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Evidence that an Isoform of Calpain-10 Is a Regulator of Exocytosis in Pancreatic β-Cells

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Calpain-10 (*CAPN10*) is the first type 2 diabetes susceptibility gene to be identified through a genome scan, with polymorphisms being associated with altered *CAPN10* expression. Functional data have been hitherto elusive, but we report here a corresponding increase between *CAPN10* expression level and regulated insulin secretion. Pancreatic β-cell secretory granule exocytosis is mediated by the soluble *N*-ethylmaleimide-sensitive fusion protein attachment receptor protein complex of synaptosomal-associated protein of 25 kDa (SNAP-25), syntaxin 1, and vesicle-associated membrane protein 2. We report, for the first time, direct binding of a calpain-10 isoform with members of this complex. Furthermore, SNAP-25 undergoes a Ca^{2+}-dependent partial proteolysis during exocytosis, with calpain protease inhibitor similarly suppressing both insulin secretion and SNAP-25 proteolysis. Based upon these findings, we postulate that an isoform of calpain-10 is a Ca^{2+}-sensor that functions to trigger exocytosis in pancreatic β-cells. (*Molecular Endocrinology* 19: 213–224, 2005)

AS GENOME SCANS uncover novel genes implicated in polygenic disease, there is an urgent need to prove biological credibility of the candidate gene. This is especially important as, after the discovery of any new disease-associated gene, there is frequently a plethora of linkage and association studies both confirming and refuting the original report. The molecular genetics of type 2 diabetes (T2D) has been extensively studied with more than 20 genome scans. *CAPN10* is the only gene to be identified thus far by this approach and has been found to account for 14% of the population-attributable risk to the disease in Mexican Americans and 4–6% in other ethnic groups (1). Furthermore, subjects homozygous for a disease-associated UCSNP43 G-allele genotype correlated with a reduction in *CAPN10* mRNA level in skeletal muscle compared with heterozygotes. Many investigators have subsequently found associations between *CAPN10* polymorphism and T2D as well as insulin action, insulin secretion, aspects of adipocyte biology, and microvascular function (1–11). However, this has not always been with the same single-nucleotide polymorphism (SNP) or haplotype or the same phenotype, suggesting there might be more than one disease-associated *CAPN10* variant and that these might vary between ethnic groups and the phenotype under study.

The calpains are a family of calcium-dependant, nonlysosomal cysteine proteases. They work by partial proteolysis, resulting in either activation or inhibition of substrate function and, as such, have been postulated to be an integral component of cell signaling. As a consequence of their wide-ranging substrate specificities, calpain family members are implicated in the development of numerous disease processes including ischemic stroke, traumatic brain injury, rheumatoid arthritis, cataract formation, Alzheimer's disease, limb-girdle muscular dystrophy 2A, and T2D (1, 12–14). Pharmacological calpain inhibition has been shown to have affects on adipocytes, skeletal muscle, and pancreatic islets; all tissues important to T2D. Interpretation of pharmacological studies is far from straightforward, however; *e.g.* short-term incubation of islets (4 h) was shown to enhance the insulin-secretory response (15) whereas inhibition is seen after longer (48 h) incubation (16). Because at least 14 human calpain proteins have been identified thus far, of which eight are ubiquitously expressed, it cannot be assumed from pharmacological intervention alone that calpain inhibition is specifically affecting calpain-10. We therefore sought to address whether calpain-10

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Abbreviations: FITC, Fluorescein isothiocyanate; HRP, horseradish peroxidase; PVDF, polyvinylidine difluoride; SNAP-25, synaptosomal-associated protein of 25 kDa; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein attachment receptor; T2D, type 2 diabetes; t-SNARE, target SNARE; VAMP, vesicle-associated membrane protein; v-SNARE, vesicle-SNARE.

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might be an important determinant in insulin secretion by augmenting traditional pharmacological approaches with a combination of molecular cell biology and imaging techniques that are specific for calpain-10. This is one of the first reports detailing a molecular mechanism of action of calpain-10 pertinent to T2D and provides evidence for the involvement of an isoform of calpain-10 in exocytosis in pancreatic β-cells.

RESULTS

Calpain-10 Is Localized in Pancreatic Islets

Calpains are widely expressed, with both ubiquitous and tissue-specific isoforms. The highest expression of calpain-10 mRNA is found in human heart, followed by the pancreas (1). Using immunolocalization, in conjunction with a well-characterized anticalpain-10 antibody that has no observed cross-reactivity with other calpain species (14), we sought to examine the cellular distribution of calpain-10 using serial-sectioned pancreas. We observe labeling only within islet cells (Fig. 1) and not in either the exocrine pancreas or ductal tissue. The density of labeling was heterogeneous and localized to cells distributed throughout the islets in specimens from six nondiabetic human subjects. Calpain-10-positive staining was located within insulin-containing cells and also other islet cell types.

CAPN10 Expression Is Glucose Sensitive

Because *CAPN10* is expressed in human islets and is implicated in T2D, we sought to determine whether expression of this protein might also be influenced by glycemic environment. We cultured INS-1 pancreatic β-cells in a chronic high-glucose environment (28 mM glucose) for various periods of time and examined calpain-10 protein levels by Western blotting whole-cell lysates (normalized for protein) with anticalpain-10 antibody. Calpain-10 production rose substantially after as little as 3.5 d exposure to high glucose, with a further gradual increase continuing over the time points observed (Fig. 2).

Calpain-10 Isoforms Are Differentially Localized to Cytosol and Membrane Compartments

As a prelude to functional studies, we examined isoform prevalence and compartmentalization. This was achieved by subcellular fractionation and immunoblotting with anticalpain-10 antibody. Although it should be noted that only 25% of cytosolic sample was loaded onto the gel as opposed to 100% of membrane sample, Fig. 3A shows the major isoforms present in INS-1 cells. We observe a band of approximately 64 kDa confined to the cytosol, and bands of about 72, 54, and 26 kDa partitioning to differing extents between cytosol and membranes. The subcellular distribution of calpain-10 has been further studied using immunofluorescence (Fig. 3B). In agreement with our fractionation data, calpain-10 staining was consistent with localization to both cytosol and membrane compartments.

CAPN10 Overexpression Is Associated with Increased Regulated Insulin Secretion

Whereas glycemic environment clearly influences *CAPN10* expression, high glucose has been shown to alter the gene expression profile of at least 180 genes in pancreatic β-cells (17, 18). Given this complex pleiotropic response, we sought to determine the con-

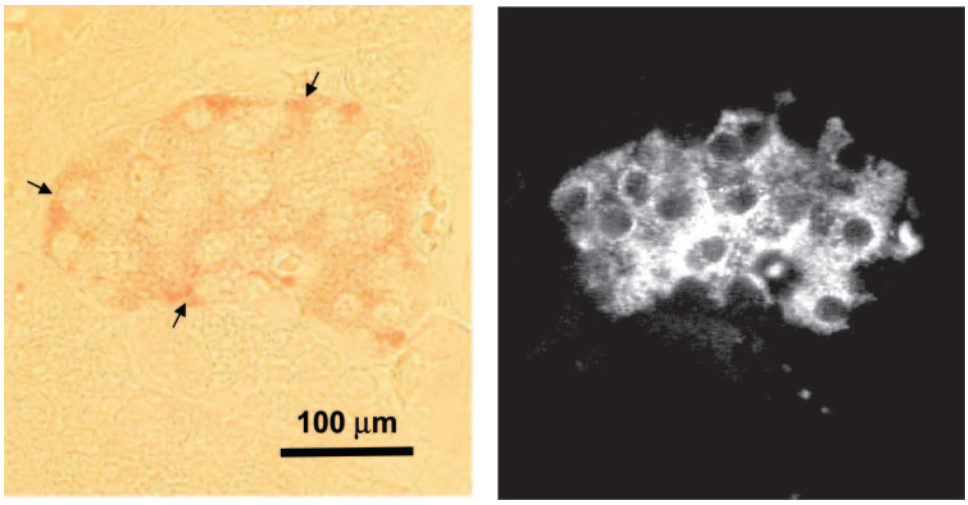

Fig. 1. Distribution of Calpain-10 in Human Islets

Serial sections of human pancreatic tissue labeled for calpain-10 (*left panel, brown*) and insulin (*right panel, white*). Calpain-10-positive staining is intraislet and largely, although not exclusively (as indicated by *arrows*), within insulin-containing cells. *Scale bar*, 100 μm.

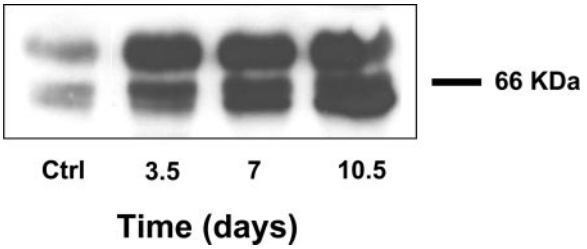

Fig. 2. CAPN10 Expression Is Glucose Sensitive

INS-1 pancreatic β-cells were cultured in either standard (11.7 mM glucose; Ctrl) or elevated glucose (28 mM glucose) for the times indicated. Calpain-10 was detected by immunoblotting with anticalpain-10 antibody in conjunction with ECL chemiluminescence. *CAPN10* expression is sensitive to glycemic environment.

sequence of elevating calpain-10 protein level alone. A cDNA vector containing human *CAPN10* was introduced into INS-1 cells, and Fig. 4A is a representative Western blot of cell lysates resulting from three such clones, along with nontransfected INS-1 control cells. As the *CAPN10* cDNA was subcloned into a vector containing Xpress tag, we were able to screen with antibody raised against this epitope, and hence pick up calpain-10 solely expressed from this vector alone without background interference from endogenous calpain-10. By selecting multiple stable clones that overexpress *CAPN10* to different extents, we have been able to gain more precise functional information than would be possible from studying populations of transiently transfected cells, in which specific expression levels cannot be disseminated from the heterogeneous population. Calpain-10 protein level varies between the three clones: clone 1 shows moderate expression, clone 19 shows strong expression, and clone 18 is intermediate between the two.

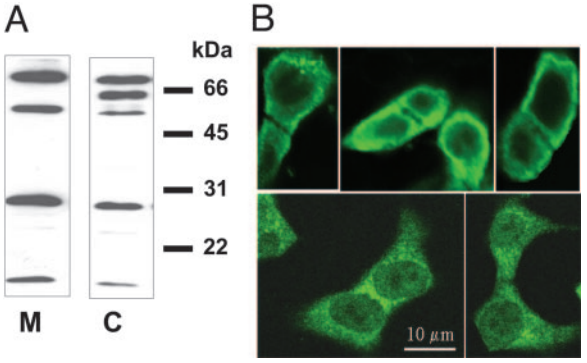

Fig. 3. Differential Association of Calpain-10 Isoforms between Membrane and Cytosol

A, Cells were scraped from dishes, homogenized, and centrifuged to separate membrane (M) and cytosol (C) fractions. Specific isoforms were detected by immunoblotting with anticalpain-10 antibody. B, Calpain-10 was visualized using anticalpain-10 antibody in conjunction with swine antirabbit FITC. Slides were mounted and fluorescence viewed under a laser scanning confocal microscope equipped with a 488-nm laser excitation filter for FITC. *Scale bar*, 10 μm. Calpain-10 exhibits both cytosolic and plasma membrane localization. Individual isoforms have different membrane affinities.

Insulin secretion from all three clones showed no increase in the low rate of unstimulated basal secretion above control (Fig. 4B, Unstim), but when we examined the effect of overexpression on the extent of secretagogue-stimulated secretion (Fig. 4B, Stim) we saw increases above control that correspond with increasing calpain-10 protein. In the case of clone 19, stimulated secretion of insulin was more than double that of control ($P = 0.005$). This effect was not due to any nonspecific stimulatory action of calpain-10 on the secretory pathway *per se*, because, were this the case, we would have seen an enhancement of basal secretion from overexpressing cells. Likewise, the increased insulin secretion is not a reflection of increased insulin production, as this was seen not to change (data not shown). The regulated secretory pathway in pancreatic β-cells is composed of two elements, ionic and nonionic (19). The ionic component is induced by the opening of Ca^{2+}-channels in response to membrane depolarization (which in itself is a result of the closure of ATP-dependent K^+-channels). The nonionic component is less well characterized but results from an intracellular glucose-induced augmentation of secretion that does not involve ion channel modulation. By comparing the stimulatory effects of addition of secretagogue cocktail to cells in the presence (Fig. 4B) or absence (Table 1) of extracellular Ca^{2+}, we have been able to determine the effects of *CAPN10* overexpression on the regulated pathway. In the absence of extracellular Ca^{2+}, insulin secretion was no longer enhanced by *CAPN10* overexpression when cells were stimulated with secretagogue cocktail, indicative of calpain-10 functioning on the ionic component of the regulated secretory pathway in pancreatic β-cells.

To determine how overexpression might affect isoform distribution and intracellular localization, cell extracts were analyzed by Western blot using antibody raised against endogenous calpain-10 (Fig. 4C). Densitometry revealed that the greatest increase in calpain-10 was observed for the approximately 54-kDa membrane (Fig. 4C; *white arrow*) and 64 kDa cytosolic (Fig. 4C; *black arrow*) isoforms, 100% and 64% increases, respectively. This extent of overexpression lies comfortably within the range of expression we observed in response to high glucose (Fig. 2).

Calpain Inhibition Also Inhibits Insulin Secretion

CAPN10 overexpression has a significant impact in increasing the extent of Ca^{2+}-dependent stimulated secretion (Fig. 4B), but is calpain itself essential for exocytosis of insulin? To address this question we examined the effects on secretion of the cysteine protease inhibitor E64, which is known to inhibit the calpain family, including calpain-10. Preincubation (48 h)

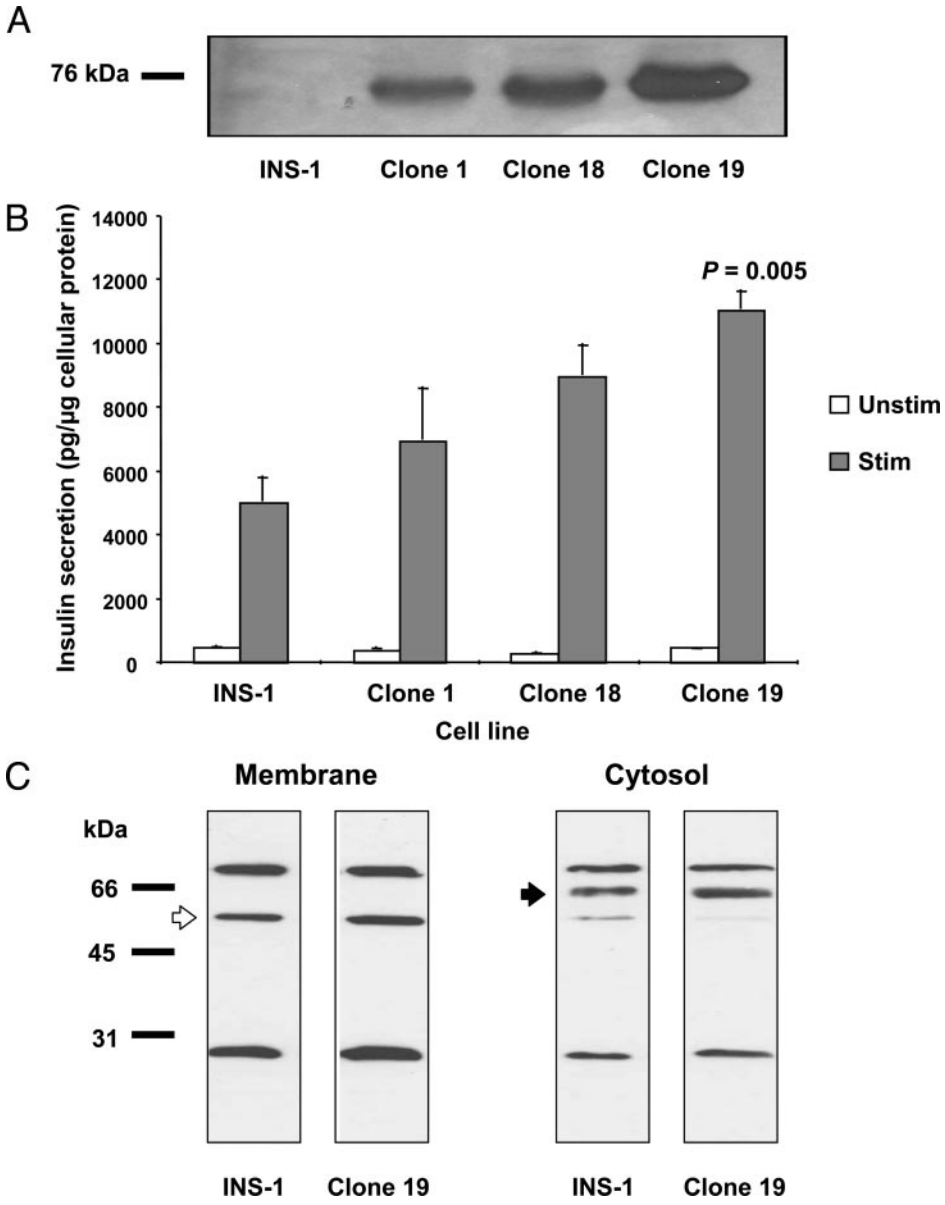

Fig. 4. *CAPN10* Expression Influences Insulin Secretion

A, Calpain-10 protein level in selected clones of stably transfected INS-1 cells. The presence of tagged calpain-10 emanating from the *CAPN10*-containing pcDNA3 vector was detected by immunoblotting with anti-Xpress antibody and quantified after ECL chemiluminescent detection. Individual clones vary in their extent of *CAPN10* expression. B, Insulin secretion under unstimulated (Unstim) and stimulated (Stim) conditions. Cells were incubated for 3 h in Krebs-Ringer solution plus or minus insulin secretagogue cocktail. Supernatant was collected and insulin secretion quantified by RIA. Cellular protein content was assayed from unstimulated cells and used to normalize secretion data. Increasing *CAPN10* expression level corresponds with increasing regulated insulin secretion. C, Cells were scraped from dishes, homogenized, and centrifuged to separate membrane (M) and cytosol (C) fractions. Specific isoforms were detected by immunoblotting with anticalpain-10 antibody. Overexpression was most pronounced for the approximately 54-kDa membrane isoform (*white arrow*) and the approximately 64-kDa cytosolic isoform (*black arrow*). Increasing *CAPN10* expression corresponded with increasing regulated secretion.

of INS-1 cells with E64 had no effect on basal secretion (Fig. 5A, Unstim) but markedly reduced stimulated secretion (Stim) in both control and overexpressing cells (Clone 19); this inhibition was dose dependent with 200 μM causing 47% inhibition from control cells and a 33% inhibition from overexpressing clone 19 cells, and 1 mM E64 resulting in complete inhibition of stimulated secretion from both sets of cells.

We next sought to determine whether E64 inhibition might reflect a direct action on the secretory granule fusion machinery docked at the plasma membrane (which mediates immediate exocytosis, sometimes

Table 1. In the Absence of Extracellular Ca^{2+} Insulin Secretion Is No Longer Augmented by *CAPN10* Overexpression

	Insulin Secretion in the Absence of Extracellular Ca^{2+} (pg insulin/μg cellular protein)			
	INS-1	Clone 1	Clone 18	Clone 19
Unstim	23.4 ± 5.0	13.5 ± 2.6	17.6 ± 0.5	15.2 ± 38.3
Stim	149.6 ± 20.9	77.7 ± 11.1	129.4 ± 38.3	111.8 ± 14.5

Cells were incubated for 3 h in Krebs-Ringer solution minus Ca^{2+} plus or minus insulin secretagogueue cocktail. Supernatant was collected and insulin secretion was quantified by RIA. Cellular protein content was assayed from unstimulated (Unstim) cells and used to normalize secretion data. *CAPN10* expression level does not correlate with the extent of insulin secretion in the absence of extracellular Ca^{2+}. Stim, Stimulated.

also referred to as "first-phase secretion"), or the machinery involved in mobilization of newly formed or recycling granules ("second-phase secretion"). This was achieved by repeating E64 experiments over a shorter (30 min) stimulation period in which the total amount of insulin secreted will represent all granules undergoing immediate (first-phase) exocytosis, but a much smaller fraction of those undergoing second-phase secretion. Therefore, if calpain inhibition affects purely second-phase secretion, we should observe a diminished percentage inhibition by E64 over this shorter stimulation period. Conversely if we observe potent inhibition during this period, while not ruling out the possibility of additional actions at more distal sites, this will nonetheless reflect an action of cysteine protease on protein(s) involved in processes integral to immediate exocytosis, such as the fusion of predocked granules. As preincubation with 200 μM E64

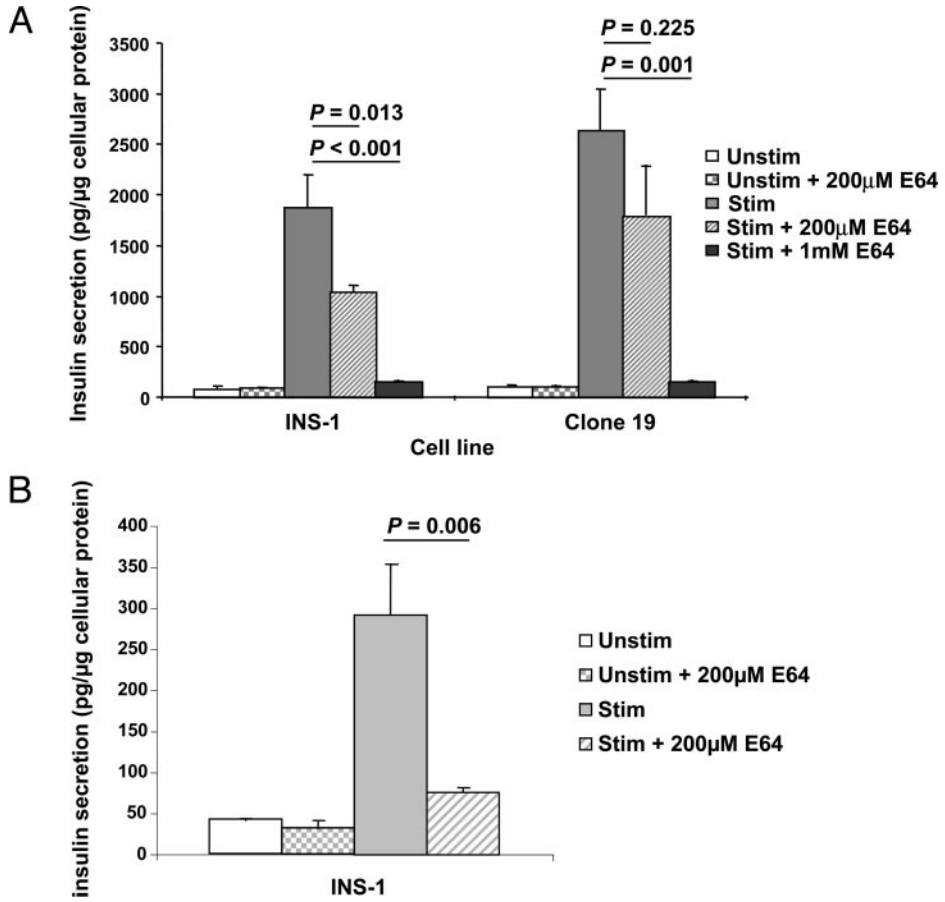

Fig. 5. Calpain Inhibition Abolishes Regulated Insulin Secretion

A, Cells were preincubated for 48 h in the presence of E64 where indicated and then incubated in Krebs-Ringer solution plus or minus insulin secretagogue cocktail for 3 h (in the continued presence or absence of E64). Supernatant was collected, and insulin secretion was quantified by RIA. Cellular protein content was used to normalize secretion data. B, Cells were treated as in panel A, except for receiving a shorter 30-min stimulation period. Insulin secretion shows dose-dependent inhibition by the calpain inhibitor E64.

results in 86% inhibition of stimulated secretion (Fig. 5B) over the shorter stimulation period, this indicates that we are, in fact, looking at inhibition of immediate exocytosis.

Association of Calpain-10 with the Exocytotic Fusion Machinery

To investigate potential interactions between membrane-associated isoforms of calpain-10 and the secretory granule fusion machinery, we immunoprecipitated cell lysates with antibodies raised against either of the plasma membrane target soluble *N*-ethyl maleimide-sensitive fusion protein attachment receptors (t-SNARE), synaptosomal-associated protein of 25 kDA (SNAP-25) or syntaxin 1, and then ran the respective samples on denaturing SDS-PAGE gels and immunoblotted with anti-calpain-10 antibody. As can be seen in Fig. 6, immunoprecipitation of either SNAP-25 or syntaxin 1 resulted in the concomitant precipitation of the approximately 54-kDa isoform of calpain-10. Similarly, immunoprecipitation with calpain-10 and subsequent blotting with antibody against t-SNARE also confirmed this positive interaction (data not shown). Although numerous SNARE-interacting proteins have recently been identified (reviewed in Ref. 20) this is, to our knowledge, the first demonstration of binding of any protease with SNARE complex in intact cells.

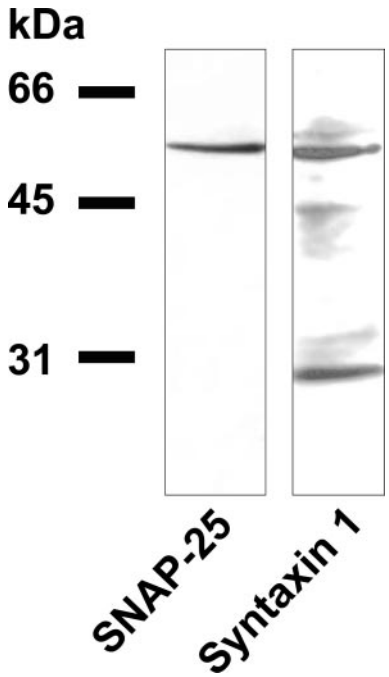

Fig. 6. Association of Calpain-10 with SNARE Complex Family Members

Cell lysates were immunoprecipitated using antibody raised against either SNAP-25 or syntaxin 1. Calpain-10 binding to the respective plasma membrane t-SNARE was examined by immunoblotting with anticalpain-10 antibody, in conjunction with ECL chemiluminescence. There is coimmunoprecipitation of the approximately 54-kDa isoform of calpain-10 with SNAP-25 and syntaxin 1.

SNAP-25 Is Partially Proteolysed during Exocytosis

How might the presence of calpain-10 in the plasma membrane SNARE complex drive exocytosis? To address this question, we first sought to ascertain whether any members of this complex were proteolysed during exocytosis. Whereas neither syntaxin 1 nor the vesicle soluble *N*-ethyl maleimide-sensitive fusion protein attachment receptor (v-SNARE) vesicle-associated membrane protein 2 (VAMP2) showed any sign of proteolysis (data not shown), this was not the case for SNAP-25. Under resting conditions (Fig. 7A; Unstim) SNAP-25 was found largely as a single band of approximately 29 kDa [consistent with previous observations of SNAP-25 in rat islets (21)]. When cells were stimulated to secrete, subsequent immunoblotting with antibody against the N terminus of SNAP-25 (Fig. 7A; *left panels*) revealed a significant proteolysis of membrane-associated SNAP-25 during a 30-min secretagogue stimulation, which was shown by densitometry to represent a turnover of 6–10% of the total cellular pool of SNAP-25. This did not occur when extracellular Ca^{2+} was absent from the media (Fig. 7B; *left panel*, *right hand lane*), thereby implicating the involvement of a Ca^{2+}-dependent protease in the proteolysis of SNAP-25. When we repeated this experiment using an antibody raised against the C terminus of SNAP-25, we observed a different result (Fig. 7A; *right panels*). Although there was evidence of limited proteolysis under resting (Unstim) conditions, in this case, rather than seeing full degradation of SNAP-25 during stimulated secretion (Stim), we saw membrane-associated SNAP-25 being cleaved to an approximately 20-kDa band. The fact that this partial proteolysis was not observed using antibody raised against the N terminus (Fig. 7A; *left panel*) enabled us to conclude that the site of cleavage was, in fact, located within the N-terminal region of SNAP-25.

Given that the approximately 54-kDa isoform of calpain-10 is bound to the SNARE complex, and that no other protease has thus far been reported to bind SNARE complex, calpain-10 might be the protease that cleaves SNAP-25. If true, then SNAP-25 proteolysis will be as similarly sensitive to the cysteine protease inhibitor E64 as was regulated secretion (Fig. 5). Western blot analysis of SNAP-25 from isolated membranes of cells preincubated with E64 clearly showed that inhibition of calpain results in the parallel inhibition of SNAP-25 proteolysis, even during a prolonged 3-h stimulation of exocytosis (Fig. 7B; *right panel*). We therefore propose that calpain-10 is the likely protease responsible for this effect.

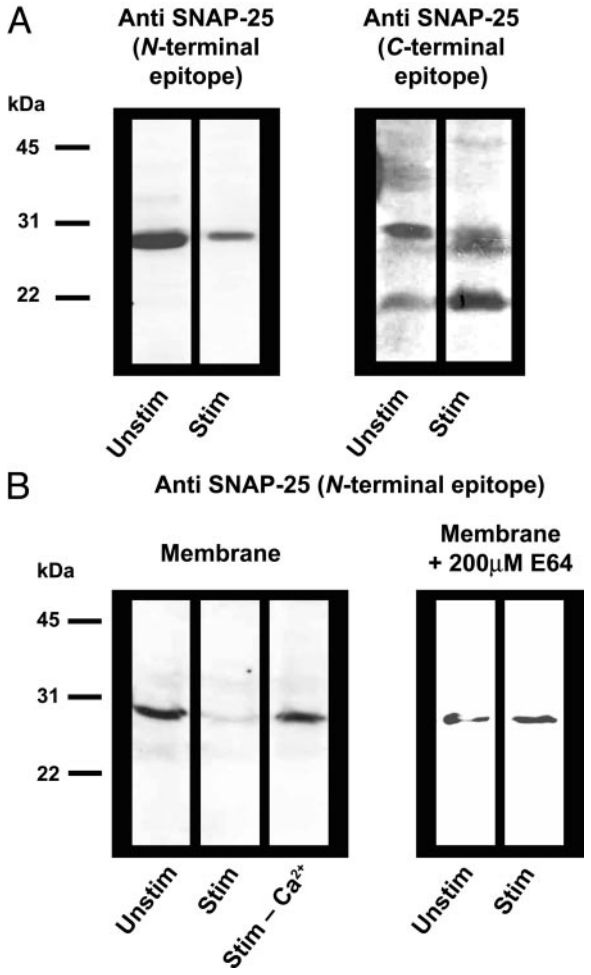

Fig. 7. Ca^{2+}-Dependent Proteolysis of SNAP-25 during Exocytosis

A, INS-1 cells were cultured with Krebs solution plus or minus insulin secretagogue cocktail. Cell membranes were isolated by subcellular fractionation. SNAP-25 protein was detected by immunoblotting with antibody directed against either the N terminus (*left panel*) or C terminus (*right panel*). B, Membranes were isolated from INS-1 cells preincubated for 48 h in the presence of E64 where indicated (*right panel*), and then Krebs-Ringer solution plus or minus insulin secretagogue cocktail for 3 h (in the continued presence or absence of E64). Membranes were immunoblotted with antibody against the N terminus of SNAP-25. SNAP-25 is proteolysed during exocytosis, with the site of proteolysis lying within the N-terminal region. Proteolysis is both Ca^{2+} dependent and sensitive to calpain inhibitor.

DISCUSSION

Since the discovery that *CAPN10* is a candidate gene that predisposes certain ethnic groups to diabetes (1), there has been extensive debate as to how it might influence glucose homeostasis. Indeed, some have questioned the strength of the association between *CAPN10* polymorphisms and T2D and concluded that functional data are required to elucidate the biological role in the pathogenesis of T2D (6). The expression of *CAPN10* was previously claimed to be ubiquitous (1). Our data indicate that, in the pancreas, calpain-10 staining is restricted to islet cells, suggesting a role in endocrine secretion. The fact that we now find that *CAPN10* expression is also glucose sensitive lends further support for a role in glucose homeostasis. Calpain-10 is likely to have multiple actions, presumably related to different molecular isoforms, metabolic circumstances, and cell type. In addition to insulin exocytosis and actin organization in adipocytes (22), it has recently been demonstrated that, under conditions of glucose starvation, calpain-10 is also a mediator of ryanodine-induced apoptosis in pancreatic β-cells (23). However, in our studies in INS-1 cells we found no evidence of calpain-10 overexpression associated with apoptosis, nor have we found any apoptosis due to a high-glucose environment.

Overexpressing *CAPN10* through introduction of a *CAPN10* cDNA plasmid has allowed us to carefully select individual β-cell clones with differing extents of overexpression. As the amount of overexpression correlates with increased regulated secretion, this again raises the question: what might its molecular target(s) be? Calpain-10 is an atypical member of the calpain family in that it lacks the calmodulin-like Ca^{2+}-binding domain at the C-terminal end of the molecule. Atypical calpains lacking this domain were therefore initially thought not to be Ca^{2+}-responsive. However, calpain-10 is composed of four domains (I, II, III, and T) of which domain III has since been shown to possess a Ca^{2+}-regulated phospholipid-binding domain (24). The C2-like region of domain III lies close to the interface with domain II, and as it can bind up to three Ca^{2+} ions (25), it could therefore either facilitate membrane binding in response to the local Ca^{2+} concentration, or alternatively, given the proximity of this site to the catalytic domain, membrane binding might instead lead to conformational changes within the catalytic site that alter the proteases Ca^{2+}-sensitivity and activation state (26, 27). Furthermore, compelling new evidence has shown that at the very heart of calpain's catalytic core are two Ca^{2+}-binding sites that lie within the actual proteolytic cleft of domain II itself (28). Together, these sites cooperatively control the enzyme's catalytic activity. Clearly then, whereas the calmodulin-like domain might play an important regulatory role for certain individual calpain family members, its presence is in itself not an essential component of this family's ability to sense Ca^{2+}.

In keeping with observations for human calpain-10 (1), we also observe a number of different isoforms of calpain-10 in INS-1 cells, presumably a similar result of differential splicing. The presence of multiple isoforms clearly gives rise to the scope for multiple calpain actions, based either upon differential isoform activation and/or localization. The situation becomes more complex when one takes into account that not only are there multiple isoforms of calpain-10, but that there are also other calpain species (and indeed other cysteine proteases) present in β-cells. It has been

proposed that the inhibition of insulin secretion observed from treating INS-1 cells with the calpain inhibitor calpeptin (29) was due to inhibition of μ-calpain-mediated proteolysis of the secretory granule protein ICA512, stimulus-evoked proteolysis of ICA512 being important for granules to mobilize from the cytoskeletal network to the cell surface. An alternative mechanism to account for calpain-inhibitory action has been proposed (16), whereby insulin secretion is inhibited by 48 h pretreatment with E64 as a consequence of impaired mitochondrial fuel metabolism. These differences might also be explained by use of transformed cell lines such as INS-1 cells as opposed to intact islets or, alternatively, could reflect an array of distinct actions of different calpain isoforms and family members.

Through subcellular fractionation we have been able to determine that calpain-10 isoforms exhibit different membrane-binding affinities. It has been suggested that calpains only become active upon membrane binding (30–32), suggesting that, at least for some isoforms of calpain-10, the principal site of action is at the plasma membrane. The regulated secretory pathway in β-cells involves a complex series of events incorporating glycolysis, changes in plasma membrane ion channel activity, and Ca^{2+}-dependent processes that enable granule relocation and release from the cell (reviewed in Ref. 33). Key molecules that operate in the late phase of the secretory pathway are the soluble *N*-ethyl maleimide-sensitive fusion protein attachment receptor (SNARE) proteins (reviewed in Refs. 34–36). They were first identified a decade ago as forming the core of the vesicle/granule fusion machinery (reviewed in Ref. 37), having helical structures that together form helical bundles between granules/vesicles (v-SNAREs) and target membranes (t-SNAREs) that facilitate membrane fusion (38–40). In β-cells granule exocytosis is mediated by the vesicle-associated membrane protein VAMP2 tethering granules to the plasma membrane through interaction with the t-SNAREs syntaxin 1 and SNAP-25. SNAREs are leading candidate substrates for calpain-10 for two reasons: 1) They facilitate regulated secretion by docking granules to specific areas of plasma membrane known as excitosomes through binding of syntaxin 1 and SNAP-25 to the L_c-type Ca^{2+} channel (41, 42). This ensures that the bound granule complex is exposed to the high levels of Ca^{2+} found immediately beneath the inner mouth of the open Ca^{2+}-channel, an environment that is clearly ideal for the operation of a Ca^{2+}-dependent protease. 2) SNAREs have been shown previously to be susceptible to proteolysis by various members of the calpain family (43–45). The fact that we now see a direct interaction between calpain-10 and the t-SNAREs, syntaxin 1 and SNAP-25, implicates the approximately 54-kDa isoform of calpain-10 in the actual exocytotic fusion event itself.

There has been a longstanding debate as to what the Ca^{2+} target might be that catalyzes exocytosis, as SNARE proteins are in themselves insufficient to account for the rapid Ca^{2+}-dependent event that represents the hallmark of secretory cells. Synaptotagmin is a candidate in neuronal cells as it has been reported to participate in synaptic vesicle fusion (46–48). However its role in β-cell secretion is far less clear as primary β-cells do not express the major synaptotagmin isoforms. Although recent work has implicated synaptotagmins V and VII as potential candidates in the exocytotic fusion event in β-cells (49), this interpretation is based primarily on localization data and has yet to be supported by direct functional studies. The emergence of calpain-10 as a component of the exocytotic granule fusion machinery complex provides us with an additional candidate for the role of exocytotic Ca^{2+}-sensor in β-cells.

Calpain-1 (μ-calpain) has been proposed to mediate cleavage of SNAP-23 (44), although in platelets cleavage occurs at the C terminus. However, because this particular proteolytic event failed to elicit any immediate effect on secretion, it was thought (based primarily on ultrastructural observations) that proteolytic action of calpain-1 is not of consequence for granule exocytosis, but instead contributes to cytoskeletal remodeling post exocytosis. It is tempting to speculate that some of the larger isoforms of calpain-10 that we find localized in the cytosol might perform a similar function. Evidence for such a role already exists in adipocytes in which calpain-10 is believed to play a role in actin reorganization during glucose transporter 4 translocation (22). It will be interesting to determine whether the differences we observe in both SNAP-23/25 cleavage site and function are based primarily upon differences in the specific SNAP isoforms, or the calpain species themselves.

As SNAP-25 is essential for regulated secretion (50), what then might be the functional significance of SNAP-25 cleavage in the β-cell? As depicted in Fig. 8, under nonstimulating conditions granules in the readily releasable pool are docked adjacent to L_c-type Ca^{2+} channels through their SNARE complex interaction. The complex of SNAP-25, VAMP2, and syntaxin 1 anchors the granule to the plasma membrane, with intact SNAP-25 (along with syntaxin 1) also inhibiting fusion of the granule and membrane (41). Our data suggest that unactivated calpain-10 also associates with this complex. We propose that secretagogue stimulation causes membrane depolarization, the associated opening of L_c-type Ca^{2+} channels, and the resultant influx of extracellular Ca^{2+} which activates calpain-10. The enzyme then cleaves SNAP-25 in a region adjacent to the N terminus. This is an area of the molecule that contains the syntaxin-binding domain (38) and, as such, proteolysis will almost certainly lead to conformational changes within the SNARE complex (Fig. 8B). Crystallography has revealed (51) that within the SNARE complex of docked vesicles/granules lies a 54-residue stretch of SNAP-25, which is open to proteolysis. Cleavage in this region would yield an N-terminal fragment of approximately 9 kDa, consistent with our current data (Fig. 7A). Ca^{2+} disrupts SNARE

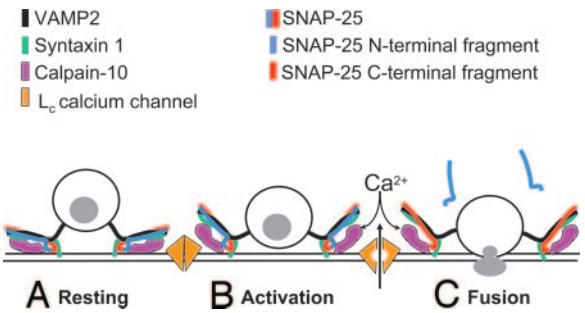

Fig. 8. Proposed Interaction between Calpain-10 and SNARE Proteins during Exocytosis

A, Docked secretory granules under resting conditions. Granules are held juxtaposed to the plasma membrane through paired binding of the granular v-SNARE protein VAMP2 with the plasma membrane t-SNAREs, SNAP-25 and syntaxin 1. In β-cells the 54-kDa isoform of calpain-10 is similarly bound with the SNARE complex; SNAP-25, syntaxin 1 (and the docked granule) are associated with the L_c-type Ca^{2+}-channel. B, Ca^{2+} influx activates calpain-10. Secretagogue stimulation results in opening of the Ca^{2+}-channel and influx of Ca^{2+} ions. This leads to activation of the Ca^{2+}-sensor calpain-10, which in turn partially proteolyses SNAP-25 and results in SNARE complex rearrangement. C, Granule fusion. The cleaved fragment of SNAP-25 is now released from the complex, freeing the way for granule fusion with the plasma membrane and simultaneously releasing granule content from the cell.

complex in native membranes (52), and as a result it has been suggested that removal of complex from the membrane interface facilitates the membrane-membrane contact and leads to fusion. Our current findings support and extend this hypothesis by identifying a 54-kDa isoform of calpain-10 bound to t-SNAREs, and therefore ideally placed to act as a Ca^{2+}-sensor to trigger SNARE rearrangement and granule fusion (Fig. 8C).

In conclusion, we have found that calpain-10 protein concentration correlates with the amount of insulin secreted from the β-cell, and that secretion is sensitive to calpain inhibition. Furthermore, at least one isoform of calpain-10 binds directly to the plasma membrane SNARE complex and is strongly implicated in SNAP-25 proteolysis. We postulate that it is this proteolytic event that allows docked secretory granules to be released by exocytosis, and hence that the 54-kDa isoform of calpain-10 is central to stimulus-secretion coupling in the β-cell. These studies give biological credibility for the investigation of *CAPN10* as a susceptibility gene in T2D.

MATERIALS AND METHODS

Materials

Human *CAPN10*, subcloned into pcDNA3.1/HisA vector (Invitrogen, Carlsbad, CA), was generously provided by Graeme I. Bell (University of Chicago). Antibodies used were as follows: mouse anti-Xpress (Invitrogen, mouse anti-SNAP25 (BD Biosciences, San Diego, CA), mouse antisyntaxin 1 (Sigma, St. Louis, MO), rabbit anti-VAMP2 (Affinity Bioreagents, Golden, CO), goat anti-SNAP-25 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anticalpain-10 (purified as described in Ref. 14), goat antirabbit-horseradish peroxidase (HRP) (Bio-Rad Laboratories, Inc., Hercules, CA), rabbit antigoat-HRP conjugate (DAKO Corp., Ely, UK), goat antimouse-HRP conjugate (DAKO Corp.), peroxidase-conjugated antirabbit antisera (DAKO), swine antirabbit fluorescein isothiocyanate (FITC) (DAKO), and guinea pig antiinsulin (Linco Research, Inc., St. Charles, MO). Insulin RIA kit was from Linco Research and, unless stated otherwise, all other reagents were from Sigma (St. Louis, MO).

Immunohistochemistry for Calpain-10 Localization in Islet Cells

Postmortem pancreas was obtained within 10 h of death after permission from relatives. Specimens were fixed in 10% formaldehyde in 0.9% sodium chloride and processed for wax embedding and light microscopy. Immunolabeling for calpain-10 and insulin was made on 5-μm thick sections on glass slides. Anticalpain-10 antisera (14) 1:40 dilution and insulin (ICN, Thame, UK) were applied overnight, and antibody-binding sites were identified with peroxidase-conjugated antirabbit antisera and diaminobenzidine hydrochloride as substrate and fluorescently labeled antiguinea pig antisera.

Tissue Culture and Generation of Stable *CAPN10* Clones

INS-1 pancreatic β-cell line was cultured using standard protocols. Cells were transfected using Lipofectin (Invitrogen) and grown for 7 d under standard conditions, after which they were subjected to antibiotic selection with 100 μg/ml geneticin (Invitrogen) for 3 wk. At the end of the selection period more than 99% of mock-transfected cells died, whereas significant numbers of transfected cells grew in a healthy manner. Individual clonal colonies were selected for subsequent screening.

Screening Clones for *CAPN10* Expression

Cell homogenates were boiled in Laemmli sample buffer (after normalizing samples for cellular protein content) and separated on 10% SDS-PAGE gels. Protein was transferred onto polyvinylidine difluoride (PVDF) membrane using a Hoefer TE 70 semidry transfer unit (Amersham Pharmacia, Little Chalfont, UK), and the presence of tagged calpain-10 emanating from the pcDNA3.1 vector was detected by immunoblotting with anti-Xpress antibody (1:1500). Antibody binding was detected using HRP-conjugated secondary antibody (1:1000) and visualized using ECL plus chemiluminescent detection and Hyperfilm ECL (Amersham Pharmacia). Band intensity was quantified using an IS-1000 Digital Imaging System (Alpha Innotech Corp., San Leandro, CA).

Insulin Secretion Assay

Cells were seeded onto poly-D-lysine-coated six-well plates and allowed to grow to near confluency (2×10^6 cells per well). They were then washed with Krebs-Ringer solution (125 mM NaCl, 1.2 mM KH_2PO_4, 5 mM KCl, 2 mM $MgSO_4$, 1.67 mM glucose, 0.1% BSA, 25 mM HEPES; pH 7.4) and incubated for 3 h in Krebs-Ringer solution plus or minus insulin secretagogue cocktail (10 mM glucose, 1 μM phorbol 12-myristate 13-acetate, 1 mM isobutyl-methylxanthine, 1 mM tolbutamide,

10 mM leucine, 10 mM glutamine) plus or minus 1 mM extracellular Ca^{2+}. Supernatant was collected and spun down to remove cell debris, Complete protease cocktail (Roche, Basel, Switzerland) was added, and insulin content was determined using a Cobra II γ-counter (Hewlett-Packard, Palo Alto, CA) following standard RIA protocol. Cellular protein content was assayed from lysed unstimulated cells as per BCA kit protocol (Pierce Biotechnology Inc., Rockford, IL) and used to quantify secretion data.

Calpain-10 Immunofluorescence

INS-1 cells were cultured on permanox or poly-L-lysine-coated eight-well Lab-Tek chamber slides (Nalgene-Nunc, Rochester, NY). After incubation, cells were fixed in 4% paraformaldehyde in phosphate buffer, permeabilized with 0.1% Triton X-100 in PBS containing 5% swine serum at 4 C, and incubated overnight with anticalpain-10 (1:30 dilution). Calpain-10 was visualized with swine antirabbit FITC (1:30) for 1 h and washed in PBS. After removal of the wells, the slides were mounted in Vectashield (Vector Laboratories, Inc., Burlingame, CA), and fluorescence was viewed under a Leica TCS-NT laser scanning confocal microscope equipped with a 488-nm laser excitation filter for FITC.

SNARE Proteolysis Plus or Minus E64

Equal numbers of INS-1 cells were cultured to 80% confluence in 6-cm diameter petri dishes. Each dish was incubated for 3 h with Krebs solution plus or minus secretagogue stimulation cocktail in the presence and absence of a 48-h preincubation with 200 μM E64. The cells in each dish were then scraped into 5 ml PBS and spun at 90 $\times$ *g* for 5 min. Each pellet was resuspended in 100 μl buffer A (10 mM 2-[N-morpholino]ethanesulfonic acid NaOH, pH 7.4; 10 mM $CaCl_2$; 0.2 mM phenylmethylsulfonylfluoride), and cells were homogenized by passing through a 25-gauge syringe needle eight times. The homogenates were centrifuged at 500 $\times$ *g* at 4 C for 10 min to produce postnuclear supernatants. Each postnuclear supernatant was further centrifuged at 23,000 $\times$ *g* at 4 C for 30 min. The pellet containing the membrane fraction was resuspended in distilled water, and Laemmli sample buffer was added. Samples (normalized to protein from whole-cell homogenates) were separated on a 15% SDS-PAGE gel and transferred to PVDF membrane, and SNARE proteins were detected by Western blotting as described above.

Subcellular Fractionation of Calpain-10 Isoforms

INS-1 cells were grown to 80% confluence in flasks and then trypsinized and spun at 90 $\times$ *g* for 5 min. Each pellet was resuspended in 150 μl buffer A (see above) and homogenized by passing through a 25-gauge syringe needle eight times. Homogenates were separated into membrane and cytosol fractions as described above. Samples were normalized to total protein, separated on 15% SDS-PAGE gels, and transferred to PVDF membrane, and protein was detected using anticalpain-10 antibody.

Coimmunoprecipitation of SNAREs and Calpain-10

INS-1 cells were grown to 80% confluence in poly-D-lysine-coated six-well plates. Media were discarded and cells lysed (100 mM NaCl; 1% Triton X-100; 0.2% Na deoxycholate; 0.1% SDS; 10 mM EDTA; 25 mM Tris, pH 7.4). Protease inhibitor was added, and a 50 μl aliquot of the mixture was assayed for total protein content. Homogenates were precleared with zysorbin, and then 40 μl protein G were added along with anti-SNAP-25 or antisyntaxin 1 antibody. After overnight rotation at 4 C, beads were washed with immunoprecipitation buffer, proteins were separated on 15% SDS-PAGE gel, and Western blotting was performed as detailed previously.

Statistical Analysis

Analysis between two groups was by independent samples *t* test. Comparison between more than two groups was by one-way ANOVA, followed by Dunnett *post hoc t* test. All experimental data were gathered from a series of independent experiments (n = 6) and are expressed as mean + SEM.

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