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The Recruitment of ERK, Cortactin, and WASP in the Process of Enzymatic Secretion of Zymogen Granules Following CCK Stimulation

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ABSTRACT

Cellular secretion has been extensively studied in physiology. It is the process whereby cellular products are released outside the cell for with the pancreatic secretion of digestive enzymes being the best example. The secretory vesicles are thought to move by cytoskeletal rearrangements in the cell to reach the plasma membrane. The goal of this research was to identify proteins that might contribute to cytoskeletal rearrangement and determine whether they assemble at zymogen granules during secretion. These protein integral components include extracellular signal-regulated protein kinase (ERK), Wiskott-Aldrich Syndrome protein (WASP), and cortactin. This research indeed confirmed the presence of all three protiens on the zymogen granule following stimulation with CCK (2nM). ERK became apparent on the zymogen granule during a time course after stimulation with 2nM CCK. The ERK phosphorylation at the zymogen granule was also shown to be dependent on protein kinase C (PKC) and phosphatidyl inositol 4,5 bisphosphate (PI3K). The presence of cortactin and WASP was observed on the zymogen granule and provides a potential role in the cytoskeletal rearrangement necessary to traffic the vesicles to the plasma membrane. It is proposed, through this research, that ERK phosphorylates cortactin at the zymogen granule. Cortactin will subsequently activate WASP, which then stimulates cytoskeletal rearrangements through the Arp2/3 complex. These changes in the cytoskeleton are likely necessary to move the vesicle to the plasma membrane for secretion.

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INTRODUCTION

Human physiology is a very complicated yet masterfully organized system. All components, or organs, work in conjunction with one another to produce physiological responses. One key player of this unified process is the pancreas, functioning in a dual role as an endocrine and exocrine gland. The pancreas secretes numerous enzymes necessary for digestion (exocrine) and hormones to regulate glucose concentration and metabolism (endocrine). The gland itself is divided up into three sections and lies horizontally within the abdominal cavity. The head region of the organ is attached via connective tissue to the duodenum of the small intestine. The duodenum and the pancreas also associate by means of a common blood supply.

The functional units of the endocrine pancreas are the islets of Langerhans. Within the islets of Langerhans, there are different types of endocrine cells in the islets and include α, β, D, and PP cells which secrete glucagon, insulin, somatostatin, and pancreatic polypeptide, respectively. Blood flows into the pancreas via branching of the celiac and superior mesenteric arteries and exits the pancreas via the portal vein to the liver. The acini, and the ducts they envelope, are surrounded by distinctly separate capillary systems.

The exocrine pancreas exhibits grape-like clusters of acini, with each containing an intercalated duct responsible for draining the acinus and a terminal acinar portion. This being the smallest duct in the unit, it then converges into larger ducts until it reaches the main pancreatic duct. Pyramidal epithelial cells found within the acini are responsible for the synthesis of the digestive enzymes in pancreatic juice. The acini have a polarized structure. The nucleus is not found in the center of the cell, but rather at the base of the cell. In addition, mitochondria can be found throughout the cell, and the Golgi apparatus and zymogen granules are situated mostly in

the apical region. These granules contain the digestive enzymes that will be used to promote breakdown of macromolecules in the intestine.

The pancreatic juice itself is a mixture of contributions from both the duct cells and the acini cells. The digestive enzymes originate in the acini, as described above, and the duct cells produce a bicarbonate alkaline secretion. The purpose of this combination is to ultimately neutralize the chime that is arriving at the small intestine from the stomach during digestion. The digestive enzymes are contained in secretory vesicles that fuse with the apical membrane during exocytosis. These vesicles are known as zymogen granules and store a number of active and precursor enzymes. Proenzyme precursors include trypsinogen, chymotrypsinogen, procarboxypeptidase, proelastase, and prophospholipase A. Lipase, α-amylase, ribonuclease, and deoxyribonuclease are already in their active states when they are packaged in the zymogen granules. The fact that proteolytic enzymes are not fully active prevents the enzymes from digesting the pancreatic tissue itself. Upon fusion with the plasma membrane and release of enzymes into the pancreatic duct, the vesicle membranes are rapidly recycled. These precursor enzymes are activated once in the duodenum and jejunum components of the small intestine. Trypsinogen is converted into its activated form of trypsin in a reaction catalyzed by intestinal enterokinase. The activation of some initial tryspin initiates a positive feedback that can then catalyze the conversion of more trypsinogen into trypsin. In addition, its proteolytic behavior allows it to convert the other enzyme precursors into their active states. Once activated, the trypsin and chymotrypsin work to break down proteins, phospholipase A breaks down phospholipids, ribo- and deoxyribonucleases hydrolyzing RNA and DNA respectively, and amylase breaking down carbohydrates.

The release of enzymes by the acinar cells is regulated by hormones and neurotransmitters. Hormones can include secretin and cholecystokinin (CCK), while acetylcholine is a parasympathetic neurotransmitter promoting release. Secretin is responsible for triggering the release of the alkaline component of pancreatic juice, and CCK is responsible for stimulating the release of the enzymatic component. The regulatory mechanisms for these two events are the source of much scientific investigation. Sophisticated feedback control mechanisms regulate the release of these digestive enzymes into the intestine. Many of the secretion details have already been identified, but a great deal remains to be elucidated. The cascade of events that extends from stimulation to secretion was the subject of this investigation. More specifically, components within the secretory cascade (ERK and cortactin) were analyzed in detail to determine their significance and role in the process independently as well as in conjunction with one another.

CCK acts through a 7-transmembrane G protein-coupled receptor. Through this receptor, CCK is able to activate multiple signaling cascades and therefore regulate many cellular functions in the pancreas. The ligand bound CCK receptor activates a heterotrimeric G protein from the Gq family. Upon activation, the subunits dissociate, with the α-subunit then activating a phospholipase C-β (PLCβ). PLCβ then creates IP3 and diacylglcerol (DAG) by cleaving phosphatidylinositol 4,5-bisphosphate. Each component goes on to activate another member of the stimulation cascade. Once IP3 binds to its intracellular receptor, it triggers the release of intracellular calcium. DAG is necessary to activate protein kinase C (PKC) isoforms. Both Ca2+ and PKC work together to arbitrate the secretion of digestive enzymes via zymogen

granules. CCK has also been shown to activate another Gq subunit (Gs) which ultimately leads to the increase in the amount of cAMP present (Dabrowski etal., 2002).

The transport of secretory vesicles to the plasma membrane takes place on actin filaments once the vesicles have been transferred from microtubules. Microtubules and actin filaments produce a directionality of movement by having + and – ends to which the vesicles migrate. The + barbed end of these filaments attach themselves to the plasma membrane; this serves as the highway for vesicle transport. Both actin filaments and myosin motors have been implicated in the exocytic vesicle transfer in secretory cells. The subapical actin filaments are likely responsible for regulating exocytosis of vesicle contents and the membrane retrieval that takes place after secretion (Doctor et al, 2002). Microtubules specifically appear to deal with the longer range transport, while actin acts as a local roadway for short distance moving. Arp 2/3, an actin associated protein, complex is responsible for severing the actin uncapped barbed ends of the actin filaments and creating new barbed ends. Once the Arp 2/3 complex is formed, it triggers new filament formation for branching networks, and intitates cortical actin polymerization that takes place during plasma membrane reorganization. Arp 2/3 also aids in the formation of the regulatory actin structures on vesicles. Actin nucleation promoting factors (NPFs) provide the stimulus for Arp 2/3 complex activation. WASp, N-WASp, and Scar/WAVE are all members of the WASP family proteins and are all powerful activators of the Arp 2/3 complex (Daly, 2001).

Cdc42 and Rac are other proteins that initiate cortical cytoskeletal rearrangement via the activation of Arp 2/3. Cdc42 and Rac require assistance from the Wiskott-Aldrich Syndrome protein (WASp) superfamily in order to initiate cortical actin polymerization. N-WASp contains

a carboxyl-terminal motif that regulates binding to Arp 2/3 complex (Weed et al., 2000), while the WASp proteins serve as a target of Cdc42/Rac cortical-actin polymerization, triggering binding and activation of the Arp 2/3 complex via stimulation of actin nucleation activity (Parsons and Weed, 2001). A number of other actin binding proteins are recruited during cytoskeletal regulation, one of which being cortactin (p80/p85). Cortactin has been referenced in signaling mediated by multiple extracellular stimuli (Huang et al., 1997). Cortactin is a multi-domain structure that is expressed in almost all mammalian tissues.

The N-terminal section of cortactin binds to F-actin and initiates the activation of Arp2/3 complex, ultimately leading to actin filament assembly (Welch et al., 1998). This N-terminus also targets cortactin to the actin reassembly near the membrane. Using negative staining and electron microscopy, it was found that most F-actin strands became thicker and formed bundle-like structures in the presence of cortactin and only single strands in cortactin's absence, suggesting an influential role (Huang et al., 1997). The C-terminus SH3 domain interacts with the regulators of the actin reassembly components such as dynamin, WASP interacting protein (WIP), and N-WASP (Kinley et al., 2003). Cortactin has also been shown to have the ability to activate Arp2/3 alone. Even though cortactin acting alone elicits a weaker response than going through N-WASP, it does show that cortactin can have a direct effect on Arp 2/3 (Weaver et al., 2001). N-WASP activates the Arp 2/3 complex by a transient interaction that increases the association of the Arp 2/3 with an existing actin filament; the N-WASP can then be released from the complex by replacement with cortactin. The high affinity of cortactin for the activated Arp 2/3 complex is needed to release WASP from the branching site and increase stabilized nucleation sites for rapid actin filament growth (Uruno, 2003). Cortactin may bridge sites of actin reorganization with signaling complexes and, in addition to recruiting other proteins that

may regulate Arp 2/3 actin polymerization (Weed et al., 2000). Cortactin may also perhaps contribute in organizing transmembrane receptor networks, for example the tight junction complex (Weed and Parsons, 2001). Cortactin is also involved with a second type of cellular junction, the adherins junction, whose formation is vital in tissue organization of developing organisms (Yap et al., 1997).The results suggest that Arp 2/3 activation and actin network stabilization is a conserved cellular function of cortactin. Cortactin's interaction with the scaffolding proteins and dynamin suggest a link between transmembrane receptor complexes and membrane vesicle dynamics to specific areas of active actin polymerization. Therefore, cortactin could act as a significant mediator for ligands to regulate the cytoskeletal reorganization.

Significantly, CCK stimulation of the pancreas is able to initiate a signaling cascade that results in the phosphorylation of proteins in the ERK cascade. The ERK cascade transmits signals from cell-surface receptors to specific downstream targets via phosphorylation. The two terminal signaling components of this cascade are extracellular signal-regulated protein kinase 1(ERK 1) and ERK2. If this cascade were to be blocked, then growth-stimulating agents would yield unsuccessful cell proliferation (Dabrowski etal., 1997). The CCK-induced ERK cascade has been shown to stimulate the formation of a Shc-Grb2 complex, utilizing the tyrlosyl phosphorylation of Shc. Daulhac etal.(1999) suggest that Shc is a Src substrate, and that Src is activated by CCK. Once the Src-type tyrosine kinases are active, they then proceed to phosphorylate precise tyrosine locations on the Shc protein. The activated Shc is now able to form a complex with growth-factor-receptor-bound protein 2(Grb2). Grb2 has the role of pulling a model Ras-guanyl nucleotide exchange factor, known as SOS, to the cellular membrane and activating the molecule, utilizing its SH3 domain. SOS is a guanine nucleotide exchange factor and will cause GTP to replace GDP on Ras. The function of activated Ras is to attach to Raf

kinases with high affinity and cause the resultant migration to the cellular membrane, ultimately leading to the activation of Raf. Signaling by Ras has been associated with such processes as cell division, cell adhesion, differentiation, cell survival, and significanctly, cytoskeletal rearrangements (Chan et al., 1999). Raf kinases will phosphorylate and activate the downstream substrate MEK. There exists two MEK isoforms, both of which are able to activate another downstream substrate, ERK, via phosphorylation of a Thr-Glu-Tyr motif. MEK is a part of a unique class of dual-specificity kinases that have the ability to phosphorylate both threonine and tyrosine residues (Dhanasekaran and Premkumar, 1998). ERK is a serine/thronine kinase with over 50 different identified substrates. The range of substrates includes cytoskeletal proteins, other kinases, phosphatases, enzymes, and transcription factors. The substrate target and physiological response is a result of a cell-specific downstream substrate combination and subcellular compartmentalization (Garcia etal, 2002).

Substrate recognition by members of the ERK cascade is enhanced by their high binding affinity. Generally, the substrate proteins contain MAPK-docking sites. These sites are located a distance away from the phosphorylation site. These two sites, docking and phosphorylation, create a bipartite recognition strategy and leads to the discriminatory nature of ERK when choosing substrates. Eamples of an ERK substrate include the phosphorylation of STAT3, part of a group of cytoplasmic transcription factors that translocate to the nucleus after tyrosine phosphorylation at the plasma membrane (Chung et al., 1997). ERK1 and ERK2 can also form a stable complex with the microphthalmia-associated transcription factor (MITF), a tissue-specific MAPK substrate found in melanocytes. This particular substrate coordinates gene expression in such processes as stem cell maintenance, differentiation, melanogenesis and cell survival (Goding, 2000).

An important study (Campbell et al.,1999) determined that EGF (epidermal growth factor)-induced activation of the Ras-Raf-MEK-ERK pathway is responsible for the cortactin serine and threonine phosphorylation. A study that inhibited the MEK component of the cascade found that the cortactin activation still took place, implying that the true regulator had to be somewhere downstream of MEK. Upon reviewing peptide mapping data, ERK ½ has been implicated as the protein responsible for phosphorylating cortactin at serine-405 and serine-418 ERK consensus sites within the proline-rich domain. Because these sites are located in the SH3 binding sites, one proposed theory is that ERK-mediated serine phosphorylation may regulate an intermolecular interaction between the cortactin SH3 domain and sequences within the proline-rich domain. This would then allow the SH3 domain to bind with other ligands. Other serine/thronine kinases may also play a role in the phosphorylation of cortactin based on the number of kinases activated by MEK.

In cells that are considered cortactin over-expressing, there is a sustained ERK activation. The potentiating of ERK signaling in these cells may provide further evidence for cortactin's role in cell migration since cortactin phosphorylation by ERK has been shown in recent studies to increase the capacity of cortactin to boost N-WASP-mediated actin polymerization (Martinez-Quiles et al., 2004).

Cytoskeletal reorganization is an important feature in both processes of exocytosis and endocytosis. Cortacin has also been shown to associate with the regulators of endocytic vesicle fission. In addition, the partnership with Arp 2/3 works with endosomal vesicles as well, solidifying the possibility of cortacitn's actin nucleation activity (Timpson et al., 2005). Cortactin couples Arp2/3 complex-mediated actin dynamics to endocytosis and vesicle

movement by binding to dynamin 2. The cortactin-dynamin 2 complex can, therefore, regulate the organization of actin filaments associated with membranes.

It has been previously determined that cortactin regulates membrane associated actin filaments, with WASP aiding in the initiation of cortical actin polymerization. ERK has also been shown responsible for cortactin serine and threonine phosphorylation. If these components are vital to the process of cellular secretion, then those components should be present in the zymogen granule. This study confirms the fact that WASP, ERK, and cortactin are all present on the zymogen granule and that their increases during granule exocytosis. A model can then be formulated which incorporates these known components.

MATERIALS AND METHODS

Isolation of Pancreatic Acini

Pancreatic tissue was harvested from male Sprague-Dawley rats ranging from 125-190g. Light ether anaesthesia followed by cervical dislocation was used to euthanize the rat. The procedure was approved by the NIU IACUC committee (protocol #221). Dissection of the pancreas from the rat followed swiftly. Fat was removed from the pancreas, minced, and transferred to a 50ml culture tube containing 20ml of Ringer solution (Medium 199, including: 10mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid [HEPES], 1mg/1ml bovine serum albumin [BSA], trypsin protease inhibitor [.1mg/ml], and 0.1 mM phenyl methyl sulfonyl fluoride [PMSF]). A shaking water bath set at 37 degrees C was then used to incubate the tissue for 10 minutes. The cell suspension was given sufficient time to settle and the supernatant

removed. Prior to experimental treatment, the pancreatic cells were washed and resuspended in 20ml of Ringer.

Chemicals

All required reagents, antibodies, agonists, and inhibitors used during the course of this project were purchased from various biochemical companies in the United States. Protease inhibitors including phenyl methyl sulfonyl fluoride (PMF), (Sigma Chemical Company, St. Louis, MO), trypsin inhibitor (Gibco BRL, Inc.), and leupeptin were used as supplements to the incubation solution. Cholecystokinin, a known physiological agonist for pancreatic secretion, was purchased from Calbiochem-Novabiochem Corp. LaJolla, CA.

Primary antibodies including polyclonal rabbit anti-ERK1 IgG, polyclonal rabbit anti-ERK2 IgG, and polyclonal rabbit anti-WASP and anti-Cortactin were purchased from Santa Cruz Biotechnology, Inc. Horseradish preroxidase, labeled anti-rabbit IgG secondary antibody was also purchased from Santa Cruz Biotechnology, Inc.

Protein Assay

Protein assays were conducted using the Bio-Rad Bradford Protein Assay Kit with bovine serum albumin as a standard. Protein fractions were incubated in 4 ml of a 1:4 dilution of protein dye stock solution for 15 minutes at room temperature. Absorbencies were measured with a Beckman DU-60 sepectrophotometer at a wavelength of 595nm.

Amylase Assay

Amylase assays were performed by a modified version of Bernfeld (1955), in order to determine the extent of secretion from the pancreatic acini during treatment. The disaccharide standard used in this study was maltose. Samples of 25 µl were incubated with 75µl of amylase resuspension buffer (0.2% Trito X-100, 50 mM Tris, 100 mM NaCl, and 10 mM CaCl2) and 100 µl substrate (1% amylopectin, 40 mM MOPS, 60 mM NaCl) at 37 degrees C for 30 minutes in a shaking water bath. The reaction was halted with the addition of 0.2 ml stop-development reagent (1% 3,5 dinitrosalicyclic acid, 0.4 N NaOH, 1.06 M sodium potassium tartarate). The resulting fractions were then heated din a dry bath for 7 minutes at 95 degrees C. The fractions were cooled and diluted with the addition of 2.6 ml of water. Individual absorbencies were read with a Beckman DU-60 spectrophotometer at a wavelength of 530 nm. Amylase release was then plotted as a function of time.

Electrophoresis and Electroblotting

Frozen samples, previously solubilized in laemmli buffer, were heated at 95 degrees C for 10 minutes after removal from -80 degree C storage. After cooling, equal amounts of each sample were then loaded into a discontinuous SDS-polyacrylamide gel (gel composition of 4% acrylamide/bis-acrylamide in the stacking gel and 12% acrylamide/ bis-acrylamide in the separating gel). The samples were electrophoresed at 100V for 60-90, or until the bromophenol blue dye front had reached approximately 1-2 mm from the bottom of the gel. After electrophoresis, gels and nitrocellulose sheets were equilibrated for 10 minutes in transfer electrode buffer (192mM glycine, 20mM Tris, 20% methanol) with the purpose of removing extraneous salts and detergents from the gel and to uniformly wet the nitrocellulose film.

Proteins were then electroblotted onto a pure nitrocellulose membrane for 1 hour at 100V at 4 degrees C. The electroblot was performed using the Mini-Trans electroblotting apparatus (Bio-Rad Laboratories).

Western Blotting and Enhanced Chemiluminescence (ECL)

After the elctroblotting process, unoccupied reactive sites on the nitrocellulose sheet were blocked by rocking with 10% nonfat dry milk in TTBS (250mM NaCl, 20mM Tris [pH 7.5] 0.05% Twen-20) for 90 minutes at room temperature. Following blocking, nitrocellulose blots were washed 6 times with a TTBS solution on a rocker at room temperature for 5 minute intervals. After washing the nitrocellulose blots were then incubated on a rocker overnight at 4 degrees C or for one hour at room temperature in a solution of 1% non-fat dry milk in specific salt concentration TBS and a primary antibody. A 1:1000 dilution was used for stock polyclonal rabbit primary antibodies. The blots were then washed six times on 5 minute intervals with TTBS at room temperature on a rocker.

Primary antibody binding to proteins was detected using a 1:2000 dilution of stock horseradish peroxidase labled anti-rabbit IgG. The blot was then incubated with the secondary antibody in 1% nonfat dry milk in TTBS for one hour at room temperature on a rocking platform. Following the incubation with the secondary antibody, the blots were again washed six times on 5 minute intervals with TTBS on the rocker. After the final wash, the bolts were then exposed immediately to lumnol developer solution (equal volumes of cyclic diacylthydrazide luminal and alkaline enhancer). This method relied upon the ability of bound horseradish peroxidase to oxidize the luminal and thereby excite the cyclic diacylhydrazide to a higher energy state. Once excited, the activated compound decays to a lower energy state and emits a

photon of light (wavelentght max = 428nm) in the process. The chemiluminescence was detected using blue light-sensitive autoradiographic ECL Hyperfilm in a darkroom. This exposure was performed in a standard film-holding cassette for 15 seconds to 30 minutes, depending on the antibody used and the amount of the starting samples. The exposed Hyperfilm was then developed and fixed in an automated film developer.

Image Scanning and Quantification

Following the western blotting procedure and enhanced chemiluminescense, protein bands on the ECL film were acquired using the computer program Adobe Photoshop 5.0. These scannedprotein bands were then quantified using the NIH Image 1.61 computer program.

RESULTS

CCK is a hormone that is known to induce the release of preformed secretory vesicles containing pancreatic enzymes (Yoshida et al., 1997). One of the signaling pathways thought to be involved is the ERK kinase system based on its reported ability to phosphorylate cortactin. This contention was verified experimentally when isolated pancreatic acinar cells were treated with 2nM CCK. Firgure 1 shows that, over time, acinar cells stimulated with CCK (2nM) exhibited significant increase in ERK phosphorylation, with the first apparent signs on zymogen granules at 10 minutes. The most dramatic increase becomes clear between the 15 minute and 20 minute time course, with about a 3 fold increase. This confirms the presence of ERK on the zymogen granule, and further confirms the fact that ERK is being phosphorylated a short time after stimulation of the cell has occurred.

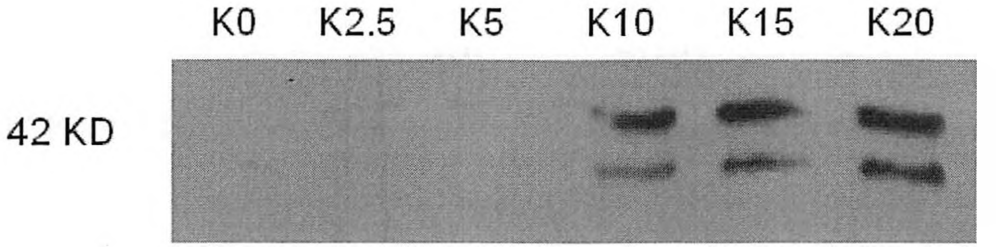
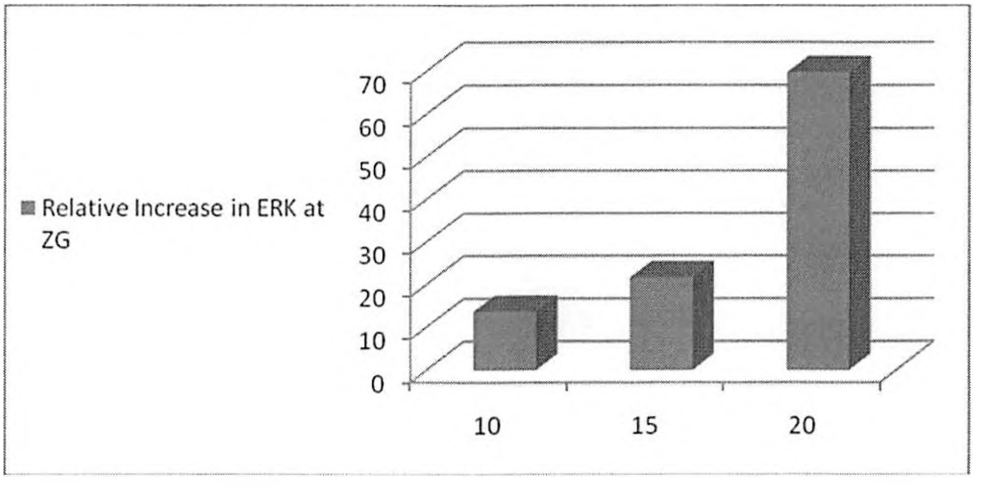


Figure 1. a) The effect of time on levels of ERK on zymogen granules following stimulation with CCK (2nM).



b) NIH imaging and quantification of Western Blot results show a significant increase in the percent of tyrosyl phosphorylation of ERK as time goes on, with the most notable increase between K15 and K20.

ERK phosphorylation was minimally detected in those isolated cells that remained unstimulated, as well as showed a significant decrease in phosphorylation when stimulation was inhibited. Figure 2 shows that the increase in ERK phosphorylation was dependent on protein kinase C (PKC). CCK stimulates tyrosyl phosphorylation of Shc and the formation of Sch-Grb2 complex through a PKC-dependent mechanisim (Dabrowski et al., 1997). GF109 blocks PKC activity and the figure shows that ERK phosphorylation decreases when the cells were treated with the inhibitor prior to stimulation with CCK. This implies that PKC must be activated in order to have substantial phosphorylation of ERK on the zymogen granules. The CCK stimulated cells exhibited twice the level of ERK phosphorylation compared to the GF109 treated cells, though both were significantly higher than that of the uninhibited control. It was postulated that the CCK receptor leads to activation of the ERK kinase cascade by protein kinase C phosphorylation of Raf (Williams et al., 2002).

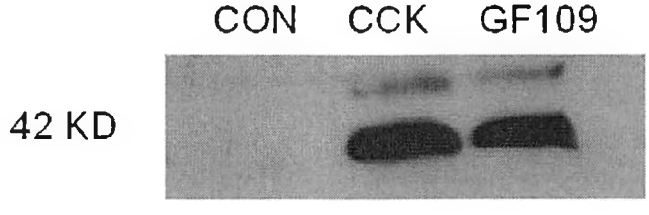
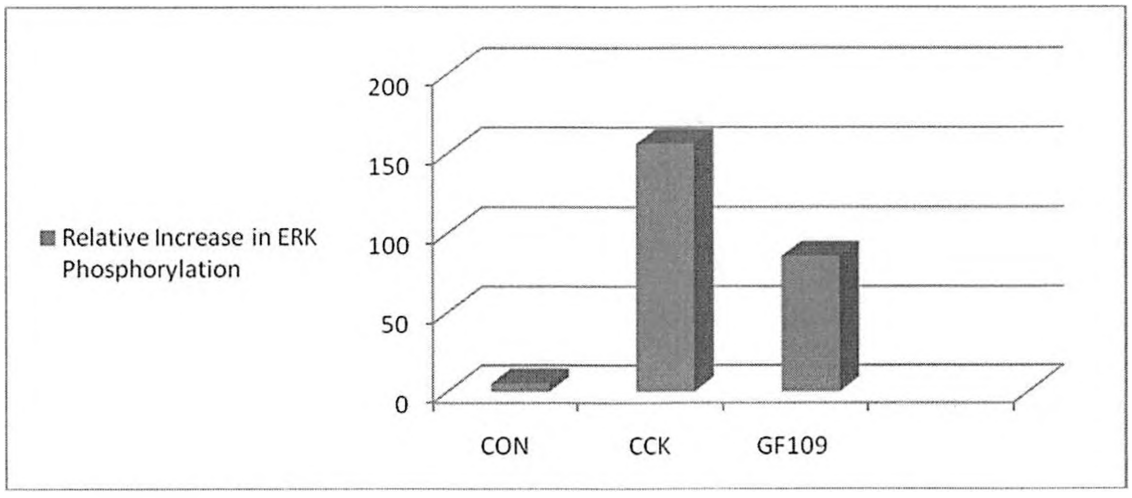


Figure 2. a) The effect of GF109 inhibition on ERK. CON represents the degree of phosphorylation in untreated pancreatic acinar cells (PAC's). CCK rpepresents the degree of phosphorylation in cells treated with CCK (2nM) for 5 minutes. GF109 represents the degree of phosphorylation in cells pre-treated with GF109 for 10 minutes prior to the addition of CCK (2nM) for 5 minutes.



b) NIH imaging and quantification of Western Blot results show CCK caused a significant increase in the percent of tyrosyl phosphorylation of ERK. GF109, a PKC-inhibitor, demonstrated inhibition of tyrosyl phosphorylation of ERK.

The increased phosphorylation of ERK as a result of CCK (2nM) stimulation is also shown to be dependent on phosphorylation of phosphatidyl inositol 4,5 bisphosphate (Figure 3). The enzyme, phosphatidylinositol3-kinase, creates lipid binding sites that can recruit proteins to the membrane. Inhibition of PI 3-Kinase activity by Wortmannin, a specific inhibitor, has been shown to block growth factor-induced cell proliferation (Daulhac et al., 1999). Figure 3 shows that Wortmannin can also block the tyrosyl phosphorylation of ERK on zymogen grandules, suggesting that the recruitment of these proteins to the membrane surface is a requirement. The difference in phosphorylation between PI3K inhibited and uninhibited samples was about seven fold. This suggests that a wortmannin-sensitive PI 3-kinase is an independent protein recruiting aspect of zymogen granule activation. Without these lipid signals, apparently the appropriate protein complexes do not form on the surface of the zymogen granule membrane.

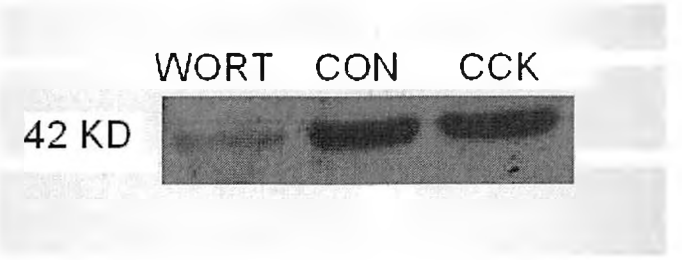
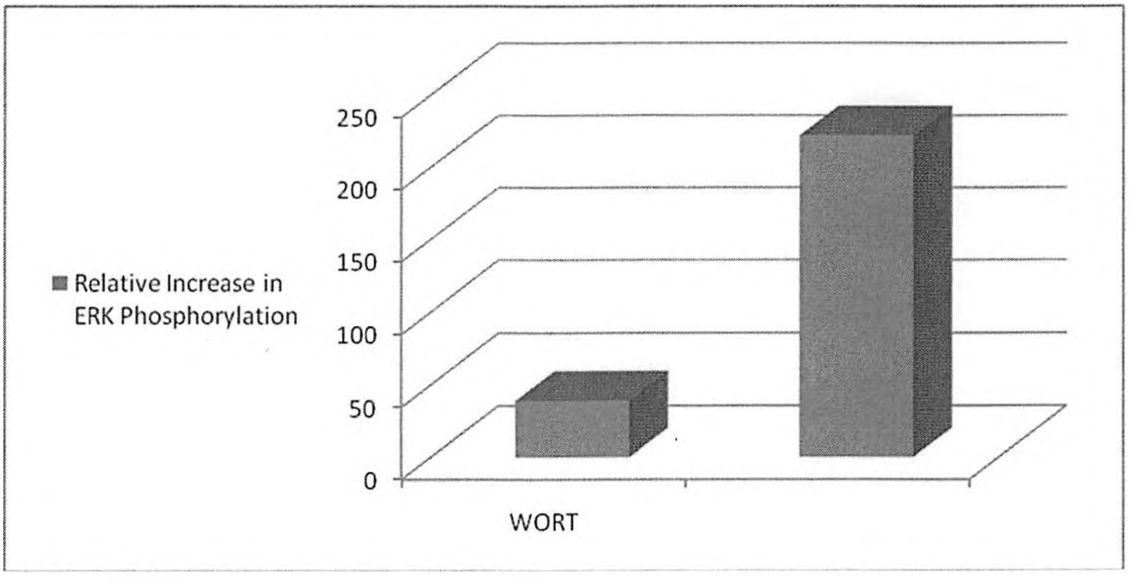


Figure 3. a) The effect of Wortmannin inhibition on ERK. CON represents the degree of phosphorylation in untreated pancreatic acinar cells (PAC's). CCK represents the degree of phosphorylation in cells treated with CCK (2nM) for 5 minutes. WORT represents the degree of phosphorylation in cells pre-treated with WART for 10 minutes prior to addition of CCK (2nM) for 5 minutes.



b) NIH imaging and quantification of Western Blot results show CCK caused significant increase in the percent of tyrosyl phosphorylation of ERK. WART inhibited tyrosyl phosphorylation of ERK.

WASP, a protein known to participate in the reorganization of actin micorofilaments was also shown to be present on zymogen granules (Figure 4). Figure 4 shows that, like ERK, the level of WASP increases on zymogen granules when cells are stimulated with CCK (2nM). When cells were treated with the PKC-inhibitor GF109, there was a dramatic decrease in the amount of WASP present. This implies that PKC stimulation by the pancreatic acinar cells is necessary to amplify the amount of WASP at zymogen granules leading to enzymatic secretion.

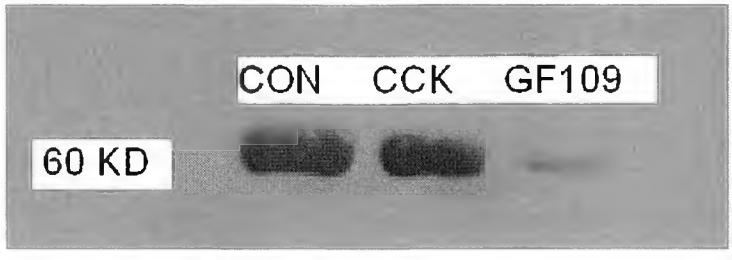
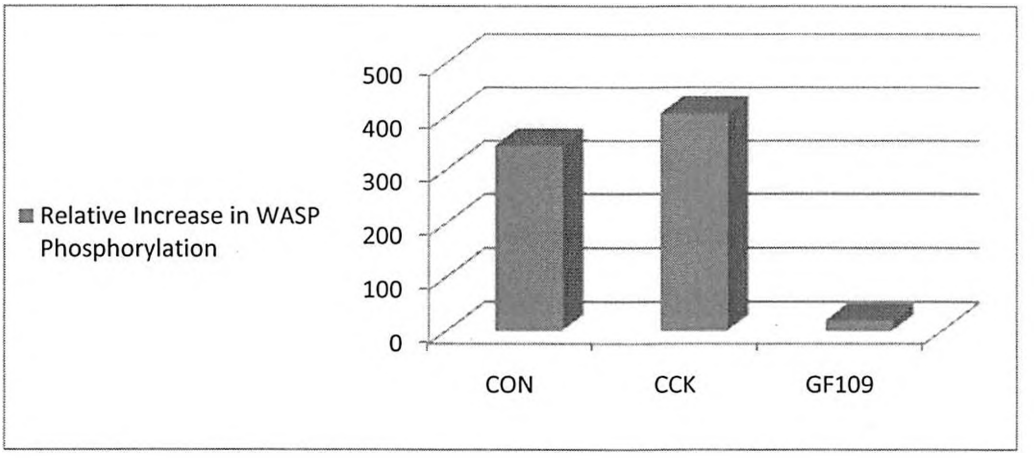


Figure 4. a) The effect of GF109 inhibition on WASP. CON represents the degree of phosphorylaytion in untreated pancreatic acinar cels (PAC's). CCK represents the degree of phosphorylation in cells treated with CCK (2nM) for 5 minutes. GF109 represents the degree of phosphorylation in cells pre-treated with GF109 for 10 minutes prior to the addition of CCK (2nM) for 5 minutes.



b) NIH imaging and quantification of Western Blot results show CCK caused an increase in the percent of tyrosyl phosphorylation of WASP. GF109 demonstrated significant inhibition of tyrosyl phosphorylation of WASP.

Cortactin is a known actin-binding protein; therefore, its presence in an acinar cell could correspond to a function in the rearrangement of the zymogen granule associated microfilaments. Figure 5 confirms the presence of cortactin on pure zymogen granules that have remained untreated. Cortactin is shown to bind to WASP and N-WASP, which then interacts with the cytoskeletal components within the cell. Cortactin-induced migration is then dependent upon the WASP family member N-WASP (Kowalski et al., 2004). Cortactin binding to and activation of the WASP family is promoted by Erk phosphorylation (Quiles et al., 2004). This sequence of events could lead to the cytoskeletal reorganization necessary to traffic the zymogen granules to the cell periphery, and therefore necessary to ensure this happens.

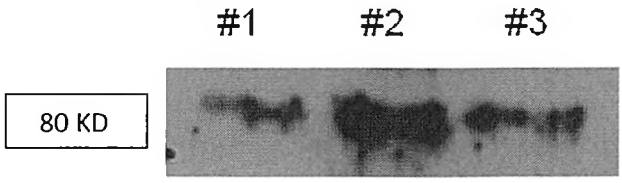


Figure 5. Each lane represents the amount of cortactin in pure zymogen granule (ZG) from pancreatic acinar cells.

DISCUSSION

The process of secretion is a vital physiological process. Many components within the cascade of events leading to secretion have yet to be fully elucidated. The identification of the key proteins their function has been the subject of many investigations to gain a complete understanding of cellular secretion. This study has shown that ERK, WASP, and cortactin are all vital components in this cascade.

It is known that zymogen granules get to the cellular membrane and release there contents outside the cell. How they physically move to the membrane still under investigation. The obvious hypothesis is that cytoskeletal reorganization takes place to physically move these granules from there position inside the cell to the cell membrane perifery. This study has shown that ERK, WASP, and cortactin all been reside or associate with the zymogen granule, and an increase in response to stimulation with CCK (2nM). This leads to the postulation that they assist in the cytoskeletal reorganization, movement of the zymogen granule, and the subsequent secretion process.

CCK receptors lead to activation of the ERK kinase cascade by protein kinase C phosphorylation of Raf (Williams et al., 2002). Therefore the present research is supported by the fact that activation of ERK was limited in the presence the PKC inhibitor GF109. The general scheme of ERK activation involves a cascade of phosphorylation events intitiated by stmimulation of the Ras proto-oncogne, which in turn allosterically activates one or more Raf family kinases (Garcia et al., 2002), leading MEK and ERK activation. Protein recruitment has also been shown to be vital to the increase in tyrosyl phosphorylation of ERK. Many proteins bind to PI 3,4,5 trisphosphate, which then results in the recruitment to the membrane. Inhibition of PI3-kinase activity by specific inhibitors or antibodies results in blockage of growth factor-induced cell proliferation (Daulhac et al., 1999). The inhibition of PI3K by Wortmannin resulted in a decrease of ERK activity. ERK phosphorylation enhances the capacity of cortactin to activate the WASP family member N-WASP (Quiles et al., 2004), and is therefore necessary to the cascade of events.

Cortactin is a known actin-binding protein provides a potential function for cytoskeletal reorganization to promote the migration of zymogen granules to the cellular periphery. The presence of cortactin was confirmed on pure zymogen granules, as seen in figure 5. In previous study, cortactin was found to be a prominent substrate for Src-related protein-tyrosine kinases and a potent F-actin cross-linking protein (Huang et al., 1997). It is, therefore, proposed that the activation of cortactin by ERK and its association with cytoskeletal structural components are necessary and responsible for the trafficking of the zymogen granules to the cell periphery for enzymatic release. For example, cortactin binds the mechanochemical GTPase dymin 2, which regulates the fission of endocytic vesicles, and the combination of cortactin and the Arp2/3 complex colocalize and associate with the endosomal vesicles (Timpson et al., 2005).

ERK and cortactin work together to help proliferate the secretion response in acinar cells. In a previous study, actin nucleation promoting factors, such as WASP, stimulate the activity of Arp2/3 (Daly, 2004). The role of the activated Arp2/3 in the complex is thought to trigger new filament formation that could be then used in as a trafficking highway for the zymogen granules to migrate to the cell perifery. The results of the present experiment confirm that WASP is present on zymogen granules in stimulated cells and is also affected by the presence of PKC, much like the presence of ERK. ERK is responsible for the activation of cortactin, and cortactin is subsequently directly responsible for the activation of WASP.

Once these components, and there suspected contributions, to secretion were identified, a working model was developed to reflect all of these elements and how they could possibly interact together. This model has been proposed in Figure 6. Stimulus first arrives to the plasma membrane receptors via hormonal stimulation, initiating a phosphorylation cascade. This cascade begins with the Src tyrosine kinase. Src is then able to phosphorylate and activate Shc. The role of Shc is to cause the Grb2 and SOS to form a complex with one another. Once the complex has been put into place, Ras is recruited and subsequently activated by GTP (see Figure 6). Ras activation then leads to the activation of Raf. It has also been found that CCK-induced activation of PKC leads to the activation of Raf, which has been shown in this research by the reduced levels of ERK phosphorylation when PKC is inhibited. Continuing the cascade (Figure 6), Raf then activates the MEK/ERK pathways. This study has proposed that once the ERK cascade has been initiated, it can then participate in a cytoskeletal rearrangement cascade, intitiated by the phosphorylation of cortactin by ERK. WASP is then activated by the amplified cortactin, which interacts with PKC to stimulate Arp 2/3 (Figure 6). This key step in the cascade is still not fully understood, but does prove an excellent future direction for the research. The

next step would, therefore, be to determine by what mechanism the Arp2/3 and the cytoskeleton is able to pull or push the zymogen granule to the membrane for exocytosis.

All of these proteins have multiple phosphorylation sites and therefore the likelihood of a more sophisticated regulation mechanism is great. For example, the CCK stimulus which leads to ERK activity also activates the Gq G-protein which in turn leads to PLCβ activity. This enzyme produces IP3 and DAG. The PKC activity, shown to be necessary for ERK and WASP (see figures 2 and 4), is another, largely unexplored, route in the control of exocytosis and secretion. The role of PKC in the regulation of cytoskeletal reorganization and zymogen trafficking is therefore viewed as important.

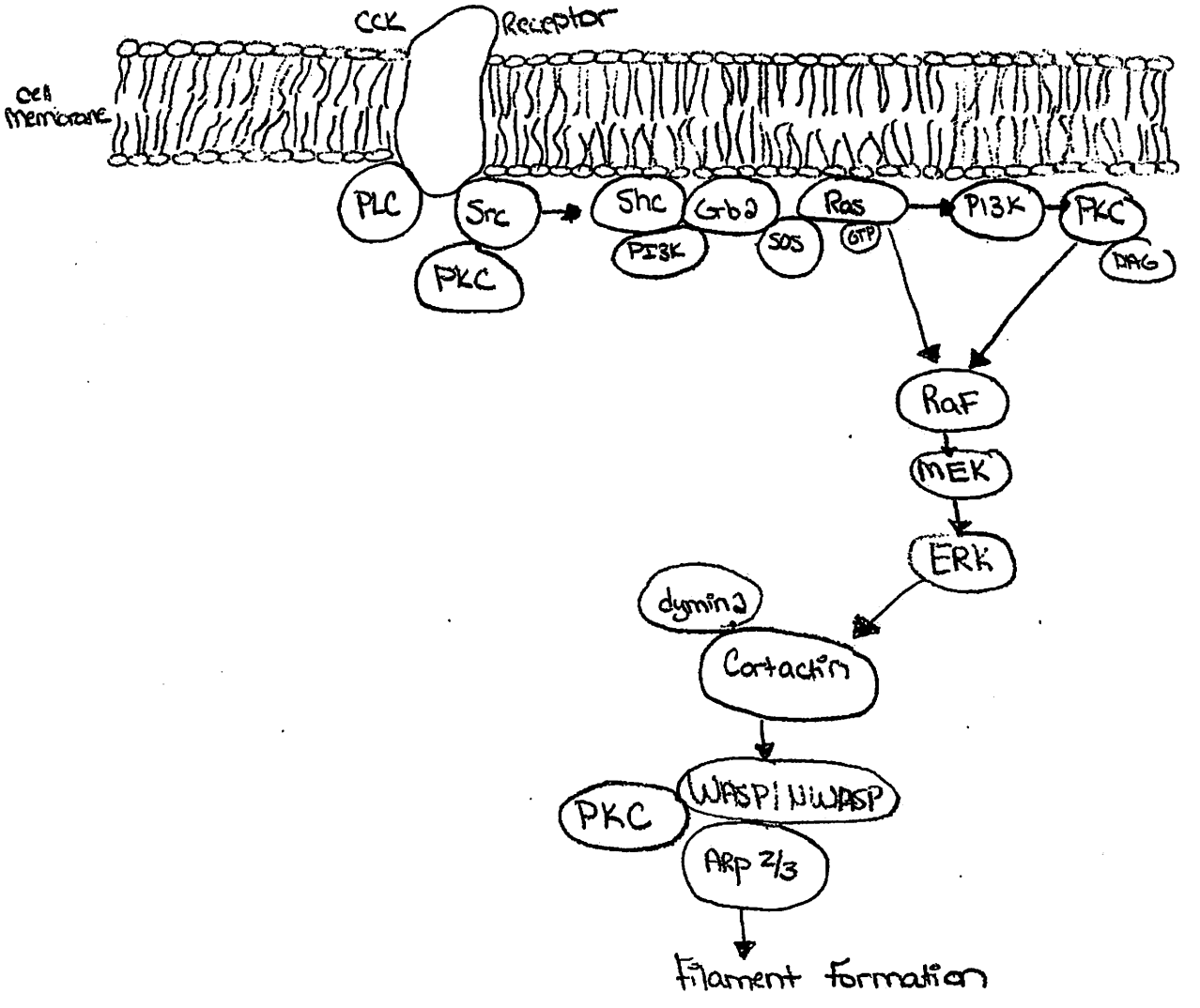


Figure 6. The proposed Model of ERK phosphorylation and its interaction with cortactin to induce filament formation.

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