RAPID COMMUNICATION

Involvement of calpain and synaptotagmin Ca\(^{2+}\) sensors in hormone secretion from excitable endocrine cells

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Abstract

The requirement for Ca\(^{2+}\) to regulate hormone secretion from endocrine cells is long established, but the precise function of Ca\(^{2+}\) sensors in stimulus–secretion coupling remains unclear. In the current study, we examined the expression of calpain and synaptotagmin in INS-1 pancreatic and GH3 and AtT20 pituitary cells, and investigated the sensitivity of hormone secretion from these cells to inhibition of the calpain family of cysteine proteases. Little difference in expression of \(\mu\)-calpain was observed between the different endocrine cells. However, AtT20 cells did exhibit an extremely low abundance of \(\mu\)-calpain and the 54 kDa isoform of calpain-10 relative to their expression in INS-1 and GH3 cells. Interestingly, secretagog-stimulated secretion from both INS-1 and GH3 cells was completely abolished following pre-incubation with the cysteine protease inhibitor E64, whereas stimulated secretion from AtT20 cells was modest and completely insensitive to E64 inhibition. These results are in stark contrast to synaptotagmin data. Synaptotagmin expression in AtT20 cells is abundant, whereas INS-1 cells express extremely low levels of this Ca\(^{2+}\) sensor, relative to the pituitary cells. We hypothesize that the expression pattern of calpain and synaptotagmin isoforms may reflect alternative mechanisms of stimulus–secretion coupling in excitable endocrine cells.

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Introduction

The Ca\(^{2+}\) requirement for stimulated secretion is well documented, although the contribution of individual Ca\(^{2+}\) sensors and their precise function in stimulus–secretion coupling remains poorly understood. Recently, the calpain family has been shown to be an important class of molecule mediating insulin secretion from pancreatic \(\beta\)-cells (Ort et al. 2001, Zhou et al. 2003, Marshall et al. 2005, Parnaud et al. 2005). Moreover, evidence has emerged for a role for calpain-10 as a Ca\(^{2+}\) sensor mediating the actual exocytotic fusion event itself (Marshall et al. 2005). However, despite \(\mu\)-calpain (calpain-1; Ort et al. 2001) and calpain-10 (Marshall et al. 2005) being specifically shown to be regulators of insulin secretion, little is known as to whether individual calpain family members play cell-specific or more general roles in endocrine secretion per se. This also raises the question, are calpains capable of interchanging function, or do they perform isoform-specific roles based upon their respective Ca\(^{2+}\) requirements for activation?

The major focus of neuronal Ca\(^{2+}\) -sensing studies has largely focused around the synaptotagmin family, and in particular synaptotagmin I (Tokuoka & Goda 2003, Yoshihara et al. 2003, Sudhof 2004). Whilst numerous questions still remain regarding the fine detail of synaptotagmin I action, nonetheless after years of debate there finally appears to be a consensus that this is the primary mediator of Ca\(^{2+}\) sensing in neuronal stimulus–secretion coupling. However, there are a number of differences between the secretory dynamics of hormone secretion and those of neurotransmission. In particular, whilst neurotransmission is an extremely rapid process (Sabatini & Regehr 1999) endocrine secretion is multiphasic, with rapid exocytosis often only a minor component of total regulated secretion (Henkel & Almers 1996). Therefore, whilst the core fusion machinery operating in all neuroendocrine cells is very similar, there are likely to be different regulatory molecules operating between the systems. The current study examines the contribution of calpain and synaptotagmin to stimulus–secretion coupling in three different endocrine cell types.

Materials and Methods

Materials

Antibodies used were obtained as follows: rabbit anti-\(\mu\)-calpain (Calbiochem, San Diego, CA, USA), rabbit anti-\(\mu\)-calpain

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(Calbiochem), mouse anti-synaptotagmin (BD Biosciences, San Diego, CA, USA), rabbit anti-calpain-10 was as described previously (Ma et al. 2002, Marshall et al. 2005), goat anti-rabbit-HRP conjugate (Bio-Rad), and goat anti-mouse-HRP conjugate (Dako, Ely, UK). E64 (Roche Diagnostics) were dissolved in methanol to make a stock of 2 × 10⁻⁵ M and stored at −20 °C. Corticotrophin-releasing factor (CRF) was diluted in sterile distilled water to form a stock solution of 10⁻⁴ M and stored at −20 °C until diluted in culture medium. Forskolin (FSK) was dissolved in sterile dimethylsulfoxide to form a stock solution of 10⁻³ M and stored at 4 °C until diluted in culture medium. All other reagents were purchased from Sigma unless otherwise stated.

Cell culture

INS-1 pancreatic β-cell line was cultured in RPMI-1640 medium (Sigma) using standard protocols. Both AtT20 and GH3 cells were cultured in DMEM (high glucose; Gibco) containing 2 mM t-glutamine and 25 mM glucose, and supplemented with 10% (v/v) horse serum (HS; Gibco), 100 U/ml penicillin-G, and 10 mg/ml streptomycin sulfate at 37 °C, in a 5% CO₂-95% air atmosphere.

Secretion

For secretion studies, AtT20 cells were seeded at 5 × 10³ cells/well and GH3 cells at 1 × 10⁴ cells/well in six-well tissue culture plates 24 h prior to incubation ± E64 (200 μM) for 48 h. For the subsequent 24 h, AtT20 cells were incubated ± CRF (10⁻⁷ M) and GH3 cells were incubated ± FSK (10⁻⁵ M) in the continued presence or absence of E64. At the end of this incubation period, media were collected and analyzed using a two-site solid-phase IRMA for adreno-corticotrophic hormone (ACTH) (Euro-Diagnostica AB, Malmo, Sweden) or a competitive RIA for growth hormone (GH) (Biocode-Hycel, Liège, Belgium). Within- and between-batch variation (% coefficient of variation) were less than 10% for both assays.

INS-1 cells were seeded at 1 × 10⁶ cells/well in six-well tissue culture plates and incubated in ± 200 μM E64 for 24 h. The cells were then washed with Krebs–Ringer solution and incubated for 3 h in Krebs–Ringer solution, ± 15 mM glucose and 1 mM extracellular Ca²⁺. Supernatant was collected and spun down to remove cell debris, complete protease cocktail was added, and insulin level measured by ELISA rat insulin assay kit (Mercodia, Uppsala, Sweden). Cellular protein content of lysed cells was assayed as per BCA kit protocol (Pierce Biotechnology Inc.), and used to normalize insulin values obtained. Media were removed from wells from each plate, and 500 μl extraction solution containing 1.5% hydrochloric acid (37%), 18.5% distilled water, 80% ethanol (95%) added to each well. After 24 h, at 4 °C, 500 μl of 0·1 M sodium hydroxide was added to neutralize the samples, which were then assayed for insulin content using standard ELISA protocols (Mercodia, Uppsala, Sweden).

FACS analysis

INS-1 cells were seeded at 1 × 10⁶ cells/well in six-well tissue culture plates and incubated in ± 200 μM E64 for 24 h. Cells were trypsinized, pelleted, and washed twice with cold PBS. From each dish, 1 × 10⁶ cells were suspended in 1 ml binding buffer, and 100 μl of this solution (1 × 10³ cells) transferred to separate tubes. Five microliters of annexin V-FITC and propidium iodide (PI; Annexin V-FITC Apoptosis Detection Kit I; BD Pharmingen, San Diego, CA, USA) were added to each tube, after which they were vortexed and then incubated in the dark for 15 min at room temperature. To each tube, 400 μl binding buffer was added and then annexin V and PI fluorescence measured by flow cytometry (Becton Dickinson FACScan; Cambridge, UK). Clumped cells and cell debris were excluded from analysis using the method of Jia et al. (2001). Staurosporine (1 μM) was used as a positive control for the presence of apoptosis (overnight stimulation).

Sub-cellular fractionation

Equal numbers of INS-1, GH3, and AtT20 cells were cultured to 80% confluence in 6 cm diameter Petri dishes, then scraped into 5 ml PBS and spun at 90 g for 5 min. Each pellet was resuspended in 150 μl buffer A (10 mM MES-NaOH, pH 7.4, 10 mM CaCl₂, 0·2 mM phenylmethylsulphonyl fluoride) and homogenized by passing through a 25-gauge syringe needle eight times. Aliquots of homogenate were taken for total cellular protein quantification using the BCA assay kit (Pierce). The homogenates were centrifuged at 500 g at 4 °C for 10 min to produce post-nuclear supernatants (PNS). Each PNS was further centrifuged at 23 000 g at 4 °C for 30 min. The supernatant corresponding to cytosol fraction was transferred to a clean tube, while the pellet containing the membrane fraction was resuspended in distilled water. Equal sample volume of 2× Laemmli buffer was added to each fraction and boiled for 5 min before storing at −20 °C.

Western blotting

Membrane and cytosol fractions were normalized for cellular protein content and separated on appropriate percentage of SDS-PAGE gels (see figure legends). Protein was transferred onto PVDF membrane using a Hoefer TE 70 semi-dry transfer unit (Amersham Pharmacia), blocked with 5% milk powder, and probed with antibodies as follows: m- and mu-calpain antibody (1:5000 incubated overnight at 4 °C), calpain-10
antibody (1:3000), and synaptotagmin antibody (1:1000). Immunoreactivity was detected using HRP-conjugated goat anti-rabbit (1:3000) or goat anti-mouse (1:1000) and visualized using ECL plus chemiluminescent detection and exposed to ECL Hyperfilm (Amersham Pharmacia).

**Statistical analysis**

All graphical data were prepared using GraphPad Prism 3.0 (GraphPad, San Diego, CA, USA) and analyzed using pre-programmed analysis equations within Prism. Secretion data were presented as normalized data pooled from multiple experiments. Where appropriate, an ANOVA was performed on data followed by Tukey’s multiple comparison test, accepting $P<0.05$ as significant.

**Results**

**Sensitivity of hormone secretion to cysteine protease inhibition**

We and other researchers have previously shown that pharmacological intervention with calpain inhibitors abolishes secretagog-stimulated insulin secretion from pancreatic β-cells (Ort et al. 2001, Zhou et al. 2003, Marshall et al. 2005, Parnaud et al. 2005). However, an understanding of the role of the calpain family in hormone secretion from other endocrine cells is less clear. In order to determine whether hormone secretion per se is either calpain-mediated or calpain independent, we pre-incubated GH3 and AtT20 pituitary cells with E64 for 48 h, an inhibitor and incubation time that have been previously shown to be highly effective in inhibiting regulated insulin secretion from both pancreatic islets (Zhou et al. 2003) and INS-1 β-cells (Marshall et al. 2005). E64 effects on secretion were determined relative to those observed in INS-1 cells, and as can be seen in Fig. 1, both GH3 and AtT20 cells also secrete hormone via a basal pathway that does not require secretagog to induce secretion. This is completely insensitive to pre-incubation with E64 (Fig. 1b and c; left panels). In marked contrast, stimulated secretion of GH from GH3 cells was completely abolished following pre-incubation with E64 (Fig. 1b; right panels). However, this was not the case with ACTH secretion from AtT20 cells, where stimulated secretion was completely E64 insensitive (Fig. 1c; right panels). Therefore, whilst GH3 cells display similar sensitivity to calpain inhibition as INS-1 β-cells (Fig. 1a), this is not the case in AtT20 cells. Our results suggest that stimulated ACTH secretion from AtT20 cells occurs via a mechanism that is independent of the calpain family, whilst stimulated secretion from GH3 cells is calpain dependent.

**E64 has no effect on cellular hormone content or apoptotic status**

In order to determine whether E64 action was specific to the regulated secretory pathway, we analyzed cellular hormone content and apoptotic status of cells treated with or without E64.

As INS-1 cells displayed the greatest secretagog-stimulated increase in secretion relative to basal, we used these cells for this series of experiments. As stimulated secretion from INS-1 cells is also extremely sensitive to E64 inhibition (Fig. 1), they should be similarly sensitive to any E64-mediated changes in insulin biosynthesis, granule storage, or apoptosis. However, as can be
seen in Fig. 2a, there is no effect of E64 upon insulin content in INS-1 cells, despite treating cells with the same concentration of E64 and for the same period of time as in the secretion experiments. Therefore, E64 inhibition of secretion is not a consequence of any action upon either the insulin biosynthetic machinery or upon insulin storage.

In order to determine whether E64 might instead inhibit insulin secretion by inducing apoptosis, we again incubated cells with or without E64, then measured their apoptotic status using FACS analysis of cells stained with propidium iodide and annexin V. Incubation with staurosporine positive control (Fig. 2b), resulted in a 79.2 ± 1.5% reduction in cell viability. By contrast, we observed no reduction whatsoever in cell viability of cells incubated for 24 h with E64. Therefore, the inhibitory action of E64 upon insulin secretion is not a consequence of any reduction in cell viability arising from a pro-apoptotic action of this cysteine protease inhibitor.

**μ-Calpain expression**

We sought to address whether the differences in secretory pathway sensitivity of different endocrine cells to E64 might arise from differences in expression of individual calpains between the different cell types. μ-Calpain has previously been shown to facilitate insulin secretion through its action on the cytoskeleton (Ort et al. 2001). Therefore, in order to address whether μ-calpain-mediated changes in cytoskeletal dynamics might reflect the differential sensitivity of hormone secretion to E64, we examined μ-calpain expression and localization. As can be seen in Fig. 3, we observed no major differences between the three cell lines with regard to μ-calpain expression. Three times more membranes were loaded onto gels relative to cytosol, but even after taking this into account there was still a surprisingly high affinity of μ-calpain for microsomal membranes, densitometric analysis revealing that there was between 57 and 60% present on microsomal membranes in all the three cell types. However, as both the expression level and the degree of partitioning between membrane and cytosol were not significantly different among the three cell types, the differences in E64 sensitivity observed in Fig. 1 were not a reflection of any differences in either μ-calpain expression or localization.

**m-Calpain expression**

As can be seen in Fig. 4, there is an abundant expression of m-calpain in INS-1 and GH3 cells. Moreover, densitometric analysis revealed that ~8% is associated with INS-1 microsomal membranes and ~15% is found associated with GH3 microsomal membranes. However (despite equal loading of protein between all cell types), there is a noticeably low-relative abundance of m-calpain in AtT20 cells, with little detected on either microsomal membranes or in cytosol. This raises the possibility that m-calpain might be a key protein-mediating hormone secretion from INS-1 and GH3 cells, but not AtT20 cells. If so, its presence in INS-1 and GH3 cells could reflect the fact that these cells are susceptible to inhibition by E64, whereas AtT20 cells which possess relatively little m-calpain are not.

![Figure 2](image1.png)  
*Figure 2* Calpain inhibition has no effect on insulin content or apoptotic status. (a) INS-1 cells were incubated in the presence or absence of 24 h pre-incubation with 200 μM E64 and total insulin cellular content determined. (b) INS-1 cells were incubated for 24 h in the presence or absence of 200 μM E64. Cells were stained with annexin V-FITC and propidium iodide and apoptosis determined using FACS analysis. Staurosporine (1 μM) was used as a positive control for the presence of apoptosis.

![Figure 3](image2.png)  
*Figure 3* Immunoblot analysis of μ-calpain. Subcellular membrane and cytosol fractions from INS-1, GH3, and AtT20 cells were normalized for protein level, then subjected to 8% SDS-PAGE, electrotransferred to PVDF membrane, and western blotted with antibody raised specifically against μ-calpain. This figure is the representative of three independent experiments.
Calpain-10 expression

Calpain-10-mediated insulin secretion from INS-1 pancreatic β-cells has previously been documented (Marshall et al. 2005) and this is clearly an attractive candidate to similarly regulate hormone secretion from other endocrine cells. In order to address this possibility, we immunobotted membrane and cytosol fractions from INS-1, GH3, and AtT20 cells with a highly specific and well-characterized anti-calpain-10 antibody (Ma et al. 2001, Marshall et al. 2005). As can be seen in Fig. 5, there was little difference in the total amount of calpain-10 expression between the three cell types. However, the membrane association pattern is markedly different between cell types for some of the isoforms. In particular, there is a pronounced lack of the 54 kDa isoform in AtT20 cells, and there is also the presence of a ~60 kDa band on GH3 cell membranes. While there is at present no proposed function for the novel ~60 kDa isoform, the mere fact that it is absent in both INS-1 and AtT20 cells seemingly rules this isoform out as a candidate mediator of differential calpain inhibitor sensitivity. However, given that the 54 kDa isoform is the very isoform proposed to trigger exocytosis in pancreatic β-cells (Marshall et al. 2005), it probably performs a similar role in the secretion of GH from GH3 cells. If so, then the absence of appreciable levels of this isoform in AtT20 cells would render these cells unable to utilize this protein to mediate stimulus–secretion coupling.

Synaptotagmin expression

There are currently known to be at least 15 isoforms of synaptotagmin in vertebrates (Sudhof 2002), and previous studies have carefully documented expression levels of these individual synaptotagmin isoforms in INS-1, GH3, and AtT20 cells respectively. Therefore, rather than blotting for each synaptotagmin isoform independently we instead chose to use a polyclonal anti-synaptotagmin antibody raised against peptide sequence that incorporates the highly conserved C2AC a2 -binding domain. In this way, we sought to address whether there were substantial differences in global synaptotagmin expression between the different endocrine cells. As can be seen in Fig. 6, INS-1 cells have very little expression of synaptotagmin relative to GH3 and AtT20 cells, where the chemiluminescent signal is extremely strong. In particular, AtT20 cells display intense bands which, based upon both the extent of C2A domain homology and the migrating protein molecular weights, are assigned as synaptotagmins I, III, and V. GH3 cells also display a similar banding pattern, albeit at a lesser intensity. While sample loading was normalized to protein, in INS-1 there is only one moderately expressed band that is readily visible. As previously studies have shown that synaptotagmin III is the major synaptotagmin implicated in insulin secretion from β-cells (Mizuta et al. 1994, 1997, Brown et al. 2000), we suggest that this is the isoform we observe in INS-1 cells.

Discussion

It has previously been reported that μ-calpain mediates secretion through proteolysis of ICA512, a secretory granule...
protein linked to the cytoskeletal remodeling, which is associated with second-phase insulin secretion (Ort et al. 2001). However, as the endocrine cells we studied in this report all expressed similar amounts of μ-calpain, this is unlikely to account for the differences we observed in sensitivity to calpain inhibition. The involvement of m-calpain in hormone secretion is not as well documented, although specific knockdown of m-calpain mRNA has been reported to result in a significant reduction of regulated secretion from alveolar epithelial cells (Li et al. 1998). The report of secretagog-induced proteolysis of spectrin in alveolar epithelial cells (Zimmerman et al. 1992) offers a clue to possible action, as spectrin has been shown to be involved in the cytoskeletal rearrangement that accompanies stimulated secretion (Mercier et al. 1989). Another possible function of m-calpain might be in secretory granule maturation, as in vitro proteolysis of clathrin and adaptins has been observed in response to addition of exogenous m-calpain to pituitary cell lysates (Ohkawa et al. 2000). Were either of these mechanisms the source of the observed differential E64 sensitivity in this report then, as with μ-calpain, this would represent a role for m-calpain in the slower but more sustained phase of secretion that follows the initial rapid burst of exocytotic granule fusion.

Calpain-10 has recently emerged as a Ca\(^{2+}\) sensor in stimulus-secretion coupling, the 54 kDa isoform being shown to regulate first-phase insulin secretion (Marshall et al. 2005). Interestingly, we saw an abundance of this particular isoform in INS-1 and GH3 cells, but severely diminished expression in the cysteine protease-insensitive AtT20 cells. These findings are consistent with this isoform performing a similar function in GH3 cells to that previously reported in INS-1 cells. As such, the low abundance of this isoform in AtT20 cells would also explain why secretion from these cells was insensitive to cysteine protease inhibition. Future studies utilizing antisense oligonucleotide or siRNA technology might enable us to determine the precise roles of each of the calpains in stimulated secretion. However, given that the calpain-10 gene is subject to differential splicing, which generates as many as eight isoforms in humans (Horikawa et al. 2000), the situation is complex.

Neurotransmission and endocrine secretion both utilize soluble N-ethyl maleimide sensitive fusion protein attachment receptor molecules, or their associated homologues, during exocytotic membrane fusion. Furthermore, both processes share a similar Ca\(^{2+}\) requirement (Burgoyne & Clague 2003, Stojilkovic 2005), although the kinetics of neurotransmission are much more rapid than those of endocrine secretion. This gives rise to the possibility that different classes of Ca\(^{2+}\) sensor might therefore trigger the respective exocytotic fusion events, their differing molecular modes of action being reflected in the differing kinetics. Our results in pituitary cells are in agreement with previous studies (Jacobsson & Meister 1996, Xi et al. 1999), which showed that synaptotagmins I and III were the major species of synaptotagmin in the pituitary. The function of synaptotagmins in pancreatic β-cell secretion however is far less clear, although a role for synaptotagmins V and VII–IX has been suggested, based upon either molecular- or antibody-based techniques (Gut et al. 2001, Iezzi et al. 2004). However, in these studies, both peptide competition and RNA interference yielded only partial inhibition of secretion. As such, it is highly likely that these isoforms of synaptotagmin are not the sole Ca\(^{2+}\) sensors in stimulus–secretion coupling in these cells. Instead, perhaps they work either in combination with other synaptotagmins, or alternatively in concert with one or more additional Ca\(^{2+}\) sensor, such as calpain-10. Additionally, a number of publications have shown that AtT20 and GH3 pituitary cells both utilize the Ca\(^{2+}\) sensor synaptotagmin to secrete hormone in response to secretory

![Figure 6 Immunoblot analysis of synaptotagmin. Western blot analysis of membrane (M) and cytosol (C) fractions from INS-1, GH3, and AtT20 cells using anti-synaptotagmin antibody. The fractions were normalized for protein level before being subjected to 8% SDS-PAGE and transferred to PVDF. Migration positions of synaptotagmin (Syt) isoforms are indicated on the right. This figure is the representative of three independent experiments.](image-url)
stimulation by CRF or FSK respectively. Therefore, it is extremely likely that both cAMP and Ca\textsuperscript{2+} have active roles to play during the secretory process in these cells, and there is likely to be a cross-talk between these signaling pathways.

In conclusion, stimulated secretion from INS-1 and GH3 cells is calpain-mediated, in contrast to stimulated secretion from AtT20 cells which is calpain independent. Calpain-10, in particular, might determine sensitivity of secretory cells to E64 sensitivity, as like synaptotagmin (Schiavo et al. 1997, Gerona et al. 2000, Earles et al. 2001, Zhang et al. 2002) it also has been reported to interact with synapsosomal-associated protein (SNAP)-25 during Ca\textsuperscript{2+}-triggered exocytosis (Marshall et al. 2005). Furthermore, the proposed conformational change in SNARE protein complex caused by calpain-10 action (Marshall et al. 2005) resonates with the direct action of synaptotagmin that has been observed on neuronal t-SNAREs (Bhalla et al. 2006). However, there is no direct evidence at this time to conclusively prove or disprove whether calpain-10 and synaptotagmin perform discreet or complementary actions, but it is nonetheless tempting to speculate that the relative abundance of both calpain and synaptotagmin isoforms decrees precisely how cells perform stimulus–secretion coupling.

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