

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 adenosine triphosphate (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 salicylilalamide-A as well as a genetic approach using siRNA. Both lobatomide-B and salicylilalamide-A inhibited CER-stimulated zymogen (trypsinogen and chymotrypsinogen) activation but had no effect on amylase secretion. Lobatomide-B (0.1 μ M) 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 salicylilalamide-A reduce early pancreatitis responses.

Keywords: lobatomide, salicylilalamide, 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 adenosine triphosphatase (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 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 have brought into question the specificity of these inhibitors for vATPase-dependent proton pumping activity. Teplova and colleagues examined the effects of bafilomycin on mitochondrial function

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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 (iNOS) inhibition. Bafilomycin causes the degradation of I κ B and phosphorylation of JNK resulting in the activation of nuclear factor-kappaB (NF- κ B) and ultimately an increase in iNOS mRNA and protein.⁷ The method by which bafilomycin causes I κ B degradation and JNK phosphorylation are unclear, but raise the possibility that these effects might not be related to vATPase inhibition. Bafilomycin⁸ and concanamycin⁹ have been shown to block chloroquine induced apoptosis in cultured cerebellar granule neurons at concentrations which do not inhibit vATPase activity (≤ 1 nM). This inhibition of chloroquine-induced apoptosis is seen with bafilomycin concentrations as high as 100 nM 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 worm¹² and marine sponge,¹³ respectively and were originally identified as antitumor compounds.¹⁰ Salicylihalamides bind to the V0 complex of the mammalian vATPase and this binding is distinct from the binding site for bafilomycin¹⁴ and concanamycin.⁴

In a previous study we showed that treatment of pancreatic acinar cells with bafilomycin or concanamycin blocked cerulein (CER)-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 CO₂ inhalation. The pancreas was

collected in buffer A: (10 mM Hepes [pH 7.4], 95 mM NaCl, 4.7 mM KCl, 0.6 mM MgCl₂, 1 mM NaH₂PO₄, 10 mM glucose, 2 mM glutamine, plus 0.1% bovine serum albumin, 1 \times MEM-amino acids (GIBCO-BRL, San Jose, CA)). The pancreas was then minced in 5 ml of buffer A and washed 2x with 5 ml of buffer A, then transferred to 12 ml of buffer-B (buffer-A + 1.3 mM CaCl₂) with 50 U/ml of type-4 collagenase (Worthington, Freehold, NJ). After incubation for one hour at 37 °C with shaking (120 rpm) under constant O₂, the preparation was filtered through a 300–400 μ m mesh (Sefar American, Depew, NY). The flow-through from the mesh was gravity pelleted and washed 2x with 12 ml of buffer B. The resulting acini were suspended in buffer B and plated at 0.5 ml 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 one hour at 37 °C with shaking (90 rpm) under constant O₂. At one hour, cells were removed from the chamber and the media exchanged for 0.5 ml of new buffer-B. At this time the vATPase inhibitors lobatomide-B, salicylihalamide-A (0.01–1 μ M) (Both inhibitors were 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 one hour prior to other treatments. After an hour CER was added to the appropriate wells at a final concentration of 100 nM and incubated for one hour. After one hour of CER treatment the contents of the wells were transferred to 1.5 ml eppendorf tubes and centrifuged 30 \times g for one minute. After centrifugation, 50 μ l 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) were prepared as follows: jetPEI (Qbiogene, Wiesbaden, Germany) was diluted 1 μ l/100 μ l in 150 mM NaCl pH 7.4. jetPEI was further diluted 8.9 μ l/25 μ l/40 pMoles of siRNA to be added. siRNA was diluted to a final concentration of 40–80 pMoles in a final volume of 25 μ l using 150 mM NaCl pH 7.4. siRNA was vortexed and allowed to sit for 10 minutes. 25 μ l of appropriately diluted jetPEI was combined with 25 μ l

of corresponding siRNA and vortexed. After vortexing the siRNA/JetPEI was allowed to sit for 60 minutes before being added (50 μ l/well) to the cells. At the same time as the siRNA addition, carbachol (1 μ M final) was added to all wells to stimulate uptake of siRNA by endocytosis.^{16,17} After 30 minutes of carbachol treatment 1 μ M 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 (100 nM) 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 plates containing pancreatic acini were placed in an incubation chamber and allowed to recover for one hour at 37 °C with shaking (90 rpm) under constant O₂. After an hour cells were removed from the chamber and the media exchanged for 0.5 ml of new buffer-B. To inhibit protein synthesis, acini were treated with 300 μ M cyclohexamide for 30 minutes. Cells were then collected and centrifuged at 30 \times g for one minute 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 1000 g for one minute. To each well of a 24-well Falcon tissue culture plate, the following were added: 100 μ l of supernatant and 350 μ l 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 μ l of 400 μ M 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 minutes). 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 (120 μ l) 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 minutes at room temperature, washed three times with blotto, and incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:2000; Sigma, St. Louis, MO) for 60 minutes at room temperature. Membranes were washed three times in TBS, and protein bands were detected using a SuperSignal West pico chemiluminescence kit (Pierce Biotechnology, Rockford, IL).

Cell morphology

Cells were isolated as above and treated with or without vATPase inhibitors (concanamycin 100 nM, lobatamide-B 1 μ M or salicylhalamide-A 1 μ M) for one hour followed by CER 100 nM for an additional hour. Cells were centrifuged at 30 \times g for five minutes and media was removed. To the cell pellets was added 1 ml of PLP fixative (10 mM NaIO₄, 75 mM Lysine, 37.5 mM NaPO₄, 2% paraformaldehyde) and placed on ice for one hour. Pellets were then washed two times with phosphate-buffered saline 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 and then stained using hematoxylin. Sections were examined for cytosolic vacuole formation and plasma membrane blebbing using an Olympus B \times 51 microscope.

Statistical analysis

Data represents mean \pm standard error of mean 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* values of <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 lobatamide-B and salicylhalamide-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 1A) and chymotrypsinogen (Figure 1B) activation to control levels at concentrations of 0.1 and 1 μ M. Salicylalahamide-A inhibited trypsinogen activation (Figure 1A) in a concentration-dependent manner, reaching significance at 1 μ M but had no effect on chymotrypsinogen activation (Figure- 1B). Neither inhibitor had any significant effect on CER-stimulated amylase secretion (Figure 2).

siRNA dependent knockdown of V1E-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 rat¹⁹ and mouse²⁰ 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 two 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 six hours and has not been detected in secretagogue-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 300 μ M cyclohexamide for 30 minutes 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 minutes. 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 5A) but dose-dependently inhibited chymotrypsinogen activation (Figure 5B). Control siRNA had no significant effect on zymogen activation (Figure 5A, 5B). 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 (Figure 7A). Cells treated with CER 100 nM (Figure 7B) showed both plasma membrane blebbing (arrows) as well as the formation of cytosolic vacuoles (arrow heads). Preincubation with the vATPase inhibitors concanamycin (Figure 7C), lobatomide (Figure 7D), or salicylalahamide (Figure 7E) prior to CER stimulation had no effect on CER-stimulated membrane blebbing 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

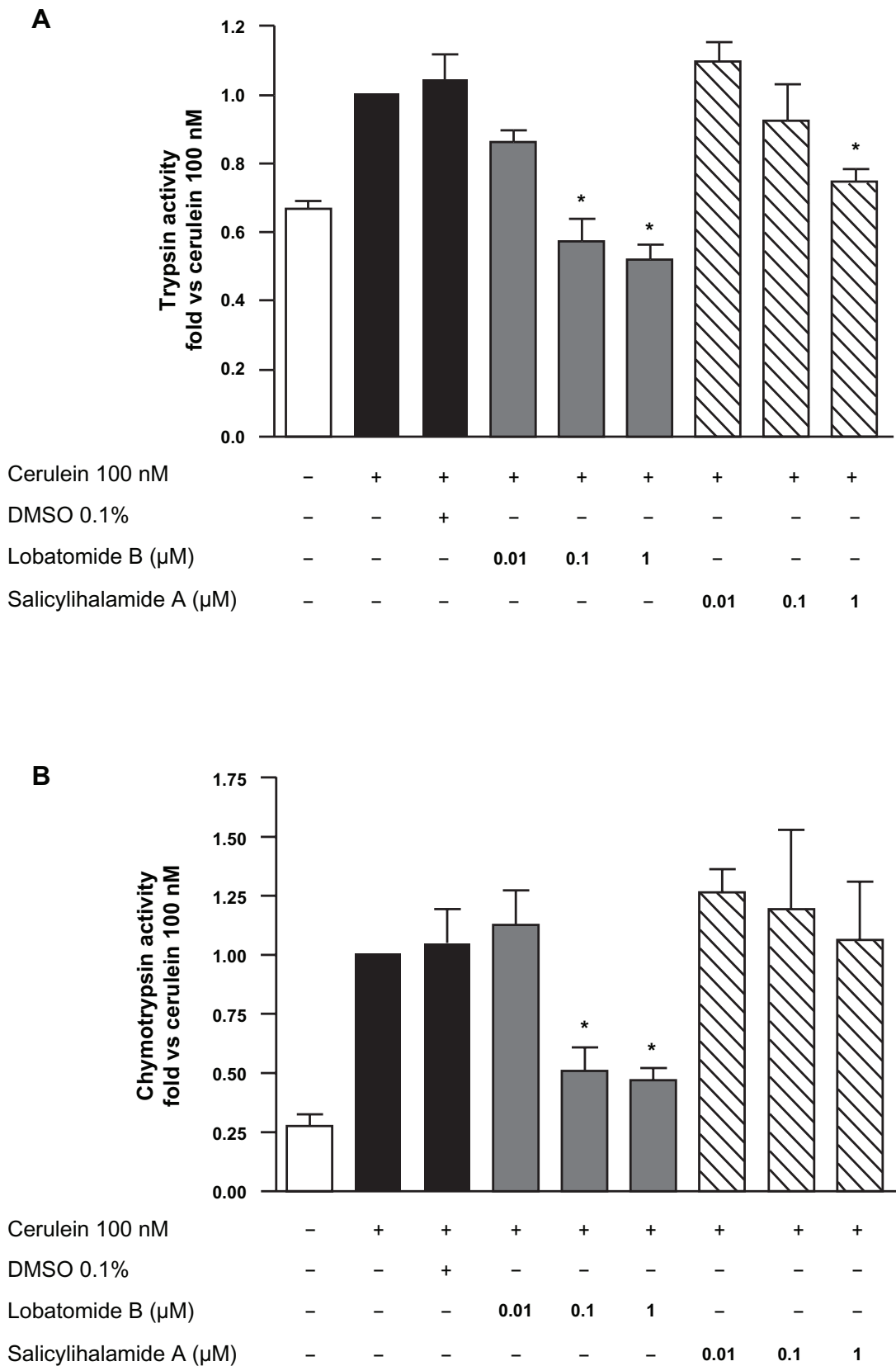


Figure 1 Lobatomide-B and salicylihalamide-A inhibit cerulein-induced zymogen activation. Acini were pretreated with lobatomide-B, salicylihalamide-A (0.01–1 μM), or carrier (0.1% DMSO) for one hour. Cerulein 100 nM was then added to the appropriate wells and acini incubated for one hour. Samples were assayed for trypsin and chymotrypsin activity. Results are expressed as fold vs. cerulein 100 nM. All samples represent the mean ± SEM of at least three experiments. **P* ≤ 0.05 vs. cerulein 100 nM.

Abbreviation: DMSO, dimethyl sulfoxide.

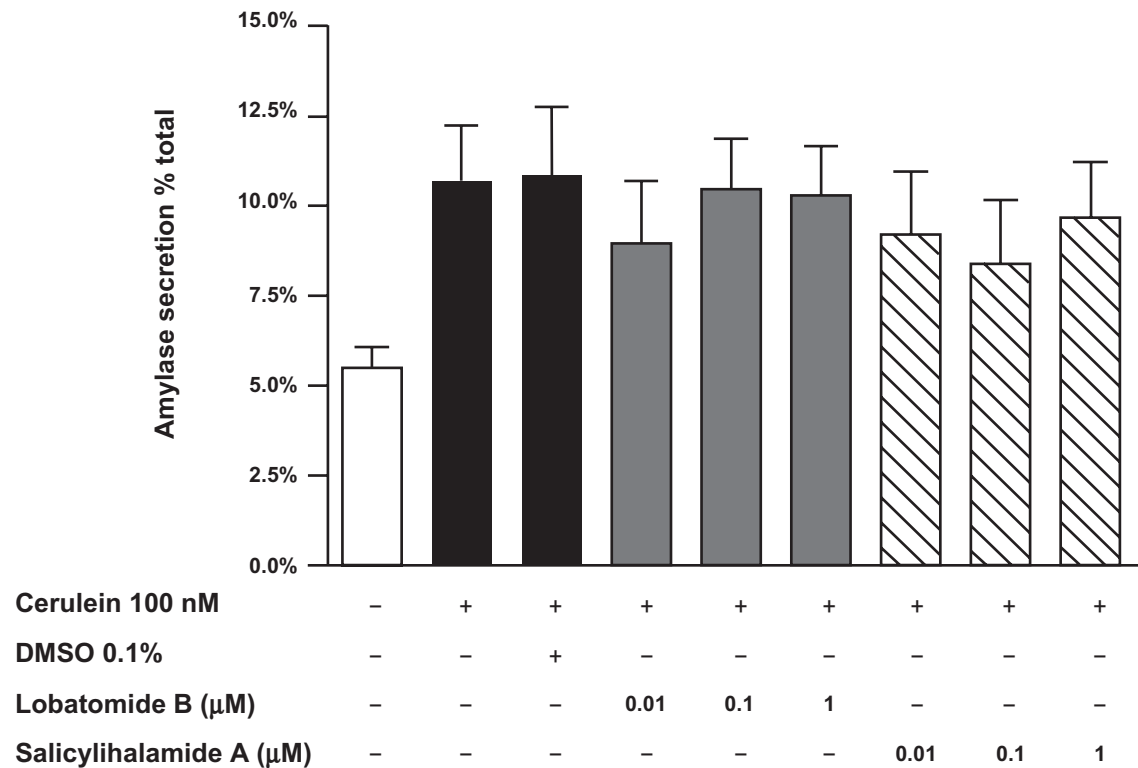


Figure 2 Lobatomide-B and salicylihalamide-A have no effect on cerulein-induced amylase secretion. Acini were pretreated with lobatomide-B, salicylihalamide-A (0.01–1 μM), or carrier (0.1% DMSO) for one hour. Cerulein 100 nM was then added to the appropriate wells and acini incubated for one hour. Samples 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 three experiments. **Abbreviations:** DMSO, dimethyl sulfoxide; SEM, standard error of mean.

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 nonspecific 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 1B) activation at concentrations equal to or greater than 100 nM. Salicylihalamide-A was not as potent causing inhibition to basal for trypsinogen activation (Figure 1A) at 1 μM but had no significant effect on chymotrypsinogen activation (Figure 1B).

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 complex.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 and colleagues¹⁰ where salicylihalamide-A was a more potent inhibitor of vATPase activity in isolated membrane preparations than lobatomides A-F, but was much less potent

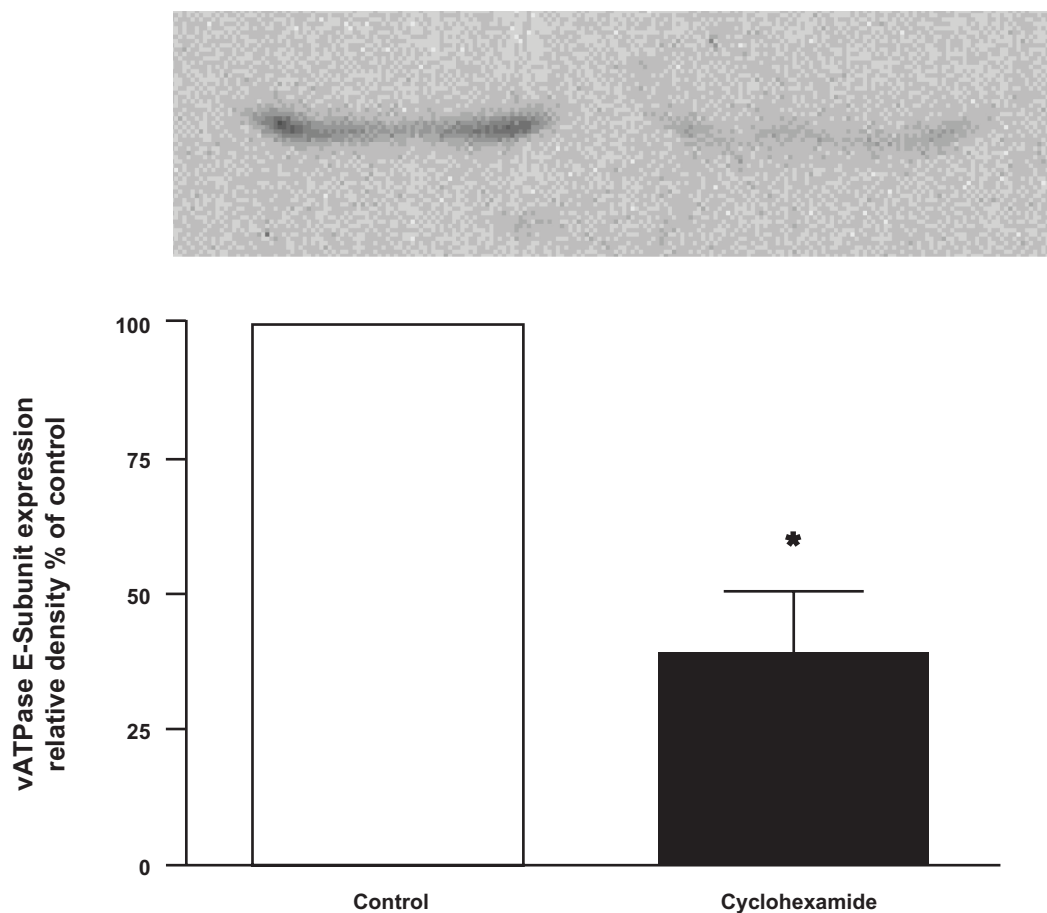


Figure 3 Inhibition of new protein synthesis knocks down vATPase E – subunit protein. Acini were treated with cyclohexamide (300 μ M) for 30 minutes. Acini were collected by centrifugation, solubilized and subjected to western blot analysis. All samples represent the mean \pm SEM of at least three experiments. * $P \leq 0.05$ vs control (no cyclohexamide). The western blot is representative of one experiment.

than lobatomide-A in its ability to inhibit cell growth in onco-gene 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 six 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 three hours (Figure 4). When we examined zymogen activation there was a significant inhibition of chymotrypsinogen activation (Figure 5B), but not trypsinogen activation (Figure 5A). 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 μ M 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

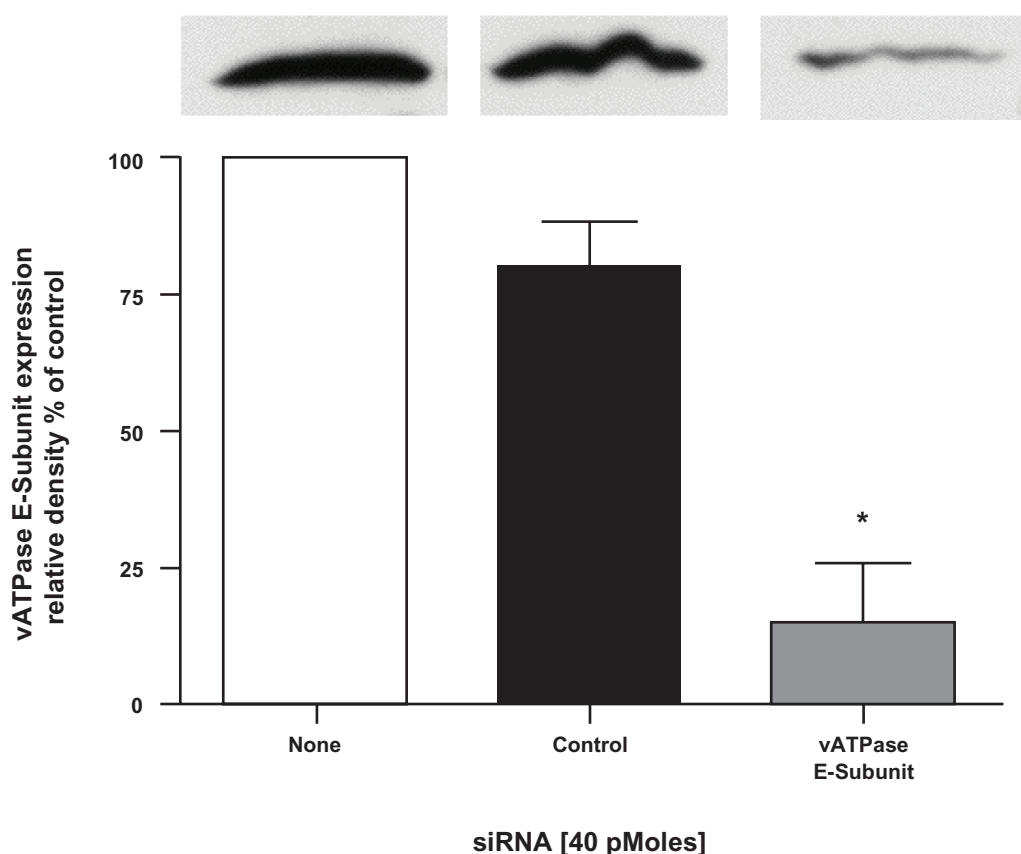


Figure 4 siRNA specific for vATPase V₁E-subunit knocks down V₁E protein content in acini. Acini were incubated with control and E-subunit-specific siRNA in the presence of carbachol (1 μ M) for 30 minutes to stimulate uptake. The effects of carbachol were neutralized by atropine treatment (1 μ M). Acini 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 \pm SEM of at least three experiments. * $P \leq 0.05$ vs no siRNA. The western blot is representative of one experiment.

Abbreviation: SEM, standard error of mean.

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 (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 upregulate their activity.

When morphology was examined we found that inhibition of the vATPase with concanamycin-A, lobatamide-B or salicylhalamide-A did not reduce CER (100 nM)-stimulated vacuole formation or membrane blebbing (Figure 7). These findings 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 NF κ B, 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 do not have nonselective 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.

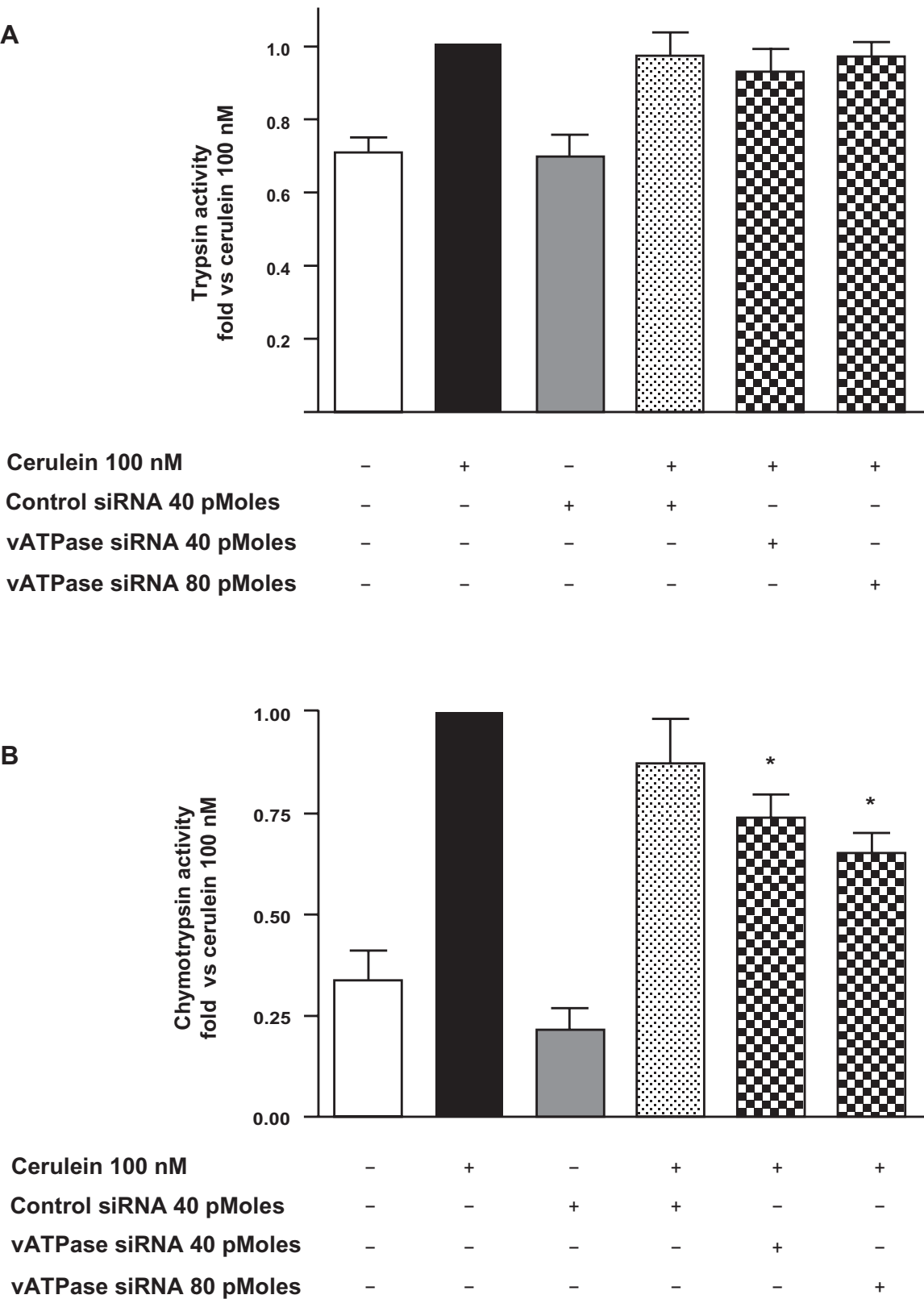


Figure 5 siRNA specific for vATPase V_E-subunit inhibits chymotrypsin but not trypsin activation. Acini were incubated with control and E-subunit-specific siRNA in the presence of carbachol (1 μ M) for 30 minutes to stimulate uptake. The effects of carbachol were neutralized by atropine treatment (1 μ M). Acini were further incubated for 2.5 hours to accomplish protein knockdown. Cerulein was then added to the appropriate wells for one hour. Samples were assayed for trypsin and chymotrypsin activity. Results are expressed as fold vs cerulein 100 nM. All samples represent the mean \pm SEM of at least three experiments. * $P \leq 0.05$ vs cerulein 100 nM. **Abbreviation:** SEM, standard error of mean.

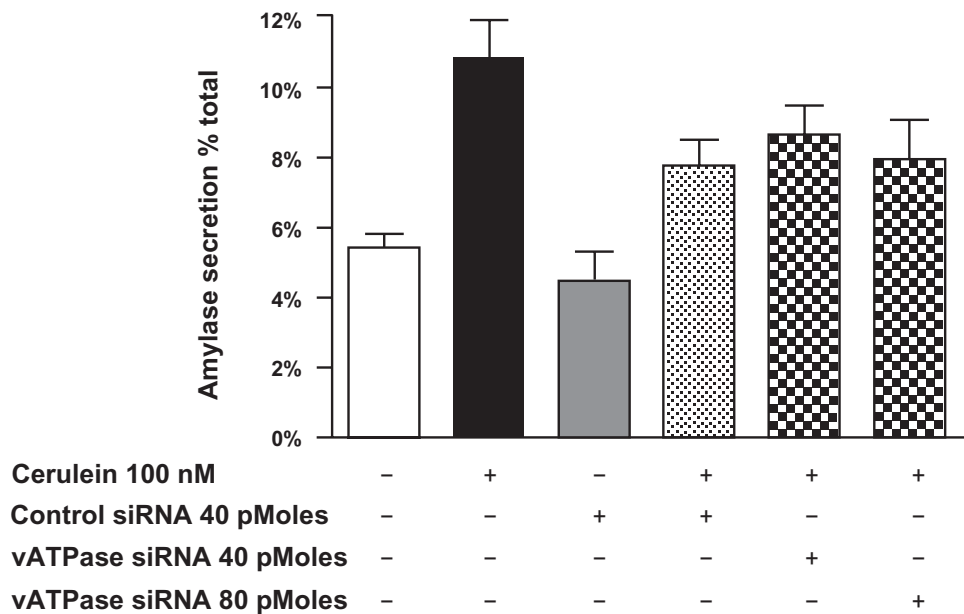


Figure 6 siRNA knockdown of vATPaseV₁E-subunit has no significant effect on cerulein-stimulated amylase secretion. Acini were incubated with control and E-subunit-specific siRNA in the presence of carbachol (1 μ M) for 30 minutes to stimulate uptake. The effects of carbachol were neutralized by atropine treatment (1 μ M). Acini were further incubated for 2.5 hours to accomplish protein knockdown. Cerulein was then added to the appropriate wells for one 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 \pm SEM of at least three experiments.
Abbreviation: SEM, standard error of mean.

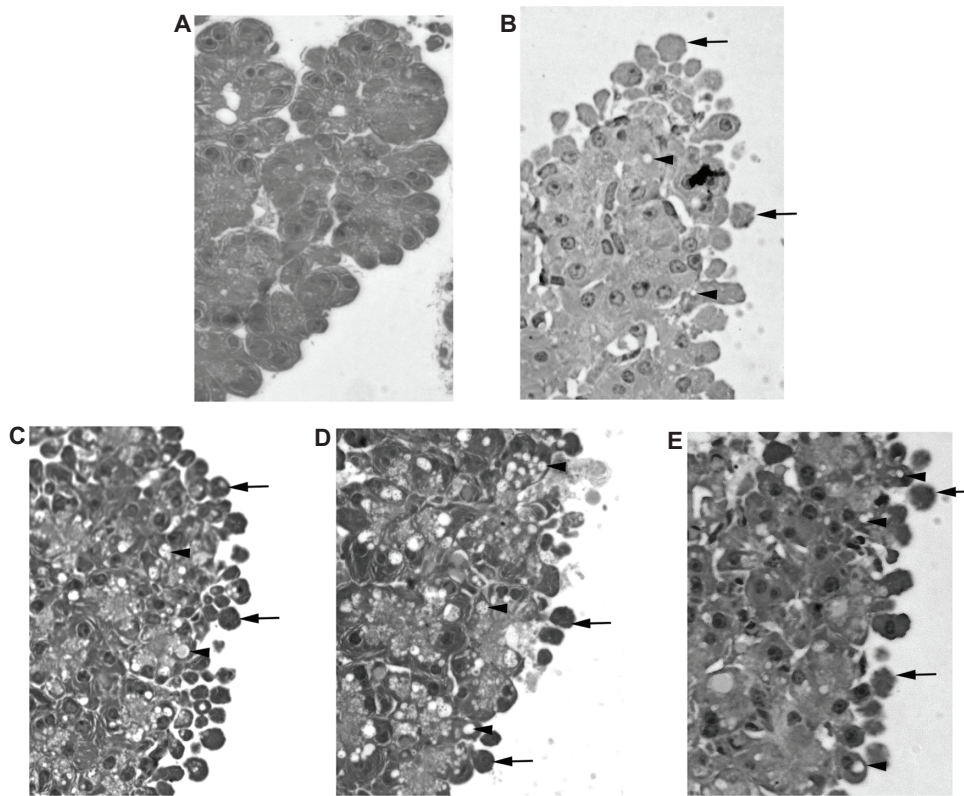


Figure 7 vATPase inhibition does not affect cerulein 100 nM-dependent changes in cell morphology. Acini were incubated with the vATPase inhibitors concanamycin-A (100 nM), lobatomide-B(1 μ M) or salicylhalamide-A(1 μ M) for one hour prior to cerulein 100 nM treatment for an additional hour. Cells were then embedded and assessed for cytosolic vacuole formation and membrane blebbing. Arrows indicate membrane blebbing and arrowheads indicate vacuoles. Images were taken at 40 \times magnification and are representative of each treatment group.
Abbreviations: A, control; B, cerulein 100 nM; C, cerulein + concanamycin; D, cerulein + lobatomide; E, cerulein + salicylhalamide.

Disclosure

The author reports no conflicts of interest in this work. 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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