formation/coculture assay originally described by Schieffele et al. in 2000 and now routinely utilized to identify and study synaptogenic proteins (such as CADM1) (13).

FIGURE 1.

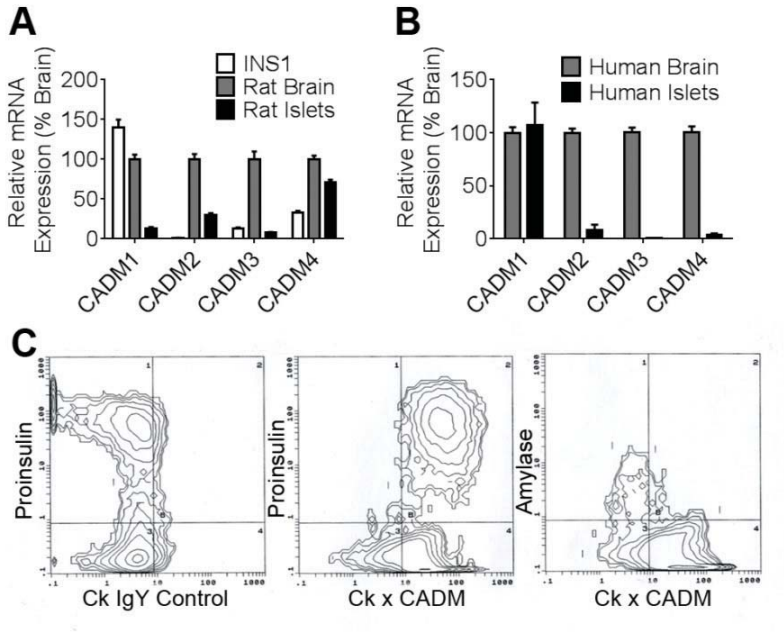

A
Relative mRNA Expression (% Brain)
200
150
100
50
0
INS1
Rat Brain
Rat Islets
CADM1
CADM2
CADM3
CADM4
B
Relative mRNA Expression (% Brain)
150
100
50
0
Human Brain
Human Islets
CADM1
CADM2
CADM3
CADM4
C
Proinsulin
Ck IgY Control
Proinsulin
Ck x CADM
Amylase
Ck x CADM

FIGURE 2.

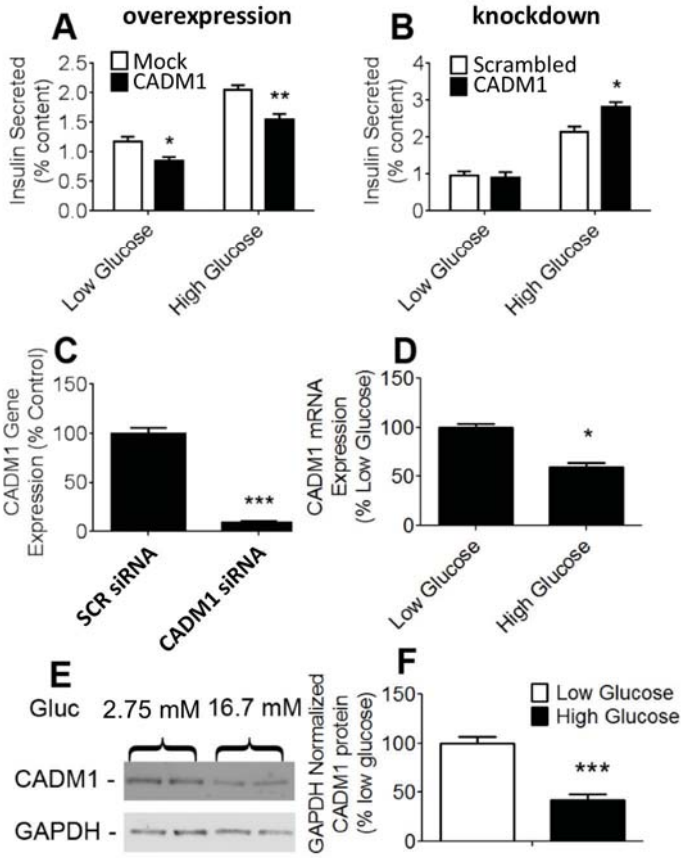


FIGURE 3.

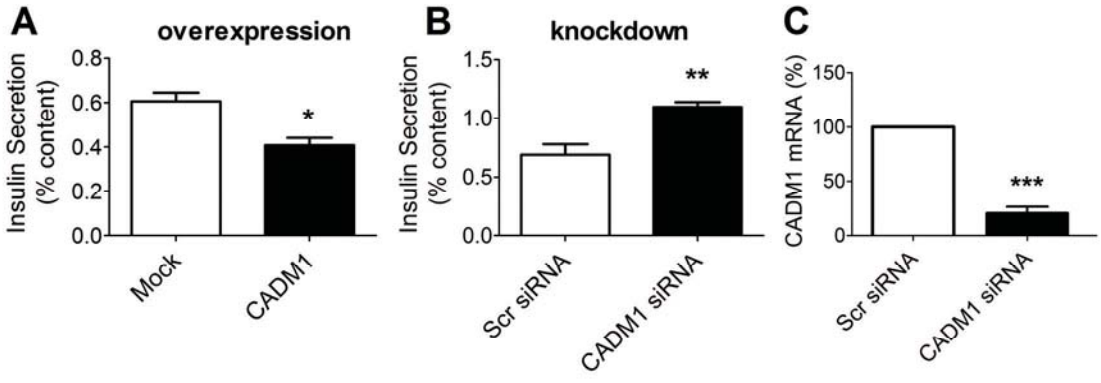


Figure 4.

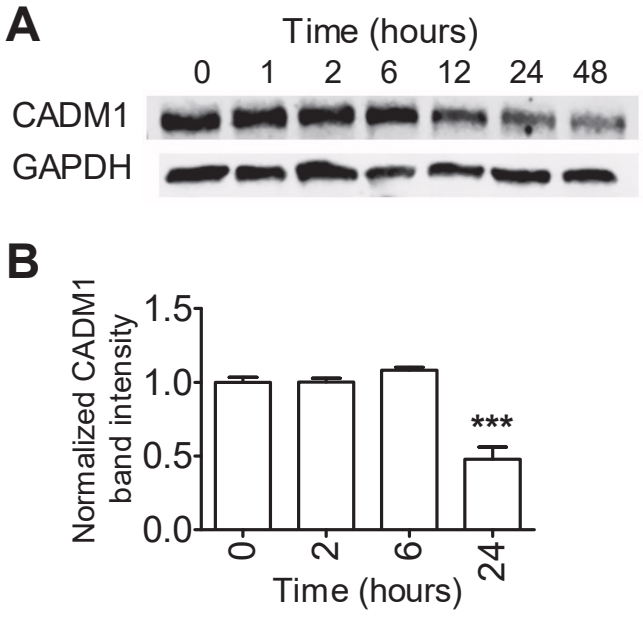

A
Time (hours)
0 1 2 6 12 24 48
CADM1
GAPDH
B
Normalized CADM1 band intensity
1.5
1.0
0.5
0.0
***
0
2
6
24
Time (hours)

Figure 5.

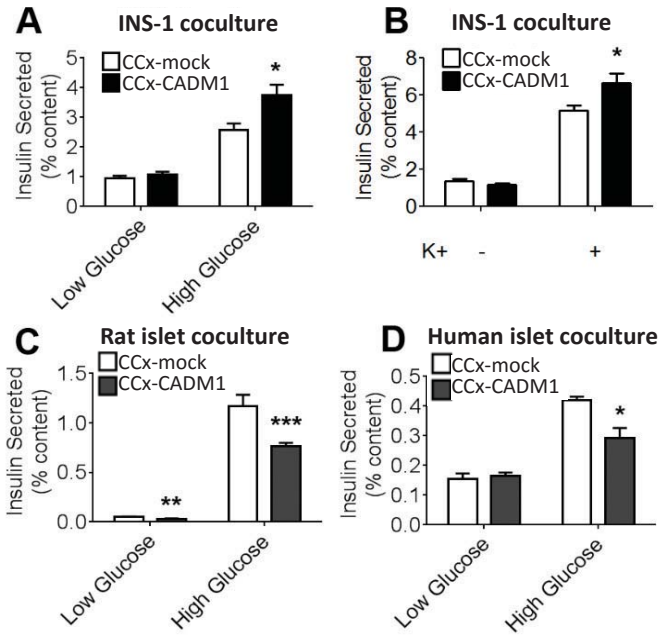

A
INS-1 coculture
CCx-mock
CCx-CADM1
Insulin Secreted (% content)
Low Glucose
High Glucose
B
INS-1 coculture
CCx-mock
CCx-CADM1
Insulin Secreted (% content)
K+ - +
C
Rat islet coculture
CCx-mock
CCx-CADM1
Insulin Secreted (% content)
Low Glucose
High Glucose
D
Human islet coculture
CCx-mock
CCx-CADM1
Insulin Secreted (% content)
Low Glucose
High Glucose

FIGURE 6.

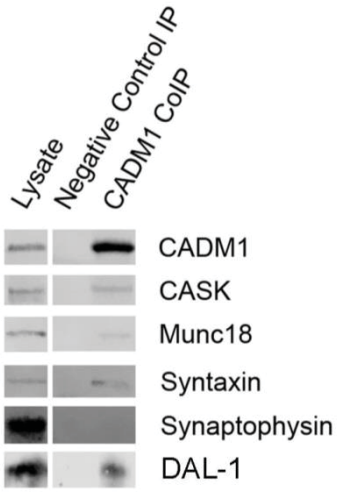


FIGURE 7.

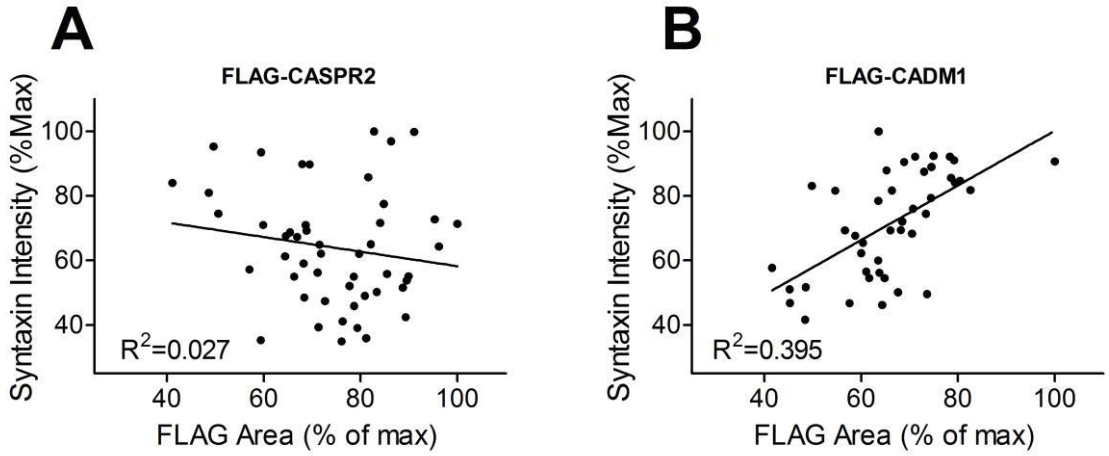


FIGURE 8.

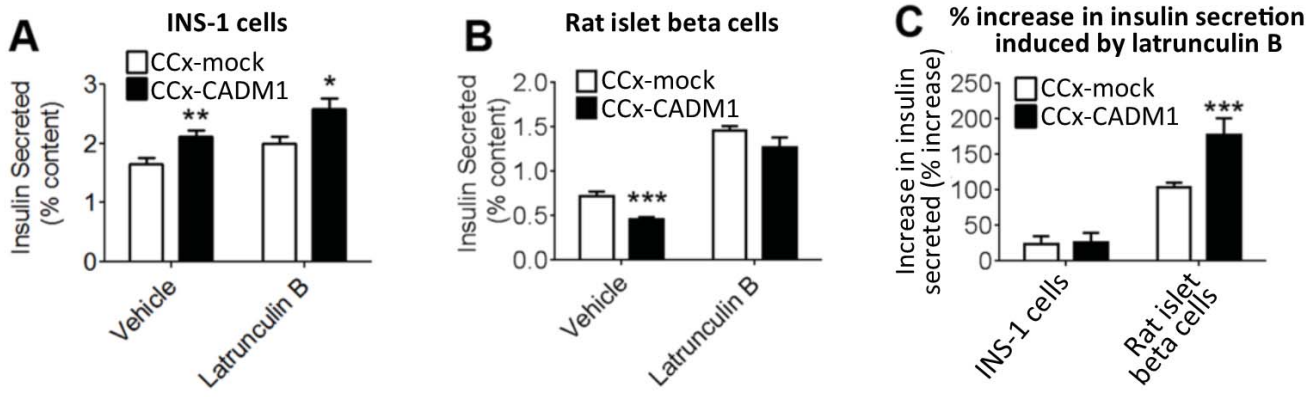
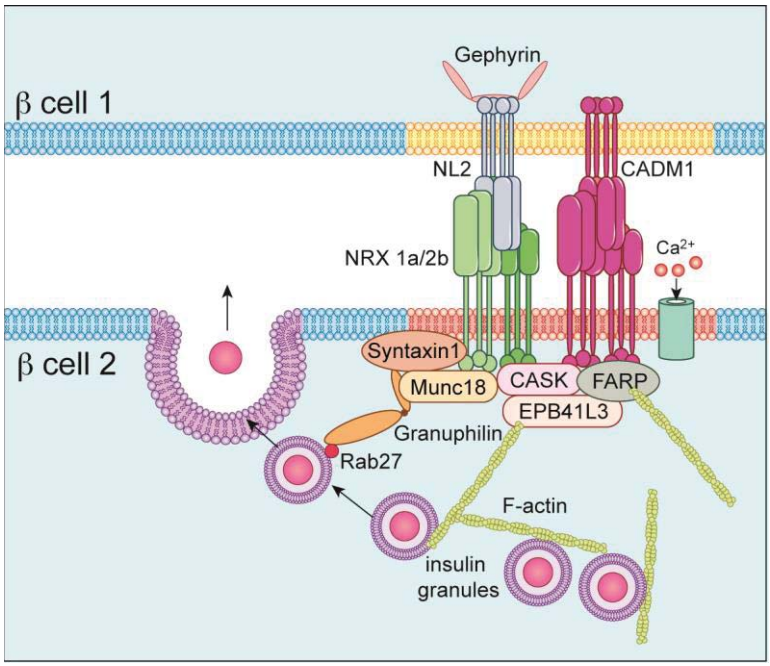
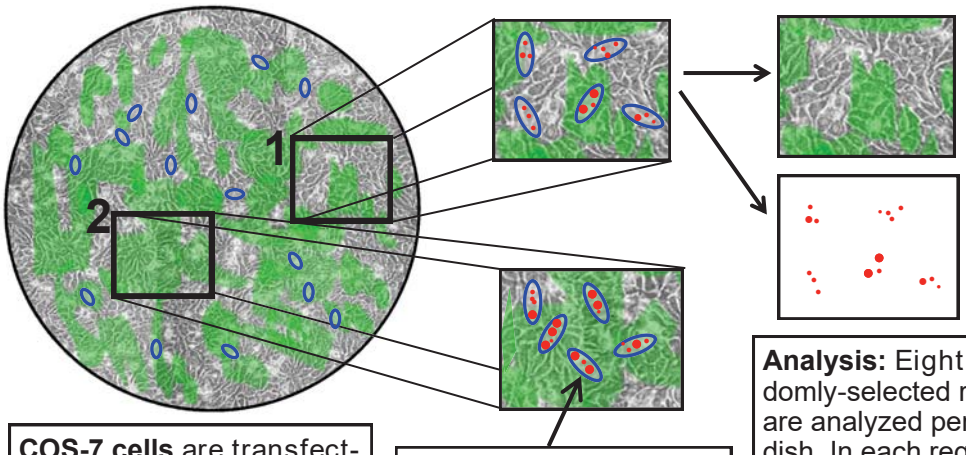

A
INS-1 cells
CCx-mock
CCx-CADM1
Insulin Secreted (% content)
3
2
1
0
**
*
Vehicle
Latrunculin B
B
Rat islet beta cells
CCx-mock
CCx-CADM1
Insulin Secreted (% content)
2.0
1.5
1.0
0.5
0.0
***
Vehicle
Latrunculin B
C
% increase in insulin secretion induced by latrunculin B
Increase in insulin secreted (% increase)
250
200
150
100
50
0
CCx-mock
CCx-CADM1
***
INS-1 cells
Rat islet beta cells

FIGURE 9.

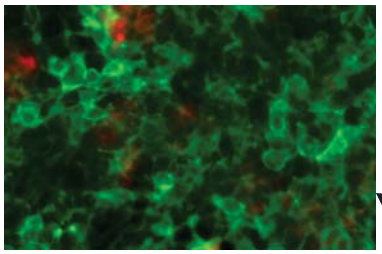

β cell 1
β cell 2
Gephyrin
NL2
CADM1
NRX 1a/2b
Ca2+
Syntaxin1
Munc18
CASK
FARP
EPB41L3
Granuphilin
Rab27
F-actin
insulin
granules



COS-7 cells are transfected with FLAG-tagged CADM1 or CASPR2 prior to co-culture for 24 h with INS-1 cells (**blue**). COS-7 cells expressing the transfected protein are stained **green** using an anti-FLAG antibody.

INS-1 β-cells are depicted in **blue**. Immunofluorescent staining is used to detect the FLAG-tagged, transfected protein in the COS-7 cells (**green**) and the β-cell protein syntaxin-1A (**red**). Stained syntaxin punctae have different intensities depending on the syntaxin content of each punctate site (i.e., degree of clustering).

Analysis: Eight randomly-selected regions are analyzed per culture dish. In each region, imaging software measures the proportion of the region covered by transfected COS-7 cells (*top*) and the immunofluorescent signal intensity of each pixel of INS1-cell syntaxin-1A staining (*bottom*). The question is: in each region, does the mean syntaxin-1A intensity correlate with the proportion of transfected COS-7 cells?



Example of immunofluorescence staining: transfected cells (green) are detected by staining for the FLAG epitope. Syntaxin in the co-cultured ß-cells stains red.