Published in final edited form as:
Open Access Anim Physiol. 2009 November 19; 2009(1): 1–11.

GENETIC AND PHARMACOLOGIC MANIPULATION OF VACUOLAR ATPASE; EFFECTS ON ZYMOGEN ACTIVATION IN PANCREATIC ACINI

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Abstract

Premature activation of inactive digestive enzymes (or zymogens) within the pancreatic acinar cell is an initiating event in acute pancreatitis (AP). We have found that this response depends on the assembly and activation of an ATP-dependent proton pump, the vacuolar ATPase (vATPase). Previously, we have shown that the classic vATPase inhibitors concanamycin and bafilomycin can inhibit zymogen activation induced experimentally by high doses of the cholecystokinin orthologue, cerulein (CER) in isolated acinar cells. Recent studies have questioned the specificity of these inhibitors. In the current study we examine the role of the vATPase in pancreatitis using the newly developed novel vATPase inhibitors lobatomide-B and salicylihalamide-A as well as a genetic approach using siRNA. Both lobatomide-B and salicylihalamide-A inhibited CER stimulated zymogen (trypsinogen and chymotrypsinogen) activation but had no effect on amylase secretion. Lobatomide-B (0.1μM) was more potent, reducing activation to baseline levels. Treatment of cells with siRNA specific for the vATPase E-subunit (V1E) significantly decreased V1E expression. V1E siRNA also significantly decreased chymotrypsinogen activation, but not amylase secretion. These studies confirm a role for the vATPase in zymogen activation and demonstrate that the novel and specific inhibitors lobatomide-B and salicylihalamide-A reduce early pancreatitis responses

Keywords

lobatomide; salicylihalamide; cerulein; siRNA

Introduction

One of the earliest events in acute pancreatitis, an inflammatory condition of the pancreas, is the aberrant intracellular activation of zymogens within the pancreatic acinar cell. Evidence suggests that pancreatic zymogen activation depends on the generation of a low pH compartment[1]. Our laboratory has shown that the proton pump, vacuolar ATPase (vATPase), is involved in pancreatic zymogen activation using the general vATPase inhibitors, bafilomycin-A1 and concanamycin-A[2]. The vATPase is found ubiquitously in the

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Disclosures The author reports no conflicts of interest in this work

membranes of eukaryotic cells and is a heteromultimeric protein complex consisting of a cytosolic V1 domain and a membrane bound V0 domain. Assembly of the soluble V1 complex onto the membrane bound V0 complex is required for proton pumping activity[3]. Classically, the involvement of the vATPase in intracellular events has been determined using the plecomacrolide antibiotics bafilomycin and concanamycin as specific inhibitors of vATPase activity. The inhibitory effect of bafilomycin and concanamycin on vATPase activity is due to their binding to the c-subunit of the V0 complex[4, 5]. Recent studies, however, have brought into question the specificity of these inhibitors for vATPase dependent proton pumping activity. Teplova et al examined the effects of bafilomycin on mitochondrial function and showed that it can function as a potassium ionophore[6]. In RAW 264.7 cells bafilomycin and concanamycin both cause an increase in nitrite production, which is blocked by nitric oxide synthase inhibition. Bafilomycin causes the degradation of IκB and phosphorylation of JNK resulting in the activation of NF-κ-B and ultimately an increase in iNOS mRNA and protein[7]. The method by which bafilomycin causes IκB degradation and JNK phosphorylation are unclear, but raise the possibility that these effects might not be related to vATPase inhibition. Bafilomycin[8] and concanamycin[9] have been shown to block chloroquine induced apoptosis in cultured cerebellar granule neurons at concentrations which do not inhibit vATPase activity (<= 1nM). This inhibition of chloroquine-induced apoptosis is seen with bafilomycin concentrations as high as 100nM but the effects are minimized due to bafilomycin's intrinsic stimulation of apoptosis[8]. Together, these findings suggest that bafilomycin and concanamycin could have cellular effects unrelated to inhibition of the vATPase.

Another class of vATPase inhibitors are the benzolactone enamides which are specific for the inhibition of vATPases from higher vertebrates[4, 10, 11]. This class of inhibitors includes the lobatomides and salicylihalamides, which were isolated from the marine tunicate worm[12], and marine sponge[13] respectively and were originally identified as anti-tumor compounds[10]. Salicylihalamides bind to the V0 complex of the mammalian vATPase and this binding is distinct from the binding site for bafilomycin[14] and concanamycin[4].

In a previous study we showed that treatment of pancreatic acinar cells with bafilomycin or concanamycin blocked cerulein induced zymogen activation[2]. Because these compounds have been recently reported to affect vATPase-independent responses, the goal of the present study was to confirm the role of the vATPase in zymogen activation using a new class of pharmacologic inhibitors and genetic knockdown of vATPase. Using these approaches, we have confirmed a role for vATPase activity in secretagogue stimulated zymogen activation.

Material and methods

Isolation of pancreatic acinar cells

All experiments and procedures using rats were performed in accordance with a protocol approved by the Veteran's Administration Institutional Animal Care and Use Committee (West Haven, CT). Pancreatic acinar cells were isolated as previously described[15]. Briefly, fasted male Sprague-Dawley rats 100–150 g (Charles River Laboratories, Wilmington, MA) were euthanized by CO_2 inhalation. The pancreas was collected in buffer A: [10 mM Hepes (pH 7.4), 95 mM NaCl, 4.7 mM KCl, 0.6 mM $MgCl_2$, 1 mM NaH_2PO_4, 10 mM glucose, 2 mM glutamine, plus 0.1% bovine serum albumin, 1× MEM-amino acids (GIBCO-BRL, San Jose, CA)]. The pancreas was then minced in 5 ml of Buffer A and washed 2x with 5ml of buffer A, then transferred to 12ml of Buffer-B [Buffer-A + 1.3mM $CaCl_2$] with 50 U/ml of type-4 collagenase (Worthington, Freehold, NJ). After incubation for 1 hour at 37°C with shaking (120 rpm) under constant O_2, the preparation was filtered through a 300–400 μm mesh (Sefar American, Depew, NY). The flow-through from the mesh was gravity pelleted

and washed 2x with 12ml of buffer B. The resulting acini were suspended in buffer B and plated at 0.5ml of cells per well in a 24-well Falcon tissue culture plate.

Treatment of acinar cells with vATPase inhibitors

The tissue culture plate containing pancreatic acini were placed in an incubation chamber and allowed to recover for 1 hour at 37°C with shaking (90 rpm) under constant O_2. At 1 hour, cells were removed from the chamber and the media exchanged for 0.5ml of new buffer-B. At this time the vATPase inhibitors lobatomide-B, salicylihalamide-A (0.01-1μM) (Both inhibitors provided by the Technology Transfer Branch of the NCI (CRADA; http://www-otd.nci.nih.gov/cradaopp.html) or the drug vehicle (0.1%DMSO) were added 1 hour prior to other treatments. After an hour CER was added to the appropriate wells at a final concentration of 100nM and incubated for 1 hour. After one hour of CER treatment the contents of the wells were transferred to 1.5ml eppendorf tubes and centrifuged 30 × g for 1min. After centrifugation, 50μl of cell free media was removed to assay for amylase secretion. The tubes containing the cell pellets and remaining media as well as those containing media alone were stored at -80°C until used for the determination of zymogen activation and amylase secretion.

Treatment of acinar cells with siRNA

Pancreatic acini were isolated as above. Both Control siRNA (Cat# SI02020076, Qiagen, Valencia, CA) and vATPase E-subunit siRNA (Cat# SI02020039, Qiagen, Valencia, CA) were prepared as follows: jetPEI (Qbiogene, Wiesboden, Germany) was diluted 1μl/100ul in 150mM NaCl pH 7.4. jetPEI was further diluted 8.9μl/25μl/40pmoles of siRNA to be added. siRNA was diluted to a final concentration of 40-80pMoles in a final volume of 25μl using 150mM NaCl pH7.4. siRNA was vortexed and allowed to sit for 10 min. 25μl of appropriately diluted jetPEI was combined with 25μl of corresponding siRNA and vortexed. After vortexing the siRNA/JetPEI was allowed to sit for 60min before being added (50μl/well) to the cells. At the same time as the siRNA addition, carbachol (1μM final) was added to all wells to stimulate uptake of siRNA by endocytosis[16], [17]. After 30 min of carbachol treatment 1μM atropine was added to block any further carbachol effects. Cells were incubated for an additional 2.5 hours to allow for siRNA dependent protein knockdown. CER (100nM) or medium was added for one hour. After one hour of CER treatment, cells were collected and processed for zymogen activation and amylase secretion or to assess vATPase E-subunit protein levels by immunoblot.

Inhibition of new protein synthesis

Tissue culture plate containing pancreatic acini were place in an incubation chamber an allowed to recover for 1 hour at 37°C with shaking (90 rpm) under constant O_2. After an hour cells were removed from the chamber and the media exchanged for 0.5ml of new buffer-B. To inhibit protein synthesis, acini were treated with 300μM cyclohexamide for 30 min. Cells were then collected and centrifuged at 30 × g for 1 min and cell pellet collected and further processed for immunoblot.

Fluorescent zymogen assay

Fluorescent zymogen assays were carried out as described[18]. In brief, samples were thawed, homogenized, and centrifuged at 1000g for 1 min. To each well of a 24-well plate Falcon tissue culture plate added: 100 μl of supernatant and 350 μl of trypsin assay buffer [50 mM Tris (pH 8.1), 150 mM NaCl, 1 mM CaCl2, 0.01% BSA]. The assay was initiated by the addition of 50 μl of 400 μM enzyme substrate (fluorometric trypsin substrate; Peptides International, Louisville, KY) or fluorometric chymotrypsin substrate (Calbiochem, San Diego, CA). Enzyme activity was measured using a fluorometric microtiter plate reader

(model HTS 7000; Perkin-Elmer Analytical Instruments, Shelton, CT; 380-nm excitation; 440-nm emission; 20 reads/10 min). Enzyme activity was normalized to total amylase content.

Amylase secretion assay

Amylase was assayed using a commercial kit (Phadebas Kit; Mangle Life Sciences, Lund, Sweden) as described[18]. Amylase secretion into the media was expressed as percent of total amylase [media/(media+cells)].

Immunoblot analysis

Protein in cell pellets was solubilized by adding SDS-page loading buffer (120μl) and heating at 95°C. Proteins were separated on 10% SDS-PAGE gels (Bio-Rad, Hercules, CA) and transferred to Immobilon-P membranes (Millipore, Billerica, MA). Membranes were blocked for 60 min at room temperature with Blotto [Tris-buffered saline (TBS), 5% nonfat dry milk, 0.05% Tween-20]. Membranes were then probed with primary antibody (rabbit anti-vATPase E-subunit, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA) in Blotto for 60 min at room temperature, washed 3 times with blotto, and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000, Sigma) for 60 min at room temperature. Membranes were washed in 3 times in TBS, and protein bands were detected using a SuperSignal West pico chemiluminescence kit (Pierce).

Cell morphology

Cells were isolated as above and treated with or without vATPase inhibitors (concanamycin 100nM, lobatomide-B 1mM or salicylihalamide-A 1mM) for one hour followed by CER 100nM for an additional hour. Cells were pelleted 30g for 5 min and media was removed. To the cell pellets was added 1 ml of PLP fixative (10mM NaIO4, 75mM Lysine, 37.5mM NaPO4, 2% Paraformldehyde) and placed on ice for 1 hour. Pellets were then washed 2 times with PBS and postfixed in 1.0% osmium tetroxide (Polysciences, Inc, Warrington, PA), dehydrated in ethanol in propylene oxide, embedded in 100% EPON resin, and sectioned using an ultramicrotome. Sections were examined for cytosolic vacuole formation and plasma membrane blebbing using an Olympus BX51 microscope.

Statistical analysis

Data represents mean ± SEM of at least three individual experiments unless otherwise noted, with each experiment being performed in at least duplicate. Student's *t*-test analysis was used to determine statistical significance and *P* valuesof <0.05 were assigned significance.

Results

Benzolactone enamide vATPase inhibitors abrogate CER stimulated zymogen activation

We first examined the effects of the benzolactone enamide vATPase inhibitors lobatomide-B and salicylihalamide-A on CER induced zymogen (trypsinogen and chymotrypsinogen) activation and amylase secretion in isolated pancreatic acini. Trypsin and chymotrypsin activity were measured as markers for zymogen activation. Lobatomide-B was the more potent inhibitor, inhibiting both trypsinogen (Figure 1-A) and chymotrypsinogen (Figure 1-B) activation to control levels at concentrations of 0.1 and 1μM. Salicylihalamide-A inhibited trypsinogen activation (Figure 1-A) in a concentration-dependent manner, reaching significance at 1μM but had no effect on chymotrypsinogen activation (Figure- 1-B). Neither inhibitor had any significant effect on CER stimulated amylase secretion (Figure 2).

siRNA dependent knockdown of V_1E-subunit protein expression

Most experiments utilizing siRNA are conducted for time periods exceeding 48 hours. Given this caveat, siRNA use in long term acinar cell cultures could prove problematic. Thus, it has been shown that in both rat[19] and mouse[20] acinar cells, cellular responses to secretagogues change with time. For example, significantly higher concentrations of cholecystokinin are required to stimulate amylase secretion in acini maintained in culture overnight[19]. When human pancreatic acinar cells are placed into long term culture and stained for amylase content half of the amylase immunoreactivity is lost by day 2, with no immunoreactivity by day 4. In contrast within 2 days 20-50% of cells are positive for the duct cell markers keratin-19 and CAM 17.1 (a mucin antigen) and by day 5 almost 100% of cells are positive for these markers[21]. This suggests that acinar cells in culture rapidly differentiate into a ductal cell type. Furthermore, zymogen activation is optimally examined in preparations of less than 6 hrs and has not been detected in secretagogoue-stimulated acinar cells under long-term culture conditions. Our laboratory has found that acinar cells cultured for 12 hours or more no longer exhibit a zymogen activation response (unpublished data). To determine whether vATPase proteins could be depleted in an appropriate time frame using siRNA, we first investigated the half-life of an essential vATPase protein, the V1 E subunit[22]. After acini were treated with 300μM cyclohexamide for 30 min to inhibit protein synthesis, the levels of the V1E protein were reduced by 60% (Figure 3). These results indicate that the V1E protein has a high turnover rate and that it might be susceptible to siRNA in a short time-frame. To further enhance siRNA uptake, cells were treated with low levels of carbachol to stimulate siRNA endocytosis[16], [17] for 30 min. This was followed by a 2.5 hour time period to allow for protein knock down. Control siRNA had no effect on V1E levels while siRNA specific for the V1E knocked down protein levels to 15% of control (Figure 4).

siRNA dependent inhibition of CER stimulated zymogen activation and secretion

Next we determined if V1E-specific siRNA could reduce CER stimulated zymogen activation and amylase secretion. V1E-specific siRNA had no significant effect on trypsinogen activation (Figure 5-A) but dose dependently inhibited chymotrypsinogen activation (Figure 5-B). Control siRNA had no significant effect on zymogen activation (Figure 5-A, B). Both control and V1E-specific siRNA caused a minor decrease in CER stimulated amylase secretion (Figure 6) but this was not statistically significant.

Effects of vATPase inhibition on CER stimulated acinar cell morphology

We also examined the effect of vATPase inhibitors on CER stimulated changes in cell morphology. Untreated cells showed none of the hallmark indicators of cell injury (Fig 7-A). Cells treated with CER 100nM (Fig 7-B) showed both plasma membrane blebing (arrows) as well as the formation of cytosolic vacuoles (Arrow heads). Preincubation with the vATPase inhibitors concanamycin (Fig-7-C), Lobatomide (Fig 7-D) or Salacylahalamide (Fig 7-E) prior to CER stimulation had no effect on CER stimulated membrane blebing and cytosolic vacuole formation.

Discussion

Premature intracellular activation of pancreatic zymogens is a key event in the onset of pancreatitis. Our laboratory has shown that reducing extracellular or intracellular pH enhances secretagogue stimulated zymogen activation both in-vitro and in-vivo[23]. In addition, the lysosomal hydrolase cathepsin-B has been shown to activate trypsinogen[24], [25] and this activation requires a low pH environment[26]. Furthermore, cathepsins have been shown to colocalize with digestive zymogens[27], [28] and the zymogen activation compartment may be lysosomally derived and/or associated with the secretory pathway [29-31].

The vATPase is associated with the regulation of pH in lysosome-related vesicles as well as other compartments. In addition to its association with intracellular compartments the vATPase has also been shown to be associated with the plasma membrane. In osteoclasts the plasma membrane bound vATPase secretes hydrogen ions resulting in bone resorption. To date the potential role of proton extrusion by plasma membrane associated vATPase on pancreatic acinar cell pathology has not been addressed. In pancreatic acinar cells CER stimulation leads to a translocation of soluble V1 subunits to membrane-associated compartments[2]. Inhibition of vATPase with the classic vATPase inhibitors bafilomycin-A1 and concanamycin-A not only inhibits CER stimulated zymogen activation[2] but also blocks the enhancing effect of an extracellular acid load in-vitro[23]. Because the classic vATPase inhibitors bafilomycin and concanamycin have been shown to have non-specific effects[6-9] the current study was undertaken to confirm our previous results utilizing the novel class of vATPase inhibitors the benzolactone enamides, which have a different mode of inhibition, as well as genetic manipulation using siRNA.

The benzolactone enamide vATPase inhibitors lobatomide and salicylihalamide were originally isolated from marine organisms[12], [13] and are specific for the inhibition of vATPases from higher eukaryotes[4], [10], [11]. Like bafilomycin and concanamycin, lobatomide-B almost completely inhibited trypsinogen (Figure 1A) and chymotrypsinogen (Figure 1-B) activation at concentrations equal to or greater than 100nM. Salicylihalamide-A was not as potent causing inhibition to basal for trypsinogen activation (Figure 1-A) at 1μM but had no significant effect on chymotrypsinogen activation (Figure 1-B). Like bafilomycin and concanamycin neither lobatomide-B nor salicylihalamide-A had a significant effect on amylase secretion (Figure 2). Salicylihalamide has been shown to bind to the V0 complex of the vATPase. Its primary effect on the assembled and active pump complex is the inhibition of proton translocation a consequence of which is an inhibition of ATP hydrolysis by the V1[14]. However the ATP hydrolysis of the uncoupled V-ATPase or the isolated V1 complex is not affected[14]. Furthermore, it has been shown that both bafilomycin and concanamycin block J-concanalid A binding to the vATPase V0 c-subunit while salicylihalamide does not block this interaction[4]. These data suggest that the binding site for salicylihalamide on the V0 complex is not the same as that for bafilomycin and concanamycin on the c-subunit but does not rule out an alternate binding site on this subunit. The effects of lobatomide and salicylihalamide on zymogen activation confirm our previous results[2]. The differences we observed in the potency of salicylihalamide and lobatomide on zymogen activation are consistent with those described by Boyd et al[10] where salicylihalamide-A was a more potent inhibitor of vATPase activity in isolated membrane preparations than lobatomides A-F, but was much less potent than lobatomide-A in its ability to inhibit cell growth in oncogene transformed cell lines.

Since all pharmacological inhibitors have potential nonspecific effects we also used a complementary genetic approach. Using siRNA specific for V1E to knockdown protein expression we then examined the effects on CER stimulated zymogen activation and amylase secretion. One of the major drawbacks of the use of siRNA in primary acinar cell culture is the time needed to affect a sufficient knockdown of protein content. Secretagogue stimulation does not reliably cause zymogen activation in acinar cells placed in culture for periods over 6 hours after isolation. Here we demonstrate that there is a rapid turnover of the V1E protein (Figure 3) and that siRNA specific for V1E can significantly knockdown V1E protein (~85%) within 3 hours (Figure 4). When we examined zymogen activation there was a significant inhibition of chymotrypsinogen activation (Figure 5-B) but not trypsinogen activation (Figure 5-A). Amylase secretion was reduced with both control siRNA and V1E siRNA but this decrease in amylase secretion was not statistically significant.

When vATPase inhibitors were used, an inhibition of activation of both zymogens was seen, but when siRNA specific to the V1E subunit was used there was only a knockdown of chymotrypsinogen activation. This difference between trypsinogen and chymotrypsinogen activation was unexpected but not unprecedented. Previous studies from our laboratory have shown that trypsinogen and chymotrypsinogen activation respond differently to various enzyme activators and inhibitors. In a reconstituted cell system it was shown that chymotrypsinogen activation was ATP dependent, inhibited by the broad-spectrum kinase inhibitor (at μM concentrations) H-89, but unaffected by the cathepsin-B inhibitor E64-d. Trypsinogen activation, on the other hand, was shown to be independent of ATP, inhibited by E64-d and unaffected by H-89[32]. In whole cell culture chymotrypsinogen activation was more sensitive to the inhibition of PKC than trypsinogen activation[33]. These data suggest that the activation of the zymogens trypsinogen and chymotrypsinogen may be via different cellular pathways. Preliminary studies from our laboratory suggest that secretagogue stimulation results in the activation of trypsinogen and chymotrypsinogen in distinct cellular compartments (C.Shugrue, unpublished observations) and our current data supports this possibility.

The siRNA knockdown lowered V1E protein levels but this resulted in only a marginal effect on trypsinogen activation as mentioned above. A number of reasons could account for this result. The compartment(s) in which zymogen activation occurs may have a significant number of V1/V0 complexes already assembled and functional; a short-term knockdown may not result in a noticeable effect on intra-compartmental pH. Additionally, these functional V1/V0 complexes may be preferentially located on trypsinogen-only containing compartments. Another factor could be that the siRNA knockdown of V1E was incomplete, reducing protein levels but not eliminating them completely. Perhaps the few remaining assembled V1/V0 complexes are able to compensate for less available functional V1 complexes and up regulate their activity.

When morphology was examined we found that inhibition of the vATPase with Concanamycin-A, lobatomide-B or salicylihalamide-A did not reduce CER (100nM) stimulated vacuole formation or membrane blebing (Figure 7). These finding suggest that these morphologic changes associated with acinar cell injury might not be related to vATPase or zymogen activation[34]. Thus, it is possible that factors such as NFκB, or changes in either cytosolic calcium or cAMP associated with CER stimulation could be vATPase independent and relate to these morphologic changes. In this study we show that inhibition of the vATPase has no effect on CER stimulated amylase secretion, suggesting that the inhibitors are not have non-selective effects on acinar cell responses (Figure 2)

In conclusion this study confirms a role for the vATPase in secretagogue stimulated zymogen activation. We also show that proteins with a rapid turnover rate can be knocked down in a timeframe in which zymogen activation can still be assessed. Lastly, we have provided evidence that trypsinogen and chymotrypsinogen activation may be differentially regulated.

Acknowledgments

The authors would like to thank Christine Shugrue for her critical reading of this manuscript and Shao Song for expert technical assistance.

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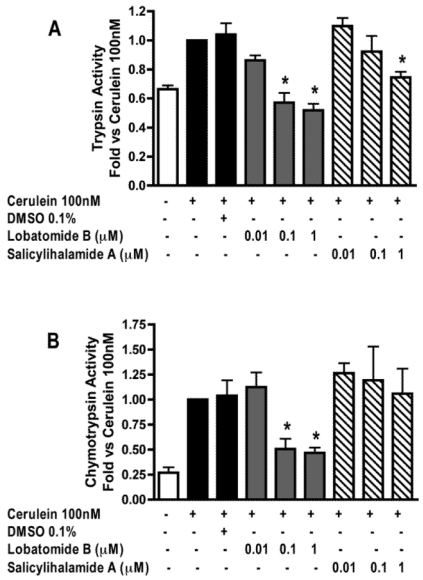

Figure 1. Lobatomide-B and salicylihalamide-A inhibit cerulein induced zymogen activation

Acini were pretreated with lobatomide-B, salicylihalamide-A (0.01-1μM) or carrier (0.1% DMSO) for 1 hour. Cerulein 100nM was then added to the appropriate wells and acini incubated for 1 hour. Samples were assayed for trypsin and chymotrypsin activity. Results are expressed as fold vs cerulein 100nM. All samples represent the mean +/- SEM of at least 3 experiments. * = p <= 0.05 vs CER 100nM.

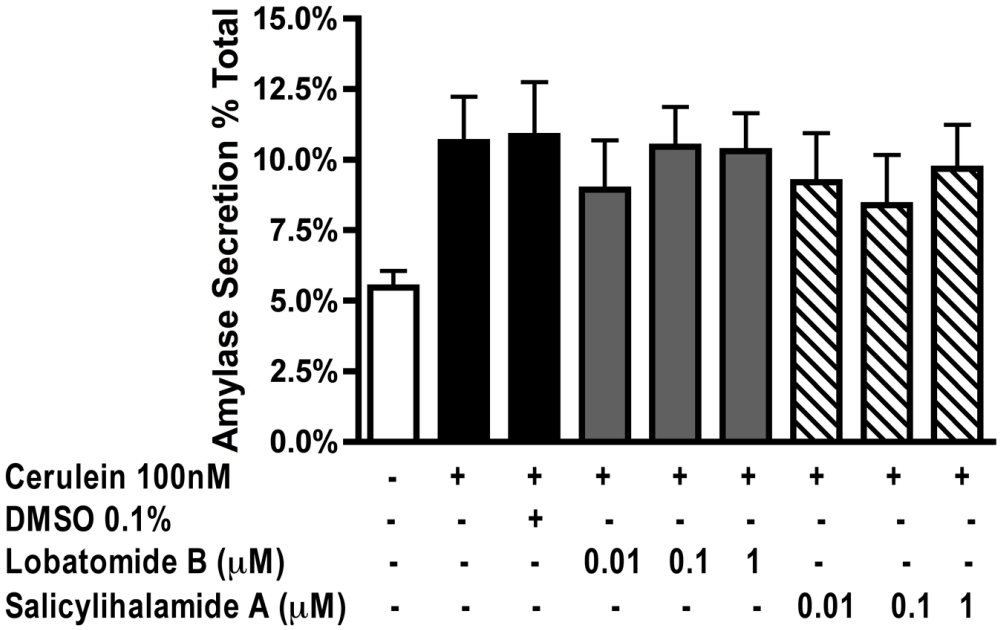

Figure 2. Lobatomide-B and salicylihalamide-A have no effect on cerulein induced amylase secretion

Acini were pretreated with lobatomide-B, salicylihalamide-A (0.01-1μM) or carrier (0.1% DMSO) for 1 hour. Cerulein 100nM was then added to the appropriate wells and acini incubated for 1 hour. Samples were assayed for secreted and total amylase activity; Results are expressed as amylase secretion as a % of total amylase. All samples represent the mean +/- SEM of at least 3 experiments.

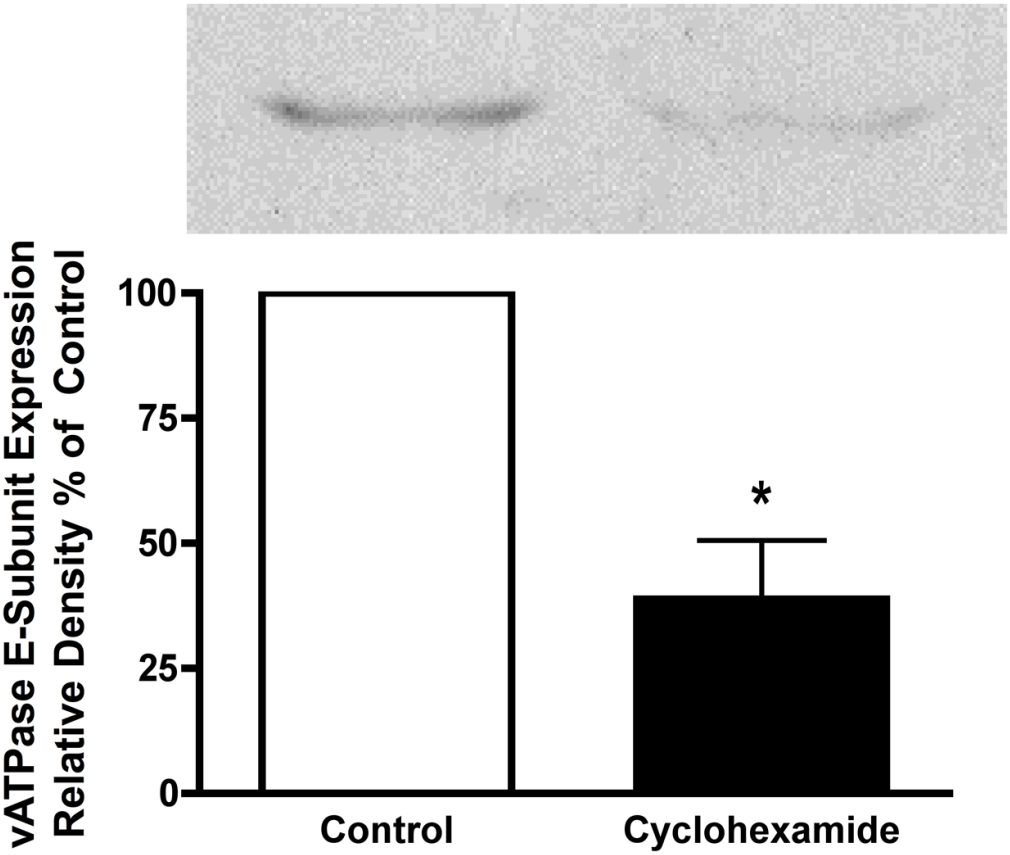

Figure 3. Inhibition of new protein synthesis knocks down vATPase E—subunit protein

Acini were treated with cyclohexamide (300μM) for 30 min. Acini were collected by centrifugation, solubilized and subjected to western blot analysis. All samples represent the mean +/- SEM of at least 3 experiments. * = p <= 0.05 vs Control (no cyclohexamide). The western blot is representative of one experiment.

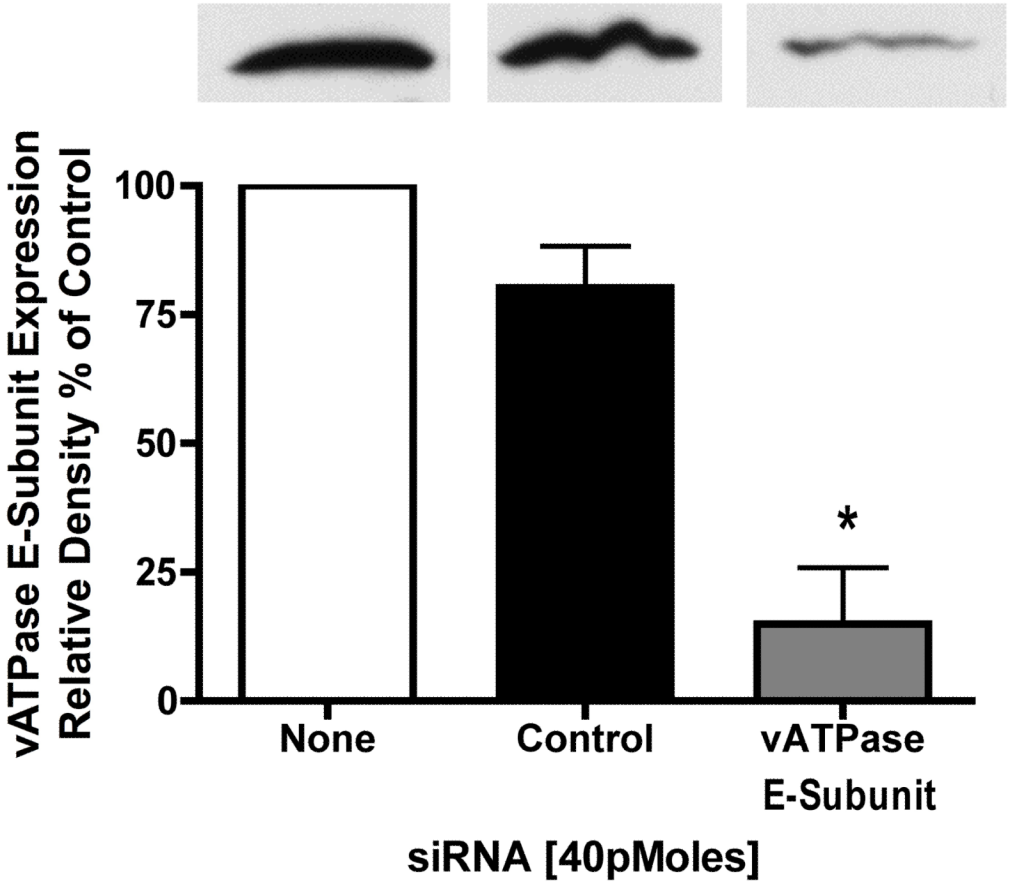

Figure 4. siRNA specific for vATPase V_1E-subunit knocks down V1E protein content in acini

Acini were incubated with control and E-subunit specific siRNA in the presence of carbachol (1μM) for 30 min to stimulate uptake. The effects of carbachol were neutralized by atropine treatment (1μM). Acini were further incubated for 2.5 hours to accomplish protein knockdown. Acini were collected by centrifugation solubilized and subjected to western blot analysis. All samples represent the mean +/- SEM of at least 3 experiments. * = $p <= 0.05$ vs no siRNA. The western blot is representative of one experiment.

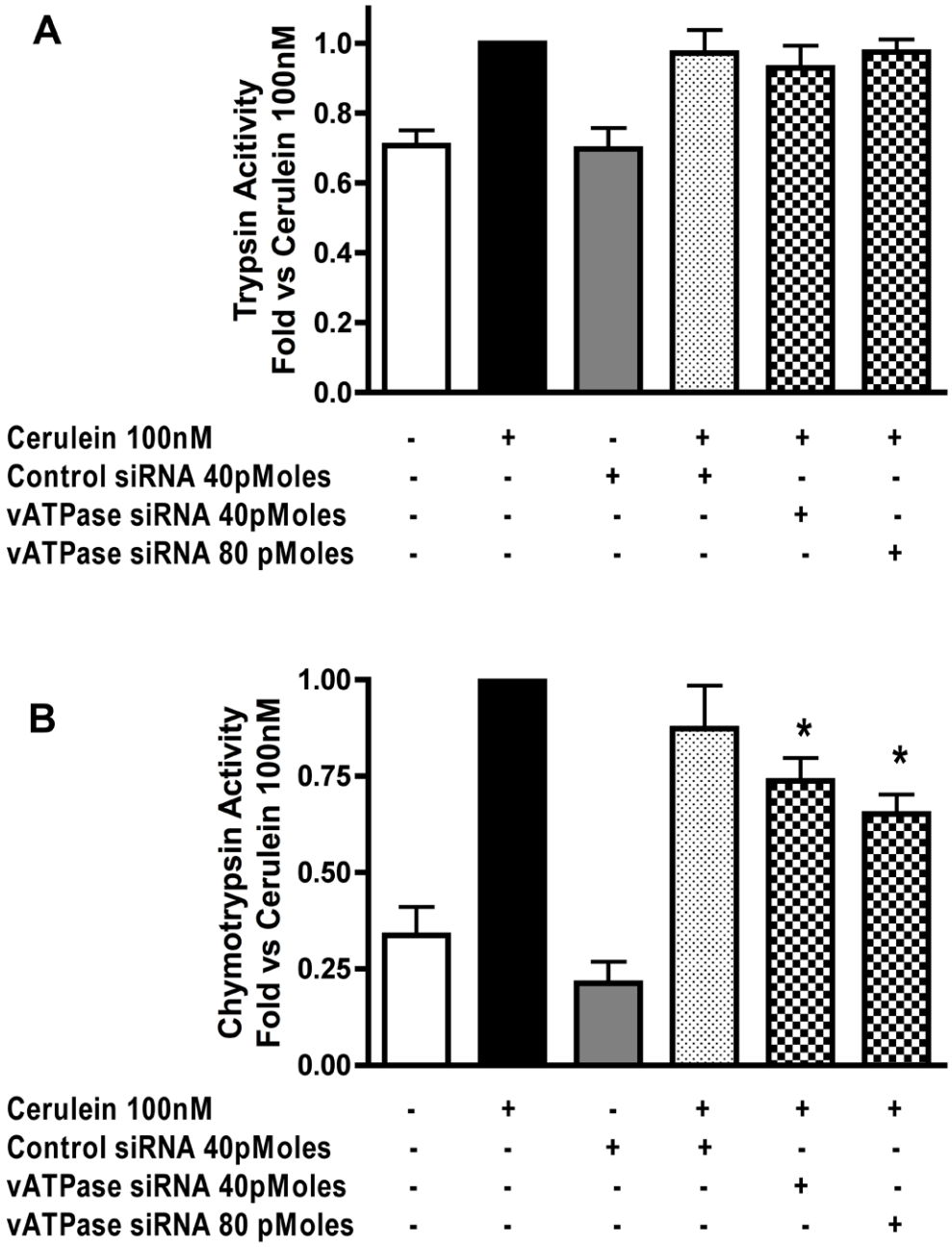

Figure 5. siRNA specific for vATPase V_1E-subunit inhibits chymotrypsin but not trypsin activation

Acini were incubated with control and E-subunit specific siRNA in the presence of carbachol (1μM) for 30 min to stimulate uptake. The effects of carbachol were neutralized by atropine treatment (1μM). Acini were further incubated for 2.5 hours to accomplish protein knockdown. Cerulein was then added to the appropriate wells for 1 hour. Samples were assayed for trypsin and chymotrypsin activity. Results are expressed as fold vs cerulein 100nM. All samples represent the mean +/- SEM of at least 3 experiments. * = p <= 0.05 vs CER 100nM.

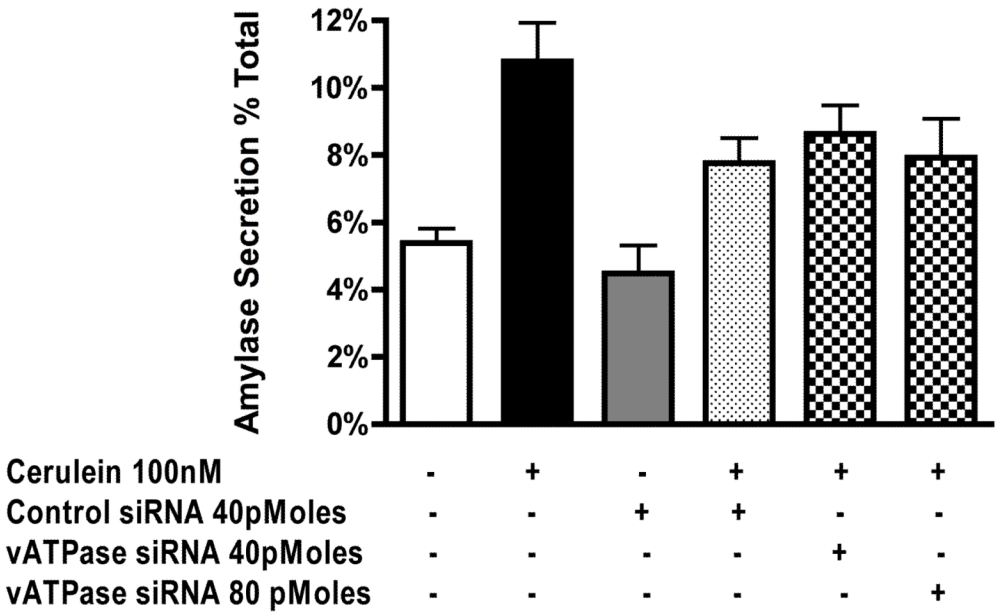

Figure 6. siRNA knockdown of vATPase V_1E-subunit has no significant effect on cerulein stimulated amylase secretion

Acini were incubated with control and E-subunit specific siRNA in the presence of carbachol (1μM) for 30 min to stimulate uptake. The effects of carbachol were neutralized by atropine treatment (1μM). Acini were further incubated for 2.5 hours to accomplish protein knockdown. Cerulein was then added to the appropriate wells for 1 hour. Samples were assayed for secreted and total amylase activity; Results are expressed as amylase secretion as a % of total amylase. All samples represent the mean +/- SEM of at least 3 experiments.

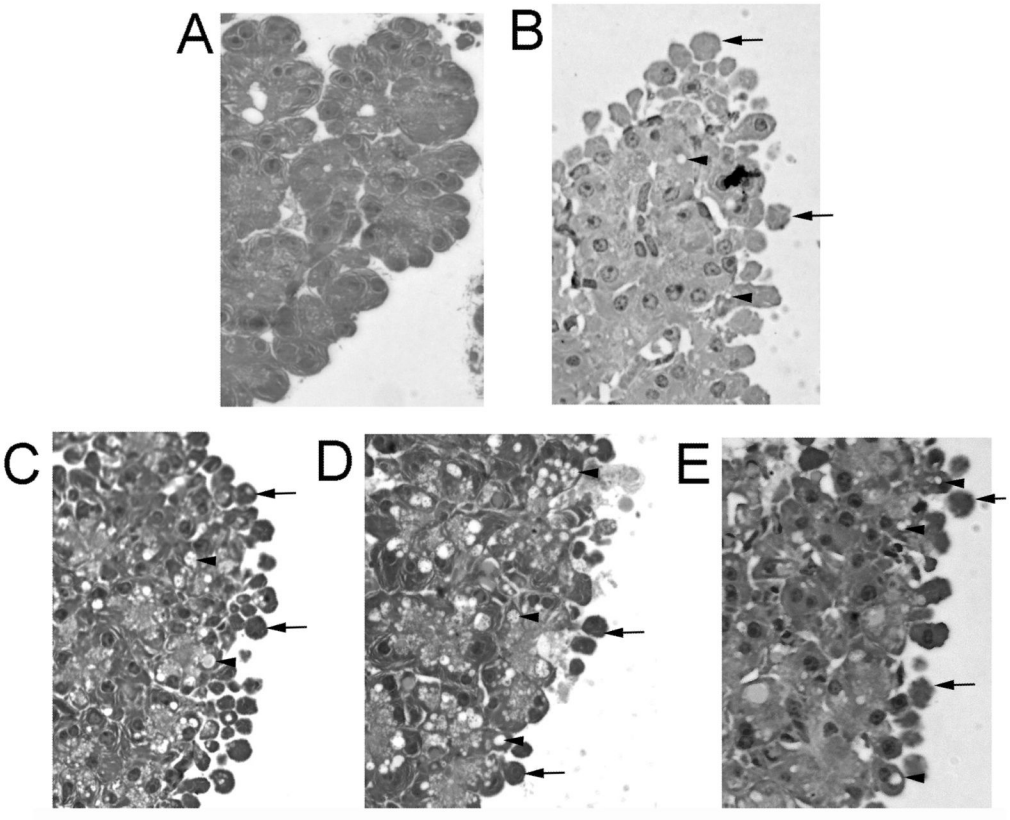

Figure 7. vATPase inhibition does not affect CER (100nM) dependent changes in cell morphology

Acini were incubated with the vATPase inhibitors concanamycin-A (100nM), lobatomide-B(1μM) or salicylihalamide-A(1μM) for 1 hour prior to CER(100nM) treatment for an additional hour. Cells were then embedded and assessed for cytosolic vacuole formation and membrane blebing. A=Control, B=CER(100nM), C=CER+Concanamycin, D=CER +Labatomide and E=CER+Salicylihalamide. Arrows indicate membrane blebing and arrowheads indicate vacuoles. Images were taken at 40X magnification and are representative of each treatment group.