SUBSTANCE P CHEMOKINE INTERACTION IN MOUSE PANCREATIC ACINAR CELLS, AND ITS IMPLICATIONS IN ACUTE PANCREATITIS

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2008

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A THESIS SUBMITTED FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHARMACOLOGY

NATIONAL UNIVERSITY OF SINGAPORE

2008

ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisor, Associate Professor Madhav Bhatia, for having given me the opportunity to do my graduate studies. I also want to thank him for his invaluable guidance, support, advice and patience.

I would like to extend my appreciation to the National University of Singapore, for providing a conducive and exciting research environment.

My thanks go to the Lab officer Miss Shoon Mei Leng as well as all my friends in the university.

I am grateful to my family for their unfailing love, affection and support.

I would also like to convey a special acknowledgement to all the animals sacrificed during the course of my study.

Thank you God!

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SUMMARY

Background: Acute pancreatitis is increasing in incidence and can be a fatal human disease, in which the pancreas digests itself and its surroundings. Inflammatory mediators such as substance P and chemokines have been shown to be critically involved in the pathogenesis of acute pancreatitis.

Aim: To investigate the functional consequences of exposing pancreatic acinar cells to the neuropeptide substance P and determine if it leads to pro-inflammatory signaling such as production of chemokines. Moreover, to investigate the mechanisms through which substance P mediates pro-inflammatory signaling in mouse pancreatic acinar cells. Furthermore, to test the significance of my *in vitro* findings in a more complex *in vivo* model of acute pancreatitis.

Results: Exposure of mouse pancreatic acini to substance P significantly increased synthesis of the CC chemokines MCP-1, MIP-1α and the CXC chemokine MIP-2. Furthermore, substance P increased NFκB activation. Blockade of the NFκB pathway significantly attenuated chemokine production, thus demonstrating that substance P-induced chemokine synthesis in mouse pancreatic acinar cells is NFκB dependent.

Substance P also induced activation of MAP Kinases ERK and JNK as well as the transcription factor AP-1. Both ERK and JNK were found to be essential for NFκB and AP-1 activation, resulting in increased chemokine production. Moreover, CP96345, a selective substance P receptor (NK1R) antagonist, attenuated the activation of ERK, JNK, NFκB and AP-1 mediated chemokine production, hence showing that chemokine production is dependent on substance P/NK1R in pancreatic acinar cells.

I also showed that substance P stimulated an early phosphorylation of the novel PKC isoform PKCδ, followed by an increased activation in MEKK1, ERK, JNK as well as NFκB and AP-1 driven chemokine production. Depletion of PKCδ decreased the activation of PKCδ, MEKK1, ERK, JNK, NFκB, AP-1 and chemokine production. Besides, PKCδ activation was attenuated by CP96345, hence showing that PKCδ activation was indeed mediated by substance P/NK1R in pancreatic acinar cells.

In addition, substance P stimulated the activation of conventional PKCα/βII which was mediated by PLC. Besides activating PKCα/βII, substance P-induced PLC increased intracellular mobilization of [Ca^{2+}] in pancreatic acinar cells. The increase in [Ca^{2+}]i resulted in the phosphorylation of PKCα/βII, ERK and JNK; consequently leading to the activation of NFκB, AP-1 and ultimately to chemokine production.

Substance P/NK1R also induced a transient increase in the activation of Src family kinases (SFKs) in pancreatic acinar cells. Moreover, substance P-induced SFKs mediated the activation of ERK and JNK, transcription factors STAT3, NFκB and AP-1 as well as MCP-1, MIP-1α and MIP-2 *in vitro*. Blockade of SFKs, both prophylactically and therapeutically, reduced the severity of acute pancreatitis *in vivo* as evidenced by a significant attenuation of hyperamylasemia, pancreatic MPO activity, pancreatic chemokine levels and pancreatic water content. Moreover, histological evidence of diminished pancreatic injury confirmed the protective effect of the inhibition of SFKs on acute pancreatitis.

Conclusions and Implications: Substance P induces chemokine synthesis in pancreatic acinar cells. The proposed signaling pathway through which substance P mediates acute pancreatitis is through substance P/NK1R - (PLC-PKCα/βII-Ca^{2+})/(PKCδ-

MEKK1)/(SFKs) - (ERK, JNK) - (STAT3, NFκB, AP-1) - (MCP-1, MIP-1α, MIP-2). A deeper understanding of the mechanisms by which substance P modulates its downstream functions will facilitate the discovery and development of novel therapeutic approaches that can target selective pathways to prevent disease progression in acute pancreatitis and/or improve treatment efficacy.

LIST OF FIGURES

LIST OF ABBREVIATIONS

AP	Acute pancreatitis
AP-1	Activator protein-1
bp	Base pair(s)
BSA	Bovine serum albumin
$[Ca^{2+}]i$	Intracellular calcium concentration
CCK	Cholecystokinin
cDNA	Complementary deoxyribose nucleic acid
CINC	Cytokine-induced neutrophil chemoattractant
DMSO	Dimethyl sulfoxide
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic
ELISA	Enzyme-linked immunosorbent assay
GDP	Guanosine diphosphate
GPCRs	G protein coupled receptors
GRO-α	Growth-related oncogene-alpha
GTP	Guanosine triphosphate
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2- ethanesulfonic acid
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
i.p.	Intraperitoneal
IL	Interleukin
IκB	I kappa B
kDa	Kilodalton
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
MEK	Mitogen-activated protein kinase Kinase
MEKK1	Mitogen-activated protein kinase Kinase kinase
MIP-1α	Macrophage inflammatory protein-1 alpha
MIP-2	Macrophage inflammatory protein-2
MODS	Multi organ dysfunction syndromes
MPO	Myeloperoxidase
mRNA	Messenger ribose nucleic acid
NEMO	NFκB essential modifier
NFκB	Nuclear factor kappa B
NK1R	Neurokinin1 receptor
PBS	Phosphate-buffered saline
PBST	0.05% Tween-20 in PBS
PCR	Polymerase chain reaction
p-ERK	Phosphorylated extracellular signal-regulated kinase
PI	Propidium iodide
p-JNK	Phosphorylated Jun N-terminal Kinase
PKCα/βII	Protein kinase C alpha/beta II
PKCδ	Protein kinase C delta

PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl Fluoride
RANTES	Regulated upon activation normal T cell expressed and secreted
RIPA	Radio-immunoprecipitation assay
RT	Reverse transcriptase
SDS	Sodium dodecyl dulfate
SFK	Src family kinase
SIRS	Systemic inflammatory response syndromes
SP	Substance P
STAT	Signal transducers and activators of transcription
t-ERK	Total extracellular signal-regulated kinase
TGF	Transforming growth factor
t-JNK	Total Jun N-terminal Kinase
TNF	Tumor necrosis factor

LIST OF ORIGINAL REPORTS

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Ramnath RD, Sun J, Bhatia M. *Involvement of src family kinases in substance P-induced chemokine production in mouse pancreatic acinar cells, and its significance in acute pancreatitis.* (Submitted)

Ramnath RD, Sun J, Bhatia M. *Role of calcium in substance P-induced chemokine synthesis in mouse pancreatic acinar cells*. Br J Pharmacol. 2008; 154: 1339-48.

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* Review article

LIST OF INTERNATIONAL CONFERENCE PRESENTATIONS

POSTER PRESENTATIONS

Raina Devi Ramnath and Madhav Bhatia
Treatment with substance P and caerulein induces chemokine synthesis in pancreatic acinar cells. 38th Meeting of the European Pancreatic Club, 2006, Tampere, Finland. A travel scholarship was awarded.

Raina Devi Ramnath and Madhav Bhatia
Treatment with substance P and caerulein induces chemokine synthesis in pancreatic acinar cells. 31st FEBS congress Molecules in Health and Disease, 2006, Istanbul, Turkey.

Raina Devi Ramnath, Jia Sun and Madhav Bhatia
Role of MAP Kinase in Substance P-induced Chemokine Synthesis in Pancreatic Acinar Cells. 39th Meeting of the European Pancreatic Club, 2007, Newcastle, UK.

Raina Ramnath, Jia Sun, Sharmila Adhikari and Madhav Bhatia
Involvement of PKCδ in Substance P-induced chemokine production in pancreatic acinar cells. 95th AAI Annual Meeting to be held in conjunction with EXPERIMENTAL BIOLOGY 2008 San Diego, CA.

Raina Ramnath, Jia Sun, and Madhav Bhatia
Participation of Phospholipase C in Substance P-induced Chemokine Production in Pancreatic Acinar Cells. Joint meeting of the European Pancreatic Club and the International Association of Pancreatology 2008 Łódź, Poland. A travel scholarship was awarded.

CHAPTER 1

GENERAL INTRODUCTION

The present chapter serves as a literature review of the topics covered in my thesis. It provides the background information that enabled its development. Following are my hypothesis and aims of this work. Subsequently, each chapter introduces and discusses the results of a main finding. Finally, conclusion consists of a general discussion, the proposed mechanisms and implications of this work.

1.1 ACUTE PANCREATITIS

Acute Pancreatitis is an inflammatory disorder of the pancreas. It varies in severity from mild to severe. Majority of the patients (80%) suffer mild pancreatitis, which is self-limiting and recover in a few days. The remaining 20% suffer a severe attack and between 30 and 50% of these will die (Neoptolemos, Raraty *et al.* 1998; Wilson, Manji *et al.* 1998; Winslet, Hall *et al.* 1992). The most common symptom is the presence of acute and constant abdominal pain.

The incidence of acute pancreatitis has increased in the past twenty years (Bhatia, Wong *et al.* 2005; Giggs, Bourke *et al.* 1988; Imrie 1997; Jaakkola, Nordback *et al.* 1993; Trapnell, Duncan *et al.* 1975). In California (1994-2001), the incidence of first time attack has increased from 33 to 44 per 100 000 adults (Frey, Zhou *et al.* 2006). Presently in USA, acute pancreatitis accounts for more than 200 000 hospital admissions yearly

(DeFrances, Hall *et al.* 2005). A similar increase is also observed in European countries (Yadav, Lowenfels *et al.* 2006).

Most cases result from excess alcohol consumption or biliary disease leading to ductal obstruction. Other minor factors include hyperlipidemia, viral infection, drugs, and hypercalcemia (Sakorafas and Tsiotou 2000). The exact mechanisms by which different aetiological factors induce acute pancreatitis are still not fully understood, but once the disease process is initiated, common inflammatory and repair pathways are brought into play. Acinar cell damage leads to a local inflammatory response but the inflammatory mediators also spill over into the general circulation. This leads to a systemic inflammatory response syndrome (SIRS), and it is this systemic response that is ultimately responsible for the majority of the morbidity and mortality (Neoptolemos, Raraty *et al.* 1998; Wilson, Manji *et al.* 1998; Winslet, Hall *et al.* 1992). Figure 1.1 illustrates the progression of acute pancreatitis and also the inflammatory mediators involved in the pathogenesis of the disease. Acute pancreatitis consists of a three-phase continuum: local inflammation of the pancreas, a systemic inflammatory response and the final stage of multi-organ dysfunction (Bhatia, Brady *et al.* 2000; Bhatia, Brady *et al.* 2002; Bhatia and Moochhala 2004; Bhatia, Neoptolemos *et al.* 2001).

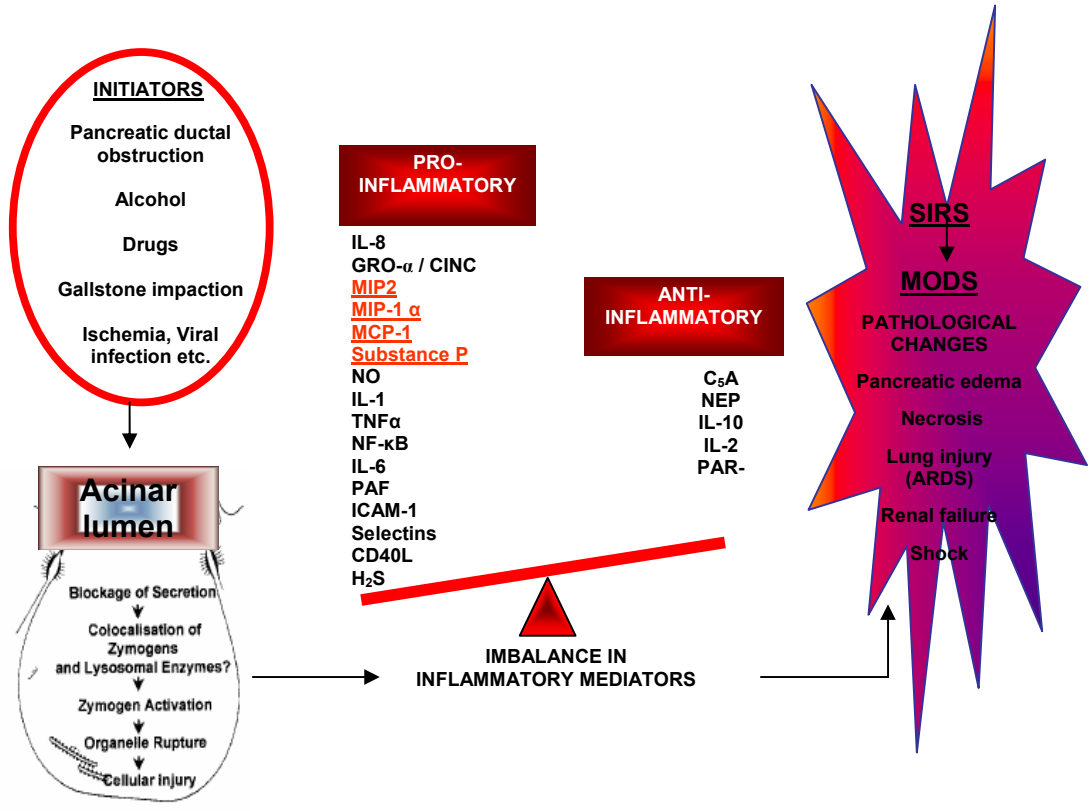


Figure 1.1 Schematic diagram of inflammatory mediators in the pathogenesis of acute pancreatitis. Activation of various digestive enzymes in pancreatic acinar cells leads to autodigestion of the pancreas and release of inflammatory mediators. The severity of acute pancreatitis is determined by an imbalance between pro- and anti- inflammatory mediators. When the inflammatory reaction is severe, it leads to pathological damages in various organs such as pancreas, lung and kidney and eventually death.

1.2 INTRA-ACINAR EVENTS IN ACUTE PANCREATITIS

The pancreas is an enzyme factory that secretes large amounts of digestive enzymes, many of which are proenzymes known as zymogens. The pancreatic acinar cell is the functional unit of the exocrine pancreas which comprises about 80% of the pancreas. The mechanism and site of initiation of pancreatitis have been a mystery. Originally, it was

believed that the pancreatic juice leaking from the pancreatic duct was responsible for the initiation of pancreatitis and that the disease began in the periductal region (Foulis 1980). Then, the observation of pancreatic fat necrosis at the time of autopsy in the patients suffering from pancreatitis led to the hypothesis that the initial event was the release of active pancreatic lipase from the acinar cells, leading to peripancreatic fat necrosis (Kloppel, Dreyer *et al.* 1986). Subsequent studies in animal models that simulate the human disease suggested that the acinar cell was the initial site of morphological damage (Lerch, Saluja *et al.* 1992). At present, it is generally agreed that the initiating events of acute pancreatitis occur in acinar cells. Thus, besides animal models of pancreatitis, isolated pancreatic acini are considered a valid model with which to investigate the pathogenesis of pancreatitis.

1.3 PATHOPHYSIOLOGY OF ACUTE PANCREATITIS IN ACINAR CELLS

Under normal physiological conditions, digestive enzymes are only activated once they have reached the duodenum. However, in acute pancreatitis premature activation of these enzymes takes place within the pancreatic acinar cells, resulting in autodigestion of the pancreas. Trypsinogen, a serine protease, is now thought to be the first enzyme to be activated; subsequently other digestive enzymes (chymotrypsin and elastase) are cleaved and activated (Gorelick, Otani *et al.* 1999; Grady, Mah'Moud *et al*. 1998; Saluja, Lee *et al.* 1999; Steer 1999). The activation of trypsinogen and other pancreatic zymogens was demonstrated in the pancreatic homogenate from animals with caerulein-induced pancreatitis (Bialek, Willemer *et al.* 1991; Grady, Saluja *et al.* 1996; Luthen, Niederau *et al.* 1995). The pancreas has a variety of mechanisms to prevent intracellular zymogen

activation and subsequent autodigestion. But in acute pancreatitis, these protective mechanisms are no longer effective or are overwhelmed (Gorelick, Otani *et al.* 1999; Grady, Mah'Moud *et al*. 1998; Saluja, Lee *et al.* 1999; Steer 1999). Hence, activated pancreatic enzymes break down cell membranes as well as tissue, causing pancreatic edema, vascular damage, hemorrhage and necrosis. The strong local inflammatory response that follows activates leukocytes and endothelial cells among others. Secreted bioactive molecules from infiltrating leukocytes contribute to local damage and subsequently to the systemic inflammatory response, which may result in multiple organ dysfunction and ultimately to death (Bhatia, Brady *et al.* 2000). A number of inflammatory mediators have been implicated in the recruitment of leukocytes into the pancreas (Bhatia, Brady *et al.* 2000). Inflammatory mediators such as substance P and chemokines along with cytokines, interleukins, intercellular adhesion molecules and platelets activating factor have been shown to play significant roles in the pathogenesis of acute pancreatitis (Bhatia, Brady *et al.* 2002; Bhatia, Ramnath *et al.* 2005; Bhatia, Saluja *et al.* 1998). My work focuses mainly on the inflammatory mediators substance P and chemokines.

1.4 SUBSTANCE P

Substance P is an 11 amino acid neuropeptide that was originally isolated and purified by Chang and Leeman from bovine pituitary glands. It is a member of the tachykinin family and has been shown to induce rapid smooth muscle contraction in guinea pig ileum and rat duodenum (Chang and Leeman 1970). Other members of the tachykinin family, sharing common carboxyl terminal Phe-X-Gly-Leu-Met-NH2 sequences in mammals,

include neurokinin A and neurokinin B (Kimura, Goto *et al.* 1984). Tachykinins are produced by three genes, preprotachykinin A (PPTA), preprotachykinin B (PPTB) and preprotachykinin C (PPTC) in mammals. Substance P is a product of the PPTA gene (Harrison and Geppetti 2001; Severini, Improta *et al.* 2002) and is localized in the central nervous system as well as in several peripheral tissues, including the entire length of the gastrointestinal tract, the pancreas as well as the colon. The effects of substance P are mediated by three different G protein coupled receptors (GPCRs), namely neurokinin (NK) 1, 2, and 3. Substance P binds with high affinity to NK1 receptor (NK1R), and with low affinity to NK2 and 3 receptors (Koon and Pothoulakis 2006).

Substance P is released from nerve endings in many tissues. Subsequent to its release from nerve endings, substance P binds to its G protein coupled receptor NK1 on effector cells, increases microvascular permeability, and promotes plasma extravasation from the intravascular to the extravascular space. It has been demonstrated that there is an elevated expression of substance P receptor binding sites in the submucosa of patients suffering from the inflammatory bowel disease (Mantyh, Gates *et al.* 1988). Patients with Crohn's disease showed increased NK1R in lymphoid aggregates, small blood vessels, and enteric neurons (Mantyh, Vigna *et al.* 1994; Mantyh, Vigna *et al.* 1995). Treatment with NK1R antagonist reduced the severity of colitis in rats. These results point to an important pro-inflammatory role of substance P and NK1R in inflammatory diseases.

1.41 Substance P in Acute Pancreatitis

Studies have indicated that substance P, acting through NK1R, plays an important role in the pathogenesis of acute pancreatitis (Bhatia, Saluja *et al.* 1998; Bhatia, Slavin *et al.* 2003; Patto, Vinayek *et al.* 1992; Sjodin and Gylfe 1992). Both genetic deletion of NK1R

and blockade of NK1R with its selective antagonist CP96345 protected mice against acute pancreatitis and associated lung injury (Lau, Wong *et al.* 2005; Saluja *et al.* 1998). The role of substance P in acute pancreatitis is further described in the following chapters.

1.5 CHEMOKINES

Chemokines are a family of small (8-10 kDa) inducible cytokines with activating and chemotactic effects on leukocyte subsets. Over 40 chemokines have been identified to date. These proteins are defined by four invariant cysteines and are classified into four subfamilies (two major and two minor) based on the relative position of the first two cysteines: CXC (α-subfamily), CC (β-subfamily), C (γ-subfamily) and CX3C (δ-subfamily) chemokines (Zlotnik and Yoshie 2000). In the CC chemokines, the first two cysteine residues are adjacent to each other. The CXC chemokines have their first two cysteine residues separated by a single amino acid. The two major subfamilies CC and CXC chemokines have been extensively investigated in various disease conditions such as acute pancreatitis.

Chemokines act as regulators of immune, inflammatory and hematopoietic processes. They play a major role in leukocyte trafficking, recruiting and recirculation. The CC chemokines [Monocyte chemoattractant protein (MCP)-1, MCP-2, MCP-3, regulated upon activation normal T cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α and MIP-1β] are believed to act on monocytes, but not on neutrophils and tend to be involved in chronic inflammation (Baggiolini, Loetscher *et al.* 1995; Wells, Power *et al.* 1996). The CXC chemokines such as interleukin (IL)-8,

growth-related oncogene-alpha (GRO-α) and the rodent CXC chemokines cytokine-induced neutrophil chemoattractant (CINC) and MIP-2 are believed to act preferentially on neutrophils and are primarily involved in neutrophil-mediated inflammation. However, recent work has shown that these narrow definitions are no longer valid (Bhatia, Proudfoot *et al.* 2003; Bonecchi, Polentarutti *et al.* 1999; Gerard, Frossard *et al.* 1997; Kobayashi, Takahashi *et al.* 2002). The key CC chemokines MCP-1, MIP-1α, RANTES and CXC chemokines, IL-8, GRO-α, CINC and MIP-2 are important in acute pancreatitis.

1.5.1 Chemokines in Acute Pancreatitis

A study of chemokine gene expression in rat pancreatic acinar cells showed an upregulated rat CXC chemokine mob-1 and CC chemokine MCP-1 mRNA expression within 1 h of cerulein induced acute pancreatitis *in vivo*. The mob-1 mRNA was also induced by either retrograde injection of bile salts or caerulein in acinar cells *in vitro* (Grady, Liang *et al.* 1997; Han and Logsdon 1999). An *in vitro* study on cholecystokinin (CCK)- and ethanol-treated rat pancreatic acinar cells demonstrated that rat pancreatic acinar cells secreted MCP-1 and RANTES in response to CCK and ethanol stimulation, suggesting a role for these two chemokines in the pathogenesis of acute pancreatitis (Yang, Demaine *et al.* 2000). It has been shown that caerulein hyperstimulation induced synthesis of MCP-1 but not CINC in rat pancreatic acinar cells (Bhatia, Brady *et al.* 2000). The synthesis is through a calcium-dependent mechanism involving NFκB activation.

The role of MCP-1 as well as two other CC chemokines MIP-1α and MIP-1β has been extensively evaluated in human AP. It was found that complicated acute pancreatitis is

associated with significantly elevated levels of local and systemic concentrations of MCP-1 and MIP-1α. A close correlation between the severity of remote organ failure and the degree of MCP-1 elevation suggests that MCP-1 might play a pivotal role in the pathological mechanism of complicated human acute pancreatitis (Rau, Baumgart *et al.* 2003). Further, MCP-1 is believed to contribute to the progression of chronic pancreatitis (which results from repetitive pancreatic injury with sustained production of various pro-inflammatory cytokines and chemokines) through monocyte/macrophage recruitment (Ohashi, Nishio 2006). Moreover, blockade of MCP-1 may reduce the development of pancreatic fibrosis in chronic pancreatitis (Zhao, Ito *et al.* 2005).

Although the mechanism of inflammation in acute pancreatitis is still not fully understood, a substantial body of evidence suggests that chemokines play a critical role in the pathogenesis of acute pancreatitis (Bhatia, Brady *et al.* 2000; Bhatia, Brady *et al.* 2002; Bhatia, Ramnath *et al.* 2005). The ability of epithelial cells (as opposed to immune and inflammatory cells) to produce chemokines has been recognized only recently (Bowden, Garland *et al.* 1994). It is now known that pancreatic acinar cells can synthesise and secrete both chemokines and cytokines (Bhatia, Brady *et al.* 2002; Grady, Liang *et al.* 1997; Gukovskaya, Gukovsky *et al.* 1997).

1.6 TEST SYSTEM: *IN VITRO* MODEL

Pancreatic acini have previously been used as a model cell type to study the mechanisms of protein secretion, hormone action, and stimulus-secretion coupling (Williams 2006). Various studies have used isolated pancreatic acinar cells (*in vitro* system) to shed light on early events in pancreatitis. Studies have shown that both isolated pancreatic acinar

cells and the pancreas respond similarly when exposed to the gastrointestinal hormone CCK. Supramaximal stimulation of pancreatic acinar cells with CCK or its analog caerulein has been commonly used as the cellular model of acute pancreatitis (Thrower, Osgood *et al.* 2008). Several *in vitro* studies conducted on pancreatic acini have substantiated the findings from animal studies. For example, in parallel to the pancreatic necrosis found *in vivo* models, diverse biochemical parameters such as cytosolic lactic dehydrogenase release, propidium iodide (PI) incorporation, and trypan blue retention have been used as markers of cellular injury in *in vitro* models (Saluja, Lerch *et al.* 2007). Besides being the site of initiation of injury in pancreatitis, pancreatic acinar cells also produce and release chemokines very early in the course of pancreatitis, which then attract and activate inflammatory cells and initiate the systemic phase of the disease. This, therefore, makes isolated pancreatic acinar cells an ideal system in which to investigate the pathogenesis of acute pancreatitis and the signaling mechanism involved.

1.7 TEST SYSTEM: *IN VIVO* MODEL

Animal models of acute pancreatitis range from mild edematous pancreatitis to severe necrotizing pancreatitis. Some examples are choline-deficient ethionine supplement diet-induced pancreatitis, bile duct obstruction model of pancreatitis and duct infusion induced pancreatitis among others. However, most studies evaluating the pathogenesis of pancreatitis have used the secretagogue induced acute pancreatitis. In this model, supramaximal doses of the secretagogue CCK or its analog caerulein are given to rodents, resulting in the induction of acute pancreatitis. Physiological concentrations of CCK/caerulein trigger normal secretion from the pancreas. It is known that physiological

doses of CCK/caerulein occur via stimulation of high-affinity CCK receptors. When supramaximal dose of CCK/caerulein is given to the animals, the excess stimulation leads to abnormally high digestive enzyme secretion, resulting in acute pancreatitis, as indicated by hyperamylasemia, edema of the pancreas acinar, cell necrosis, hemorrhage, and severe inflammation of the pancreas. This appears to be mediated by the low-affinity CCK receptors (Saluja, Saluja *et al.* 1989). What makes caerulein induced acute pancreatitis such an ideal animal model is that the histological presentation of this model is quite similar to the early phase of acute pancreatitis in humans (Dabrowski, Konturek *et al.* 1999). Among other advantages are rapid induction, non-invasiveness, high reproducibility and high applicability. Moreover, caerulein can effectively induce pancreatitis in different animals such as mice, rats, rabbits, dogs, and pigs (Chan and Leung 2007; Kahle, Lippert *et al.* 1991; Klar, Schratt *et al.* 1994; McEntee, Leahy *et al.* 1989; Renner, Wisner *et al.* 1986; Yotsumoto, Manabe *et al.* 1993). Another advantage of the CCK/caerulein-induced model is the availability of parallel *in vitro* research, where caerulein is administered to isolated pancreatic acinar cells *in vitro* to mimic CCK/caerulein induced acute pancreatitis (Chaudhuri, Kolodecik *et al.* 2005; Ueda, Takeyama *et al.* 1992)

1.8 NUCLEAR FACTOR κB (NFκB)

NFκB activation is a key mediator of the inflammatory response in pancreatitis (Chen, Ji *et al.* 2002; Jaffray, Yang *et al.* 2000; Satoh, Shimosegawa *et al.* 1999; Steinle, Weidenbach *et al.* 1999). NFκB is a ubiquitous transcription factor which is implicated in

the regulation of many genes that code for mediators of the immune, acute phase and inflammatory responses (Baeuerle and Baichwal 1997).

NFκB is usually composed of the two subunits p65 (also called RelA) and p50, although these polypeptides belong to a family of proteins that can form homo- or heterodimers with each other (Ghosh, May *et al.* 1998). In a classical pathway as illustrated in Figure 1.2, NFκB is sequestered in the cytoplasm of most resting cells through its association with an inhibitory protein called IκB. During stimulation by IL-1 or TNFα, a whole cascade of adaptor proteins and protein kinases is activated, leading to the phosphorylation of IκB by the IκB kinases α and β (IKK α / β) (Karin 1999). This depends on the regulatory protein NEMO/ IKK γ (NFκB essential modifier) associated with the complex containing two kinases, IKK α and IKK β (Akira and Takeda 2004; Hayden and Ghosh 2004). Once phosphorylated, IκB is ubiquitinated and subsequently degraded through 26S proteasome. Consequently, NFκB is freed to migrate into the nucleus, and binds to its consensus decameric sequence located in the promoter region of several genes involved in the pro-inflammatory response, encoding various immunoreceptors, cell adhesion molecules, cytokines and chemokines (Baeuerle and Baichwal 1997).

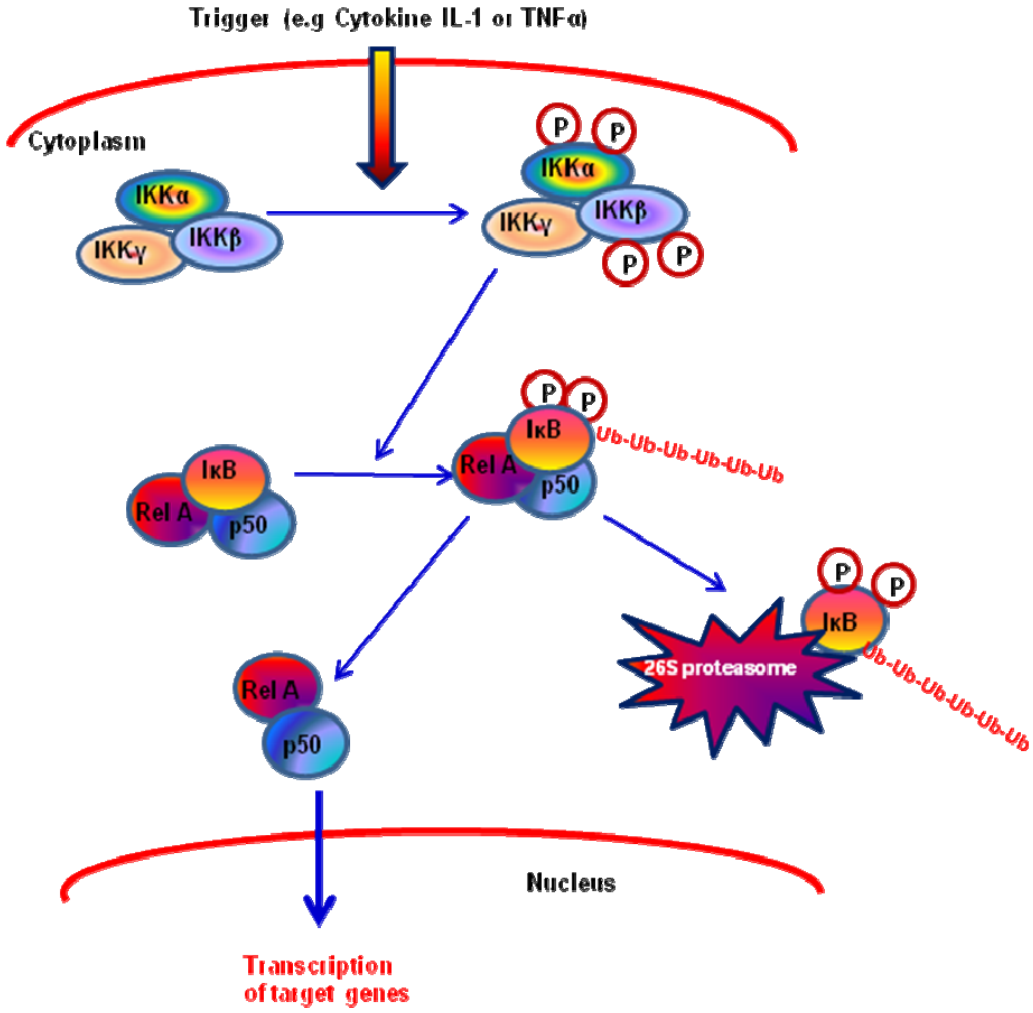


Figure 1.2 Schematic representation of the classical pathway of NFκB activation. During stimulation by IL-1 or TNFα, a whole cascade of adaptor proteins and protein kinases is

activated, leading to the phosphorylation of inhibitory protein IκB by the IKK complex. Once phosphorylated, IκB is ubiquitinated and subsequently degraded through 26S proteasome. Consequently, NFκB is freed to migrate into the nucleus to promote transcription of its target gene.

1.9 ACTIVATOR PROTEIN-1 (AP-1)

AP-1 expression is induced by multiple stimuli such as inflammatory cytokines, mitogenic growth factors, phorbol esters, oncogenes and cellular stress among others. It is activated during the cell cycle to promote cell survival, differentiation and adaptive responses.

AP-1 transcription factors belong to a large family of structurally related transcription factors that includes ATF1-4, c-Fos, c-Jun, c-Myc and C/EBP (Shaywitz and Greenberg 1999; Wisdom 1999). The members of this family, named bZIP, share a dimerization domain with a leucine zipper motif and a DNA binding domain rich in basic residues (lysines and arginines). AP-1 is composed of a mixture of heterodimeric complexes of proteins derived from the Fos and Jun families including c-Fos, FosB, Fra-1, Fra-2, c-Jun, JunB and JunD. Only Jun proteins can form transcriptionally active homodimers with AP-1 members or heterodimers with CREB/ATF members, to bind the CRE element (5'-TGACGTCA-3') (Shaywitz and Greenberg 1999). Primarily, AP-1 dimers bind to DNA on a TPA-response element (TRE) with the 5′-TGA(C/G)TCA-3′sequence (Angel, Imagawa *et al.* 1987). Phosphorylation of AP-1 family members by kinases is required for transactivation activity. The transcriptional activity of c-Jun is stimulated by phosphorylation at Ser-63 and -73 within its N-terminal activation domain (Binetruy,

Smeal *et al.* 1991; Pulverer, Kyriakis *et al.* 1991; Smeal, Binetruy *et al.* 1991; Smeal, Binetruy *et al.* 1992). It was reported that the serine/threonine kinase activity, termed JNK, binds to c-Jun and specifically phosphorylates its N-terminal sites. However there were also reports that the N-terminal sites of c-Jun are phosphorylated *in vitro* by extracellular signal-regulated kinases (ERK)1 and ERK2 (Pulverer, Hughes *et al.* 1993; Pulverer, Kyriakis *et al.* 1991)

1.10 MITOGEN-ACTIVATED PROTEIN KINASES (MAPKs)

MAPKs are a family of serine/threonine kinases activated by a cascade of intracellular phosphorylation events and transduce signals from the cell surface to the nucleus (Chang and Karin 2001; Dong, Davis *et al.* 2002; Hazzalin and Mahadevan 2002). There are three well-characterized subfamilies of MAPKs that control an array of physiological processes. It is generally believed that ERKs function in the control of cell division, Jun-N terminal kinases (JNKs) are critical regulators of transcription and p38 MAPKs are activated by inflammatory cytokines and environmental stress.

The MAP kinase cascade is one of the most ancient and evolutionarily conserved signaling pathways. A typical MAPK cascade is composed of MAPKs (e.g ERK and JNK), the kinases that activate the MAPKs is MAPK kinases (e.g MEKs). MEKs are dual-specificity kinases that recognise and phosphorylate a Thr-X-Tyr motif in the activation loop of their downstream targets, the MAPKs. MEK kinases (MEKKs), on the other hand, is located directly upstream of MEKs and serve as their activators (Schramek 2002). Thus MAPK activity is regulated through three-tiered cascades (as illustrated in Figure 1.3) composed of a MAPK, MAPK kinase (MAPKK, MKK or MEK) and a

MAPKK kinase or MEK kinase (MAPKKK or MEKK) (English *et al.* 1999). The focus of my thesis is on the MAPKs JNK and ERK. Most cells express two isoforms of JNK, 46 and 55 kDa in size and termed JNK1 and JNK2, that are highly similar in their modes of regulation (Hibi, Lin *et al.* 1993; Su, Jacinto *et al.* 1994). The two best-characterized isoforms, p42 MAPK (ERK2) and p44 MAPK (ERK1), are directly activated by phosphorylation on specific tyrosine and threonine residues. Like activation of ERK1 and ERK2 (Ahn, Seger *et al.* 1992), activation of JNK requires its phosphorylation on adjacent threonine and tyrosine residues (Drijard, Hibi *et al.* 1994). Activation of the MAPKs results in phosphorylation of various transcription factors (e.g NFκB and AP-1), other protein kinases, phospholipases, cytoskeleton-associated proteins, thus resulting in biological responses.

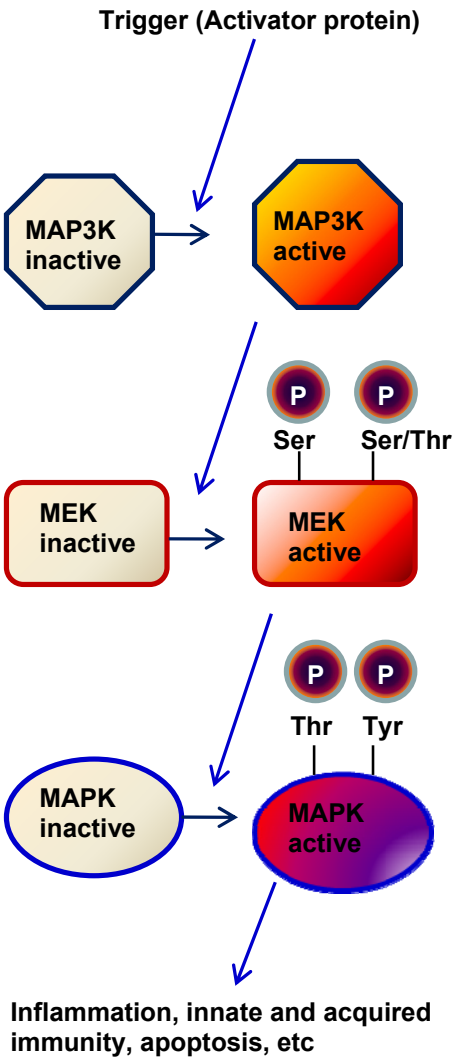


Figure 1.3 Schematic representation of a typical MAPK cascade. A typical MAPK cascade is composed of MAPKs (e.g ERK and JNK), the kinases that phosphorylate and activate MAPKs is MAPK kinases (e.g MEKs). MAP3 kinases (MEKKs) is located directly upstream of MEKs and serve as their activators.

1.11 PHOSPHOLIPASE C

Heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins) are made up of α, β, and γ subunits. They are classified according to their α subunits into four families namely Gs, Gi, Gq, and G12. They are responsible for the transduction of external signals from the receptor into biological responses. Substance P is known to activate G protein Gq (Mizuta, Gallos *et al.* 2008; Sinnett-Smith, Santiskulvong *et al.* 2000; Williams, Zou *et al.* 2007). As shown in Figure 1.4, activation of the GPCR (e.g. NK1R) induces a conformational change in the cytoplasmic domain of the receptor that results in the exchange of guanosine diphosphate (GDP) bound to the α subunit of the G protein for guanosine triphosphate (GTP) (Johnston and Siderovski, 2007; Kobilka 2007; Oldham, Van Eps *et al*. 2007; Rozengurt 2007) and hence induces its dissociation into Gα and Gβγ subunits. The resulting GTP-Gα complex consequently activates the β isoforms of phospholipase C (PLC). Once activated, it then catalyses the hydrolysis of phosphatidyl inositol 4,5 bisphosphate (PIP2) in the plasma membrane to generate two second messengers, inositol 1,4,5 trisphosphate (IP3) and 1,2,diacylglycerol (DAG) (Exton 1996; Rozengurt 1998). IP3 then binds to its intracellular receptor, which is a ligand gated calcium channel found in the endoplasmic reticulum. This leads to the release of calcium from the internal stores (Mikoshiba 1997). The other second messenger DAG directly activates PKC (Nishizuka 1995).

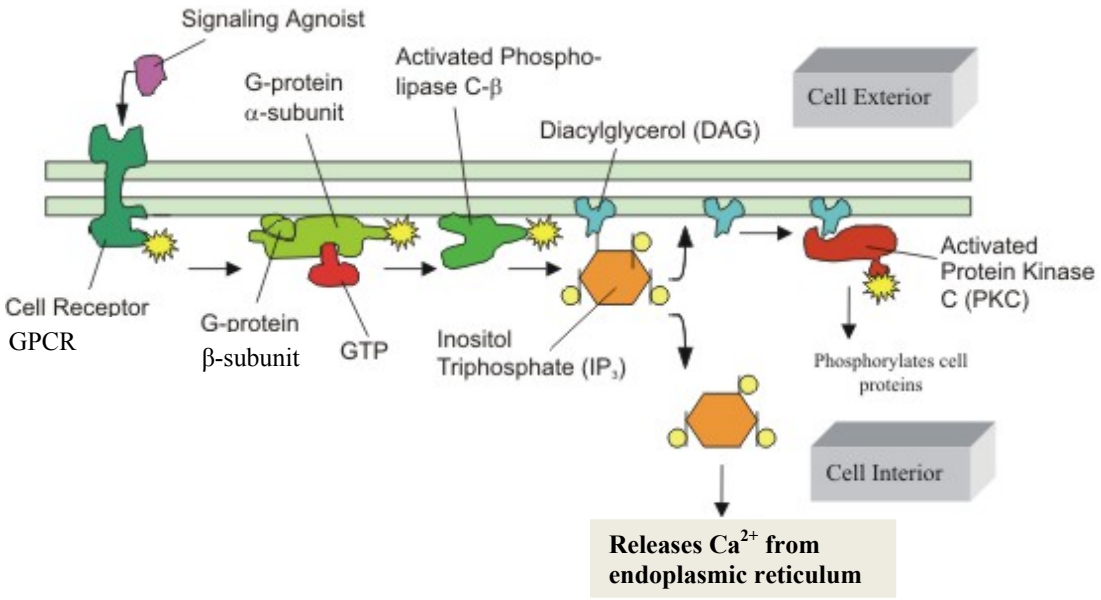


Figure 1.4 The schematic representation of PLC signaling pathway. (This figure is taken from Barron *et al.* 2002)

1.12 PROTEIN KINASE C (PKC)

PKC family of proteins consists of 12 members that are phospholipid-dependent serine/threonine kinases (Dempsey, Newton *et al.* 2000; Gschwendt 1999; Hug and Sarre 1993; Liu and Heckman 1998; Ron and Kazanietz 1999) that are involved in regulation of cellular processes including growth, migration, and inflammatory responses (Patto, Vinayek *et al.* 1992). The PKC superfamily is classified into three subfamilies based on their domain structure and their ability to respond to calcium and DAG (Newton and Johnson 1998). The three subfamilies are the calcium-dependent conventional PKCs (α, β1, β11, and γ), the calcium-independent subgroups are the novel PKCs (δ, ε, η, θ), and atypical PKCs (ζ, λ/ι, and μ) (Hug and Sarre 1993; Liu and Heckman 1998; Ron and Kazanietz 1999).

The kinase domains of each family member are basically the same, it is the subgroups that demonstrate considerable differences among their regulatory domains. The two mechanisms that regulate PKC activation are: phosphorylation of sites in the kinase domain and interactions of cofactors with the regulatory domains (Newton 1997; Newton 2003). Different cofactors activate distinct regulatory domains that characterize each subgroup. C1 and C2 modules are the main structural elements of the regulatory domains. They differ in their cofactor binding and subsequent activation mechanism for each enzyme (Webb, Hirst *et al.* 2000). For instance, conventional PKC which are activated by DAG and calcium bind to the C1 and C2 domains respectively. The novel PKC which possess only the functional C1 domains, are activated by DAG alone and do not require calcium. Atypical PKC lack a C2 module but contain a C1 module. So they do not bind to calcium and are activated by complex mechanisms involving binding of phosphoinositides and phosphorylation (Nishizuka 2001).

Once activated, PKC becomes tightly associated with the membranes. Calcium binding to the C2 domains of conventional PKC induces conformational changes that promote binding of the enzymes to the membrane lipids. Novel PKCs also bind to membrane lipids, but because they lack the calcium-binding domain, their translocation rates are much lower when compared to those of conventional PKCs. The mechanisms through which atypical PKC is targeted to distinct subcellular regions remain unknown. Moreover, PKC can bind to specific membrane proteins termed ‘receptors for activated C kinase’ (RACK) (Ron, Luo *et al.* 1995). The RACK-PKC interactions may occur between specific isoforms and is regulated by phosphorylation. The characterizations of RACK-PKC pairing have been limited to only a few PKC isoforms; some examples are

PKC α, β and δ. Many of these isoforms play a prominent role in the pathogenesis of acute pancreatitis.

1.13 CALCIUM

Calcium is an intracellular messenger that regulates several cellular functions. The calcium signals are responsible for the physiological release of inactive zymogens from the apical pole of the acinar cells into the pancreatic duct and duodenum, where the zymogens are activated. Physiological calcium signals are generally transient and localized. Global and sustained increase in cytosolic calcium levels causes abnormal intracellular enzyme activation, vacuole formation and necrosis (Criddle, Raraty *et al.* 2004; Kim, Kim *et al.* 2002; Krüger, Albrecht *et al.* 2000; Raraty, Ward *et al.* 2000; Voronina, Longbottom *et al.* 2002), all of which are important in the initiation of acute pancreatitis. Necrosis occurs as a result of excessive loss of calcium from the endoplasmic reticulum. This is mediated through specific calcium channels, inhibition of calcium pumps in intracellular stores and entry of extracellular calcium.

1.14 SRC FAMILY KINASES (SFKs)

SFKs consist of nine members. Src, Fyn, Yes, and Yrk are ubiquitously expressed, whereas the expression of Blk, Fgr, Hck, Lck, and Lyn are more restricted (Thomas and Brugge 1997). The SFKs are nonreceptor tyrosine kinases involved in signal transduction in both normal and cancer cells. All SFKs share common structural features and are composed of seven functional domains. The NH_2-terminal unique domain enables membrane attachment and is known as the Src homology (SH) 4 region. Subsequent to

the SH4 region, is the regulatory SH3 and SH2 domains, which are highly conserved and bind proline-rich and phosphotyrosyl regions, respectively. It is through these interactions that these domains participate in intra and inter-molecular regulation of kinase activity and hence determine the localization and substrate recognition of SFK (Williams, Wierenga *et al.* 1998). Next to SH3 and SH2 domains are the catalytic domain and the COOH-terminal tail. When Src is phosphorylated at Tyr-527 in its COOH-terminal tail, it interacts with its own SH2 domain, thus masking its catalytic domain and locking it in an inactive form (Brown and Cooper 1996). Once activated, Src is dephosphorylated at Tyr-527 and thus no longer interacts with its own SH2 domain. This leads to a conformational change which then activates its catalytic domain (Brown and Cooper 1996). Hence, the activity of Src protein tyrosine kinase members is upregulated by phosphorylation of the tyrosine in the catalytic region (Tyr416 for Src) and negatively regulated by phosphorylation of the tyrosine in the COOH-terminal tail (Tyr527 for Src) (Okutani, Lodyga *et al.* 2006; Williams, Wierenga *et al.* 1998). The activation of SFKs is mediated through a variety of cell surface receptors (Thomas and Brugge 1997), such as tyrosine kinase receptors, integrin receptors, and GPCRs among others (Han, Lodyga *et al.* 2005). SFKs are known effectors of activated G proteins. Inactive Src can be activated *in vitro* by the G protein subunits Gαs [bound to the nonhydrolyzable GTP analog, GTP-γS] and Gαi (Ma, Huang *et al.* 2000). Activated c-Src interacts with and activates several substrates. Studies have shown that both v-Src and c-Src are capable of activating signal transducers and activators of transcription (STAT) 3 in fibroblasts (Bromberg, Horvath *et al.* 1998; Turkson, Bowman *et al.* 1999; Yu, Meyer *et al.* 1995). Src activation of STATs is the most well characterized model.

1.15 SIGNAL TRANSDUCERS AND ACTIVATORS OF TRANSCRIPTION (STAT) 3

There are seven STAT proteins that have been discovered. STATs 2, 4 and 6 are activated by cytokines such as IFNα, IL 6, IL 12 and IL 13 respectively. STATs 1, 3, 5a and 5b are activated not only by cytokines but also growth factors and GPCR agonists (Brivanlou and Darnell 2002; Levy and Darnell 2002; O'Shea, Gadina *et al.* 2002).

All seven STAT proteins share a highly homologous domain structure. These include an N-terminal domain which is involved in protein-protein interactions, a DNA-binding domain that binds to the consensus sites upstream of its regulated genes and a SH-2 domain that interacts with tyrosine phosphorylated residues on receptors and/or kinases (Kisseleva, Bhattacharya *et al.* 2002). Activation of the STAT proteins involves a tyrosine phosphorylation cascade. STAT protein is recruited to the receptor kinase complex through the interaction of its SH2 domain with a phosphorylated tyrosine residue at the receptor or kinase complex. STATs are then phosphorylated at a single tyrosine residue in the C terminal domain. Activation occurs when the STAT proteins form hetero- or homodimers through tyrosine phosphorylation–SH2 domain interaction. The activation and dimerisation of the STAT protein also exposes its nuclear localisation signal. The STAT dimer then translocates to the nucleus. Once inside, STAT dimers interact, through their DNA binding domain, with the consensus DNA elements upstream of their target genes and hence initiate transcription (Silva 2004).

1.16 HYPOTHESIS AND AIMS

At present, there is no cure for acute pancreatitis. Besides, the mechanism of inflammation in acute pancreatitis is still not fully understood. A substantial body of evidence suggests that inflammatory mediators such as substance P and chemokines play a key role in the pathogenesis of acute pancreatitis. However, the mechanisms by which substance P and chemokines contribute to acute pancreatitis are still unknown.

In light of the existing information, I hypothesized an interaction between the two inflammatory mediators namely substance P and chemokines, and its contributions to acute pancreatitis. Substance P could be mediating acute pancreatitis through the induction of chemokines. If so, the underlying signaling mechanisms need to be investigated. Furthermore, it is important to determine the implications of these signaling pathways in a disease model of acute pancreatitis. Finally, it is crucial to show if inhibition of the signaling pathway would protect against acute pancreatitis.

To address these questions I needed a suitable model/s. It is generally accepted that the initiating events of acute pancreatitis occur in pancreatic acinar cells. Therefore, isolated pancreatic acini are considered a valid *in vitro* model to investigate the pathogenesis of acute pancreatitis.

Therefore, to investigate my hypothesis, I have used two systems namely an *in vitro* model of isolated pancreatic acinar cells and an *in vivo* model of caerulein-induced acute

pancreatitis. The *in vitro* model is a simpler system where isolated pancreactic acinar cells are used to investigate the interaction between substance P and chemokines. The *in vivo* system is a more complex model, but appropriate system to investigate the *in vivo* relevance of the *in vitro* findings.

The present study aims:

1. To investigate the interaction between the two inflammatory mediators substance P and chemokines in an *in vitro* system of isolated pancreatic acinar cells. To determine if substance P treatment leads to the activation of pro-inflammatory signals such as production of CC and CXC chemokines in pancreatic acinar cells.

2. If so, to establish the underlying signaling mechanisms through which substance P mediates chemokine production in pancreatic acinar cells.

3. Then, to test the significance of my *in vitro* findings in a more complex *in vivo* model of caerulein-induced acute pancreatitis. To determine if blockade of a signaling pathway that contributes to chemokine production would protect against acute pancreatitis.

CHAPTER 2

SUBSTANCE P TREATMENT STIMULATES CHEMOKINE SYNTHESIS IN MOUSE PANCREATIC ACINAR CELLS VIA THE ACTIVATION OF NFκB

2.1 INTRODUCTION

Acinar cell injury early in acute pancreatitis leads to a local inflammatory reaction and to the subsequent systemic inflammatory response, which may result in multiple organ dysfunction and death (Bhatia, Brady *et al.* 2000). The initial signals that recruit leukocytes into the pancreas are not completely defined, although several inflammatory mediators have been implicated (Bhatia, Brady *et al.* 2000; Grady, Liang *et al.* 1997). Inflammatory mediators such as chemokines and substance P are known to play a key role in the pathogenesis of acute pancreatitis (Bhatia, Brady *et al.* 2002; Bhatia, Ramnath *et al.* 2005; Bhatia, Saluja *et al.* 1998).

It has been shown that pancreatic acinar cells produced the CC chemokine MCP-1 in response to CCK or its analog caerulein hyperstimulation, indicating that acinar-derived MCP-1 is an early mediator of inflammation in acute pancreatitis. Treatment with bindarit, a blocker of MCP-1 synthesis, considerably decreased caerulein-induced MCP-1 production in pancreatic acinar cells (Bhatia, Brady *et al.* 2002; Bhatia, Ramnath *et al.* 2005). Moreover, both prophylactic and therapeutic treatment with bindarit protected

mice against acute pancreatitis (Bhatia, Ramnath *et al.* 2005). CC chemokine MIP-1α and CXC chemokine MIP-2 are the other chemokines involved in acute pancreatitis.

Substance P has been shown to play an important role in asthma, inflammatory bowel disease and arthritis (Bowden, Garland *et al.* 1994; Thurgston, Baluk *et al.* 1996). Substance P levels in the pancreas and pancreatic acinar cell expression of NK1R are both increased during secretagogue-induced experimental pancreatitis (Bhatia, Saluja *et al.* 1998; Jensen, Jones *et al.* 1984; Patto, Vinayek *et al.* 1992; Sjodin and Gylfe 1992). It has been suggested that the neuropeptide substance P might play a role in the evolution of pancreatic inflammatory disease such as acute pancreatitis (Bhatia, Saluja *et al.* 1998). Moreover, genetic deletion of NK1R reduces the severity of pancreatitis and pancreatitis-associated lung injury (Bhatia, Saluja *et al.* 1998). These observations indicate that substance P, acting through NK1R, plays an important pro-inflammatory role in regulating the severity of acute pancreatitis. However, the exact mechanism by which substance P contributes to the pro-inflammatory signaling in acute pancreatitis is not completely understood.

The study described in this chapter aims to examine the functional consequences of exposing mouse pancreatic acinar cells, which is known to express NK1R, to substance P. The specific aim is to determine if substance P leads to pro-inflammatory signaling such as the production of the CC chemokines MCP-1, MIP-1α and the CXC chemokine MIP-2 and establish the underlying mechanisms.

2.2 MATERIALS AND METHODS

2.2.1 Animal Ethics

All animal experiments were approved by the Animal Ethics Committee of National University of Singapore and carried out in accordance with established International Guiding Principles for Animal Research. Male Swiss albino mouse (20-30 g) were maintained in the Animal Housing Unit of this University in an environment with controlled temperature (21-24 °C) and lighting (12:12 h light-dark cycle). Standard laboratory chow and drinking water were provided *ad libitum*. A period of 2 days was allowed for animals to acclimatize before any experimental manipulations were undertaken.

2.2.2 Preparation of mouse pancreatic acini

Pancreatic acini were obtained from mouse pancreas by collagenase treatment as described previously (Bhatia, Wallig *et al.* 1998; Gerasimenko, Gerasimenko *et al.* 2002; Gukovskaya, Gukovsky *et al.* 2002; Wallig, Gould *et al.* 1988; Wallig, Kore *et al.* 1992) Briefly, pancreas from three Swiss mice (20-30 g) were removed, infused using 29G syringes with *buffer A* (in mM: 140 NaCl, 4.7 KCl, 1.13 MgCl2, 1 CaCl2, 10 glucose, 10 HEPES, pH 7.2) containing 200 U/ml collagenase and 0.5 mg/ml soybean trypsin inhibitor. The bloated pancreas were then minced with a sharp tip surgical scissors till a fine suspension was achieved, and incubated in a shaking water bath for 10 min at 37°C. The digested tissue was passed through (by centrifugation) 50 mg/ml bovine serum albumin (BSA) and washed twice with *buffer A* before further experiments. A cell

suspension of (in *buffer A*) consisting of only small clumps, around 3 to 5 acinar cells, was used to carry out the following experiments.

2.2.3 Viability of mouse pancreatic acinar cells

Viability of the pancreatic acinar cells was determined by trypan blue dye exclusion assay. One drop of 0.4% trypan blue dye was added to one drop of the isolated acinar cells and examined under the light microscope (Carl Zeiss, Oberkochen, Germany). The number of unstained cells/clumps was expressed as a percentage of the total number of cells/clumps. This process was repeated for different fields and the average was then calculated. In all experiments, cell viability was greater than 95%.

2.2.4 *In vitro* treatment with substance P

Experiments were performed to examine the effects of substance P (Sigma-Aldrich) treatment on chemokine production and NFκB activation in mouse pancreatic acini. Acini in *buffer A* (500 μl) were incubated in a shaking water bath at 37°C with substance P at a concentration of 10^{-6} M (1 μM) for 45 min. After which the supernatant was used for chemokine detection by ELISA whereas the pellet was used for NFκB isolation and detection. No gas mixture was used during incubation.

2.2.5 Chemokine detection

Pancreatic acinar cell supernatants were assayed for MCP-1, MIP-1α and MIP-2 using a sandwich ELISA, according to the manufacturer's instructions (Duoset kit; R&D Systems, Minneapolis, MN). For example MCP-1, anti-MCP-1 primary antibody was aliquoted onto ELISA plates and incubated at 4°C overnight. Samples and standards were incubated for 2 h, the plates were washed, and a biotinylated anti-MCP-1 antibody was added for 2 h. Plates were washed again, and streptavidin bound to horseradish

peroxidase was added for 20 min. After a further wash, tetramethylbenzidine was added for colour development, and the reaction was terminated with 2 M H_2SO_4. Absorbance was measured at 450 nm by using a 96-well microplate reader (Tecan Systems, San Jose, CA). The same procedure was followed for the detection of the remaining chemokines MIP-1α and MIP-2.

2.2.6 Preparation of nuclear cell extract

Nuclear cell extracts were prepared by employing a kit from Active Motif (SciMed, Asia). In brief, cells were washed, collected in ice-cold PBS in the presence of phosphatase inhibitors, to limit further protein modifications, and then centrifuged (4°C) at 24 *g* for 5 min. The pellets were resuspended in a hypotonic buffer, treated with detergent and centrifuged (4°C) at 14,000 *g* for 30s. After collection of the cytoplasmic fraction, the nuclei were lysed and nuclear proteins solubilized in lysis buffer containing proteasome inhibitors. Protein concentrations were determined by using Bio-Rad protein assay. 5 µl of sample was added to 250 µl of Bradford reagent (Bio-Rad Laboratories, Hercules, CA) and read at 595 nm after a 5 min incubation at room temperature.

2.2.7 NFκB DNA-binding activity

The binding of NFκB to DNA was measured in nuclear extracts with an ELISA-based TransAM NFκB p65 assay kit (Active Motif, SciMed, Asia). This assay uses multi-well plates coated with an unlabeled oligonucleotide containing the consensus binding site for NFκB (5'-GGGACTTTCC-3') (Parry and Mackman 1994). Nuclear proteins (5 µg) were added to each well and incubated for 1 h at room temperature to allow NFκB DNA binding. Subsequently, by using an antibody that is directed against NFκB p65 subunit, the NFκB complex bound to the oligonucleotide is detected. Addition of the secondary

antibody conjugated to horseradish peroxidase (HRP) provides sensitive colorimetric readout that is easily quantified by spectrophotometry. Absorbance was read at 450 nm within 5 min by using a 96-well microplate reader (Tecan Systems, San Jose, CA). The wild-type consensus oligonucleotide was provided as a competitor for NFκB binding to monitor the specificity of the assay. Results were expressed as fold increase over the control group.

2.2.8 NFκB inhibition

Pancreatic acini were incubated for 2 h with 50 μM of the NEMO- binding domain peptide (NBD) (Tas, de Jong *et al.* 2005) purchased from Calbiochem, or vehicle (DMSO) prior to stimulation with 1 μM substance P for 45 min. Subsequently, the supernatant was used for chemokine detection.

2.2.9 Preparation of total cell lysates

After treatment, pancreatic acinar cells were homogenized on ice in radioimmunoprecipitation assay (RIPA) buffer supplemented with 1 mM phenylmethylsulphonyl fluoride (PMSF) and the protease inhibitor cocktail (Sigma-Aldrich) containing pepstatin, leupeptin, chymostatin, antipain and aprotinin (5 μg/ml of each), and centrifuged at 4 °C for 15 min at 14,000 *g*. The supernatants were collected and stored at -80°C until use. Protein concentrations were determined by using Bio-Rad protein assay. 5 μl of sample was added to 250 μl of Bradford reagent (Bio-Rad Laboratories, Hercules, CA) and read at 595 nm after a 5 min incubation at room temperature.

2.2.10 Western blot analysis

Cell lysates (50 μg) were separated on 12% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Non-specific binding was blocked by 1 h incubation of the membranes, at room temperature, in 5% nonfat dry milk in phosphate buffered saline Tween 20 (PBST) (0.05% Tween 20 in phosphate buffered saline). The blots were then incubated overnight at 4°C with the primary antibody IκB-α (purchased from Cell Signaling Technology) at 1:1000 dilutions in the buffer containing 2.5% nonfat dry milk in PBST. The blots were then washed four times with PBST, and finally incubated for 1 h with goat anti-rabbit HRP-conjugated secondary antibody (purchased from Santa Cruz Biotechnology) at 1:2000 dilutions in the buffer containing 2.5% nonfat dry milk in PBST. The blots were developed for visualization using enhanced chemiluminescence (ECL) detection kit (Pierce, Rockford, IL). Hypoxanthine-guanine phosphoribosyl transferase (HPRT) (purchased from Santa Cruz Biotechnology) was used as the housekeeping protein. The densities of the bands were quantified using a UVP GelDoc-It Imaging Systems (Scimed, Asia).

2.2.11 Amylase estimation

Pancreatic acini were incubated with substance P or caerulein (Bachem, Bubendorf, Switzerland) (10^{-12} to 10^{-6} M) for 45 min and amylase assays were performed. Amylase activity was measured by using a kinetic spectrophotometric assay. Acinar cell supernatant samples were incubated with the substrate, 4,6-ethylidene (G7)-p-nitrophenol (G1)-1-D-maltoheptoside (Sigma, St. Louis, MO) for 2 min at 37°C, and absorbance was measured every minute for the subsequent 2 min at 405 nm (Bhatia, Brady *et al.* 2000;

Bhatia, Saluja *et al.* 1998; Pierre, Tung *et al.* 1976). The change in absorbance was used to calculate the amylase activity. Pre-treatment of acini with 50 μM of NBD peptide followed by stimulation with caerulein ranging from 10^{-12} to 10^{-7} M had no effect on amylase secretion.

2.2.12 Statistical analysis

Results are presented as means + SE with 6 replicates for each condition. Each experiment was repeated at least three times. The significance of changes was evaluated by using ANOVA and Tukey's method was used as a post hoc test for the difference between groups. A P value < 0.05 was taken as the level of significance.

2.3 RESULTS

2.3.1 Substance P induces chemokine production in mouse pancreatic acinar cells in a concentration-dependent manner

To investigate the effect of different doses of substance P on chemokine synthesis in mouse pancreatic acini, isolated pancreatic acinar cells were challenged for 45 min at 37°C with different concentrations of substance P, ranging from 0.01 to 1 μM. Subsequently, the supernatant was used to assess the levels of chemokines MCP-1, MIP-1α and MIP-2 by ELISA. As shown in Figure 2.1, substance P increases MCP-1, MIP-1α and MIP-2 production in a concentration-dependent manner. Maximal activation of MCP-1, MIP 1α and MIP-2 was observed with 1 μM of substance P. The increased in MCP-1, MIP-1α and MIP-2 synthesis was significantly higher when compared to vehicle (saline) treated control. The concentration of 1 μM (10^{-6} M) of substance P was used to carry out subsequent experiments.

2.3.2 Substance P or caerulein induces NFκB activation in mouse pancreatic acinar cells

Pancreatic acinar cells were stimulated with 1 μM of substance P or 0.1 μM of caerulein for 45 min at 37˚C. After which the pellet was used for nuclear extraction and NFκB was detected by performing an ELISA-based NFκB DNA binding assay. As shown in Figure 2.2 (a), substance P at a concentration of 1 μM significantly upregulated NFκB activation when compared to the vehicle-treated control. This finding was further confirmed by the western blot analysis. As shown in Figure 2.2 (b), when mouse pancreatic acini were stimulated with 1 μM of substance P for 45 min at 37˚C, there is an increased degradation

of total IκB when compared to vehicle-treated control. As shown in Figure 2.2 (c) 0.1 μM of caerulein significantly increased NFκB activation when compared to the vehicle-treated control. Furthermore, pre-treatment of acini with 50 μM NBD, an NFκB inhibitor, significantly attenuated the caerulein induced NFκB activation

2.3.3 Substance P- or caerulein-induced chemokine synthesis is prevented by NEMO-Binding Domain peptide (NBD), an NFκB inhibitor

I further investigated the role of NFκB on chemokine production in mouse pancreatic acinar cells. Pancreatic acini were pre-treated with NBD peptide for 2 h followed by stimulation with 1 μM of substance P or 0.1 μM caerulein. After which the levels of MCP-1, MIP-1α and MIP-2 were determined by ELISA. As shown in Figure 2.3, treatment of pancreatic acini with 1 μM substance P or 0.1 μM caerulein caused a significant production in MCP-1, MIP-1α and MIP-2. Pre-treatment of acini with 50 μM of NBD peptide followed by stimulation with 1 μM of substance P or 0.1 μM caerulein significantly attenuated the production in (2.3a) MCP-1, (2.3b) MIP-1α and (2.3c) MIP-2 when compared to substance P or caerulein-treated pancreatic acini.

2.3.4 Substance P and caerulein may act via different pathways in inducing chemokine synthesis

Studies have shown that treatment of pancreatic acinar cells with supramaximal dose of caerulein 0.1 μM leads to activation of NFκB as well as production of chemokine both *in vitro* and *in vivo* (Bhatia, Brady *et al.* 2000; Bhatia, Brady *et al.* 2002; Bhatia, Saluja *et al.* 1998). My aim was to investigate the effect of both substance P and caerulein on mouse pancreatic acini. I therefore incubated the cells with both 1 μM of substance P and 0.1 μM of caerulein for 45 minutes at 37˚C. After which the supernatant obtained was

used for chemokines MCP-1, MIP-1α and MIP-2 detection by ELISA. As shown in Figure 2.4, stimulation of acini with substance P or caerulein caused a significant increase in chemokine synthesis when compared to vehicle-treated acini. Furthermore, when mouse pancreatic acinar cells were treated with both substance P and caerulein the increased production of chemokines (2.4a) MCP-1, (2.4b) MIP-1α and (2.4c) MIP-2 production was significantly higher when compared to either substance P or caerulein-treated cells. These data show that substance P and caerulein act via overlapping, yet distinct pathways in activating chemokine synthesis.

2.3.5 Effect of substance P treatment on amylase secretion in mouse pancreatic acinar cells

Mouse pancreatic acini were treated with different doses, ranging from 10^{-12} to 10^{-6} M, of substance P. After incubation for 45 minutes at 37˚C, the supernatant was used for the amylase assay. As shown in Figure 2.5, substance P did not have any effect on amylase secretion. As a positive control, mouse pancreatic acini were also treated with different concentration of caerulein, ranging from 10^{-12} to 10^{-7} M. In accord with previously reported findings (Bhatia, Saluja *et al.* 1998), a biphasic stimulation/inhibition of amylase secretion by increasing concentration of caerulein was observed.

2.4 DISCUSSION

It is generally believed that the earliest events in the induction of acute pancreatitis lead to intra-acinar cell activation of digestive zymogens and that those enzymes once activated cause acinar cell injury (Saluja, Saluja *et al.* 1989a; Saluja, Saluja *et al.* 1989b). Recent studies have suggested that the ultimate severity of the resulting pancreatitis may be determined by events that occur subsequent to acinar cell injury, such as inflammatory cell recruitment, activation, generation, release of cytokines and other chemical mediators of inflammation including substance P and chemokines (Dusetti, Ortiz *et al.* 1995; Gross, Leser *et al.* 1993; Scholmerich 1996). Substance P has been detected within the pancreas and it has been suggested that it may act as a neurotransmitter for sensory afferent nerves in the pancreas. Receptors for substance P have also been detected on guinea pig pancreatic acinar cells (Sjodin, Dahlen *et al.* 1991; Sjodin, Viitanen *et al.* 1994; Song, Iwashita *et al.* 1988) and now it is known that mouse pancreatic acini also express NK1R (Bhatia, Saluja *et al.* 1998). Substance P has been shown to activate the transcription factor NFκB in macrophages (Marriott, Mason *et al.* 2000). Intra-pulmonary administration of substance P results in rapid activation of NFκB in lung tissues. Its administration also results in the appearance of the neutrophil-attracting CXC chemokine, MIP-2, in bronchoalveolar lavage fluid (Okaya, Holthaus *et al.* 2004).

Although pancreatic acinar cells have earlier been shown to express NK1R (Bhatia, Saluja *et al.* 1998; Lau and Bhatia 2006; Lau, Wong *et al.* 2005), the mechanism by which substance P induces synthesis of chemokines in acute pancreatitis is not yet known. To that end, I investigated the effect of substance P on chemokine synthesis, such

as CC chemokines MCP-1, MIP-1α and CXC chemokine MIP-2 in mouse pancreatic acini. Furthermore, I showed that the increased chemokine synthesis was mediated by the activation of NFκB. I demonstrated that substance P induced chemokine synthesis in a concentration-dependent manner. Substance P at a concentration of 1 μM significantly increased NFκB activation resulting in significant chemokine synthesis when compared to its vehicle-treated control.

NFκB activation is a key mediator of the inflammatory response in pancreatitis (Chen, Ji *et al.* 2002; Jaffray, Yang *et al.* 2000; Satoh, Shimosegawa *et al.* 1999; Steinle, Weidenbach *et al.* 1999). The importance of NFκB response stems from its ability to up regulate the expression of chemokines/cytokines and other inflammatory molecules that are induced in human and experimental pancreatitis (Bhatia, Brady *et al.* 2000; Norman 1998). The mechanism and regulation of NFκB response, in relation to substance P, in the pancreatic acinar cell are however not well understood. To my knowledge, this is the first time that a neuropeptide is shown to induce activation of transcriptional activator NFκB as well as chemokine synthesis in mouse pancreatic acinar cells. To prove that the effect of substance P was dependent upon NFκB activation and chemokine synthesis, I pre-treated the acini with NEMO-binding domain peptide (NBD). NBD is a short cell-permeable peptide, spanning IKKβ. This peptide was already shown to disrupt the association of NEMO with IKKβ *in vitro*, to block TNFα-induced NFκB activation, and to effectively ameliorate responses to various inflammatory stimuli *in vivo* (May, D'Acquisto *et al.* 2000). Pre-treatment of mouse pancreatic acini with NBD completely attenuated the chemokine synthesis induced by substance P. This shows that the

increased in chemokine synthesis induced by substance P was mediated by NFκB activation.

It is known that treatment with supramaximal dose of caerulein, CCK analog, induces NFκB activation as well as chemokine synthesis in pancreatic acini both *in vitro* as well as *in vivo* (Bhatia, Brady *et al.* 2002; Bhatia, Ramnath *et al.* 2005). Similar observation was made when mouse pancreatic acini were treated with supramaximal dose of caerulein, there was a significant up regulation in NFκB activation. Furthermore, I showed that pre-treatment with NBD significantly attenuated caerulein-induced NFκB activation.

In order to investigate the combined effect of substance P and caerulein in mouse pancreatic acini, the cells were incubated simultaneously with substance P and caerulein. Substance P and caerulein independently activated the synthesis of chemokine MCP-1, MIP-1α and MIP-2 in mouse pancreatic acini. However, when cells were treated with both substance P and caerulein the increased in chemokine synthesis was significantly higher when compared to cells treated with either substance P or caerulein alone. Moreover, substance P had no effect on amylase secretion. This is in contrast to caerulein which is known to produce a dose dependent response in amylase secretion (Bhatia, Saluja *et al.* 1998). Studies characterizing acinar cell secretion in the presence of increasing caerulein concentrations have revealed a typical biphasic dose-response relationship, with stimulation at low caerulein concentrations and inhibition at supramaximally stimulating concentrations (10^{-7} M). Treatment of mouse pancreatic acini with different concentration of substance P did not have any effect on amylase secretion unlike that observed with caerulein treatment. These results suggest that substance P and

caerulein act via overlapping, yet distinct, pathways to stimulate chemokine synthesis in pancreatic acinar cells.

In conclusion, substance P induced synthesis of CC chemokines MCP-1, MIP-1α and CXC chemokine MIP-2 via a NFκB dependent pathway. This is the first direct evidence of the role of substance P, acting via NK1R present on mouse pancreatic acini, in inflammation and points to the mechanism by which substance P contributes to inflammation in acute pancreatitis.

Figure 2.1 (a)

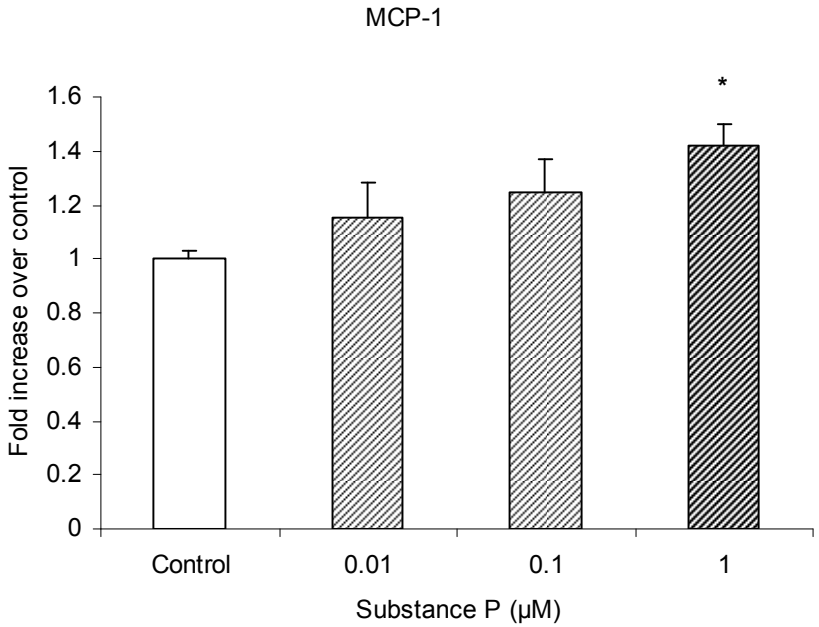


Figure 2.1 (b)

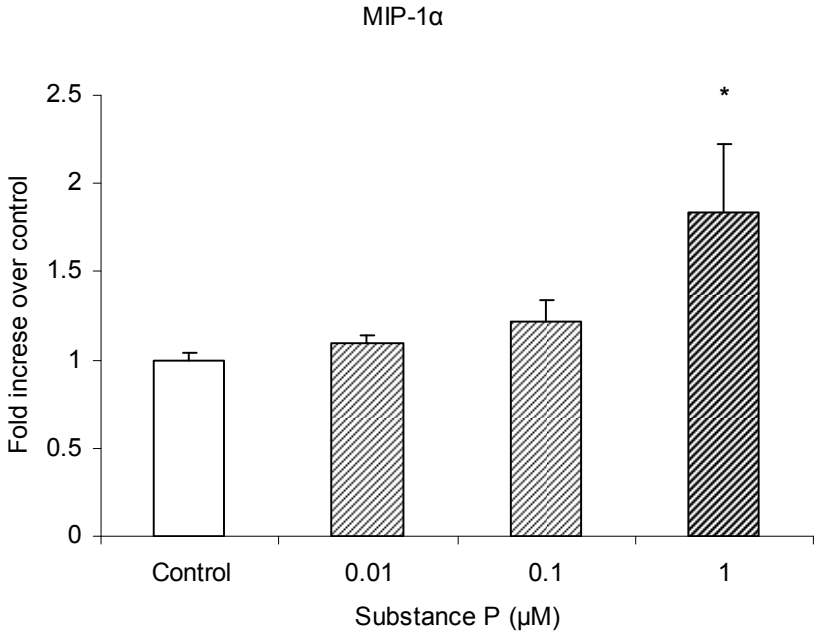


Figure 2.1 (c)

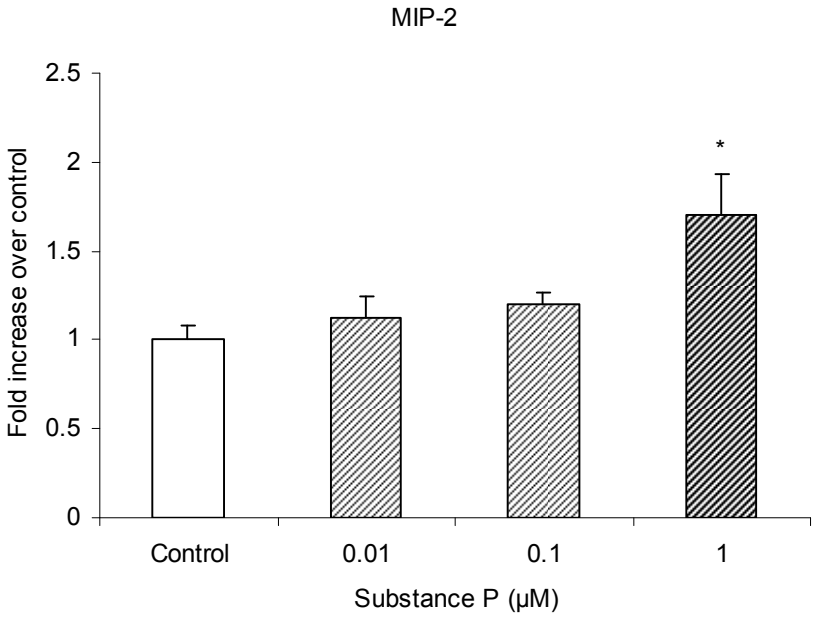


Figure 2.1 Substance P (SP) induces chemokine production in a concentration-**dependent manner in mouse pancreatic acinar cells.** Pancreatic acini, obtained from three mice, were incubated for 45 minutes at 37°C with different concentration of SP ranging from 0.01 to 1 μM. After which the suspension was centrifuged. The supernatant obtained was used for (a) MCP-1, (b) MIP-1α and (c) MIP-2 detection. Results shown are the means + SE. *$P < 0.05$ when 1 μM SP treated acini were compared with vehicle-treated acini.

Figure 2.2 (a)

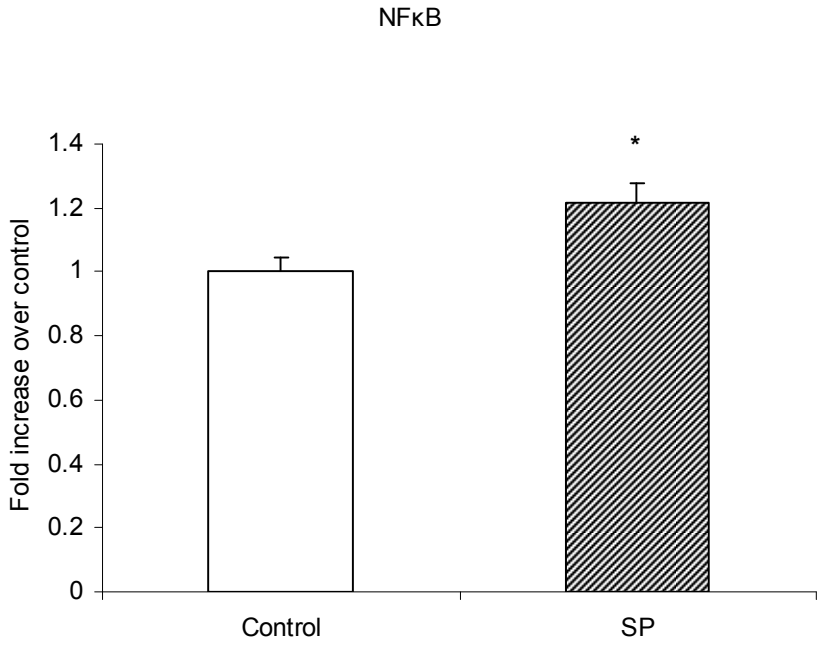


Figure 2.2 (b)

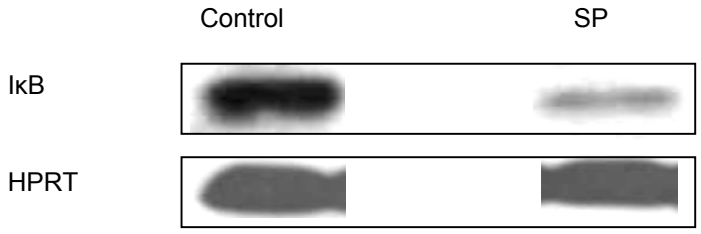


Figure 2.2 (c)

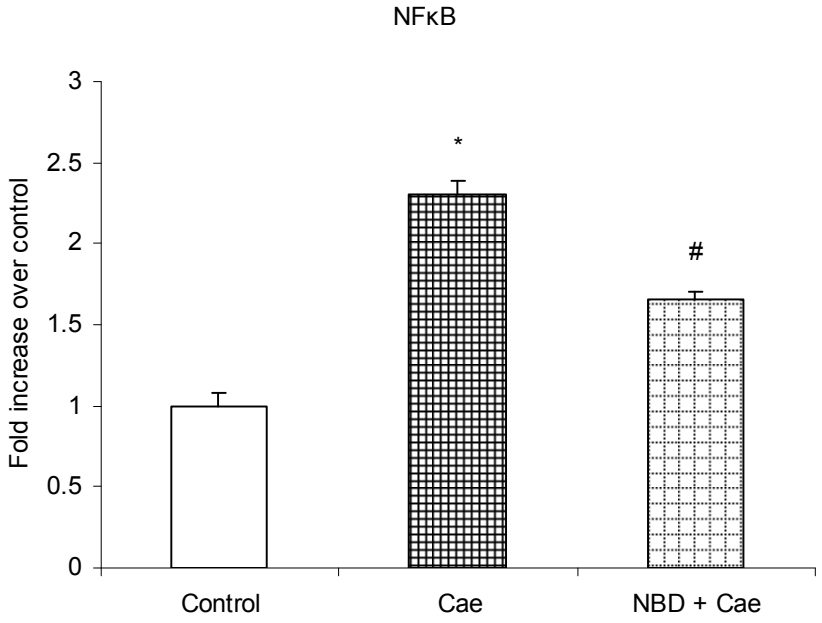


Figure 2.2 Substance P (SP) or caerulein (Cae) induces NFκB activation in mouse pancreatic acinar cells. Pancreatic acini, obtained from three mice, were incubated for 45 minutes at 37˚C with 1 μM SP or 0.1 μM Cae. Acini were separated from incubation medium by centrifugation. (a) The pellet (acini) was used for NFκB extraction and detection. MW in (kDa) for IκB is 39 and HPRT is 24. Results shown are the means + SE. *$P < 0.05$ when 1 μM substance P treated acini were compared with vehicle-treated acini. (b) Western blot analysis was performed as described in Materials and Methods. (c) *$P < 0.05$ when 0.1 μM Cae treated acini were compared with vehicle-treated acini, # $P < 0.05$ when NBD and Cae-treated acini were compared with Cae alone stimulated cells.

Figure 2.3 (a)

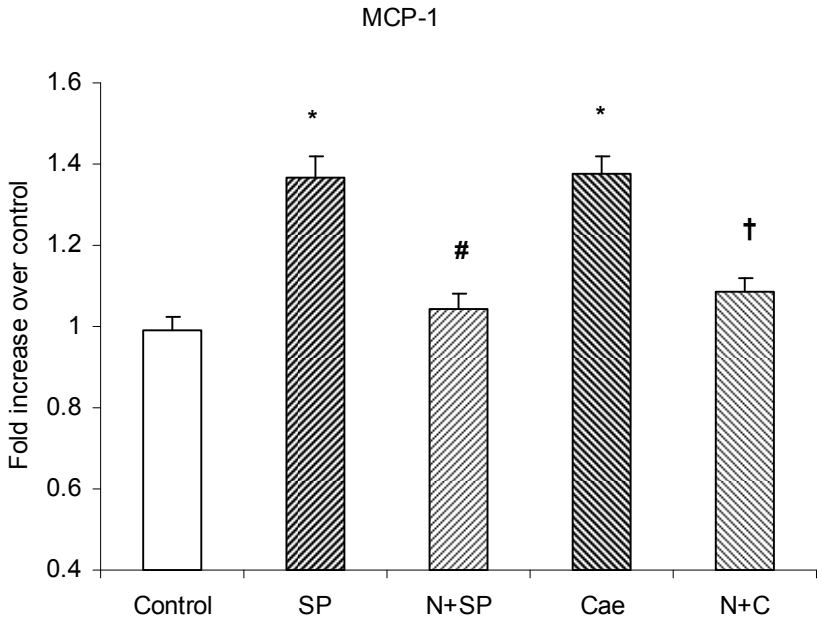


Figure 2.3 (b)

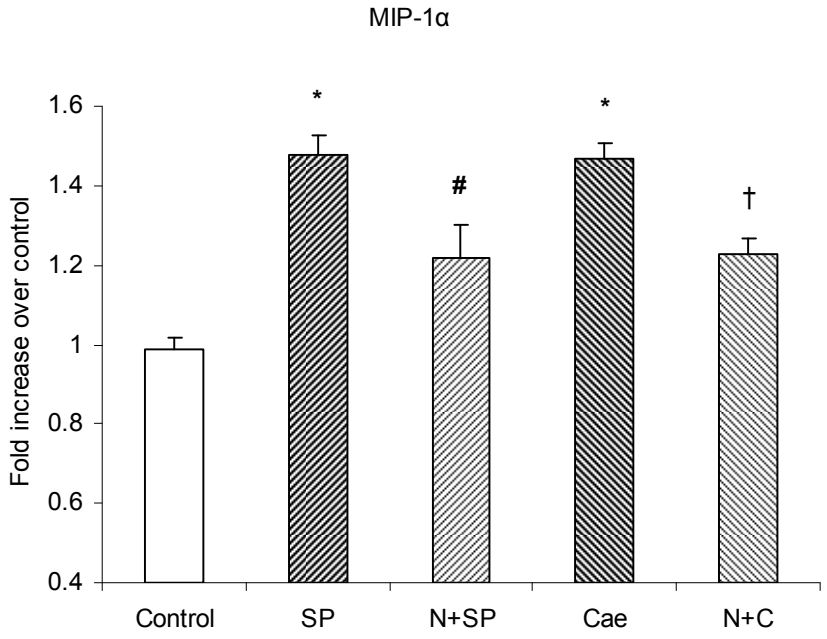


Figure 2.3 (c)

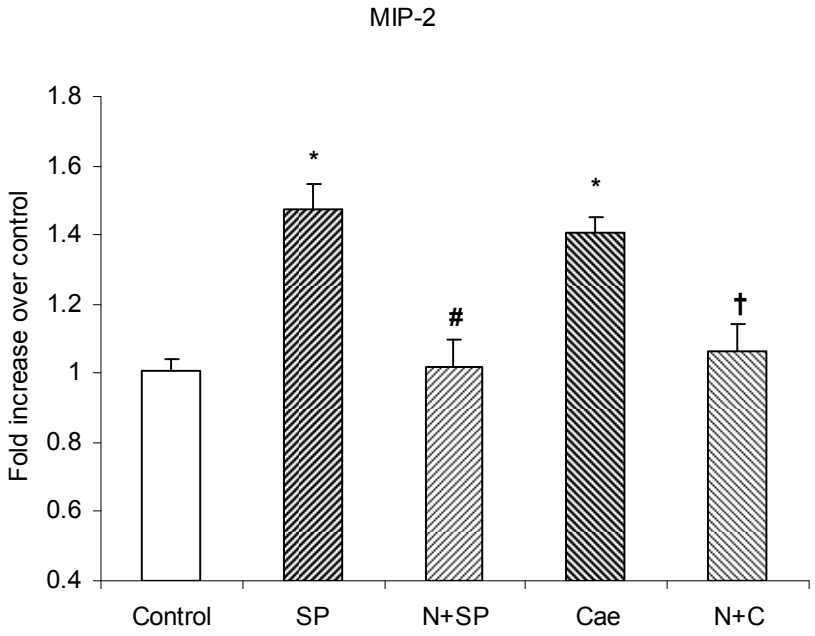


Figure 2.3 Substance P (SP) or caerulein (Cae)-induced chemokine synthesis is abolished with NEMO-Binding Domain peptide (NBD), an NFκB inhibitor. The effect of NFκB inhibitor, NBD, on (a) MCP-1, (b) MIP-1α and (c) MIP-2 production after being stimulated by SP or Cae. Freshly isolated mouse acini, obtained from three mice, were pre-incubated with or without NBD, 50 μM, for 2h followed by stimulation with 1 μM SP or 0.1 μM of Cae for 45 minutes at 37˚C. (a) MCP-1, (b) MIP-1α and (c) MIP-2 levels in conditioned media were measured by ELISA. Results shown are the means + SE. *$P < 0.05$ when SP or Cae-treated acini were compared with vehicle-treated acini. # $P < 0.05$ when NBD and SP-treated acini were compared with SP alone stimulated cells. † $P < 0.05$ when NBD and Cae-treated acini were compared with Cae alone stimulated cells.

Figure 2.4 (a)

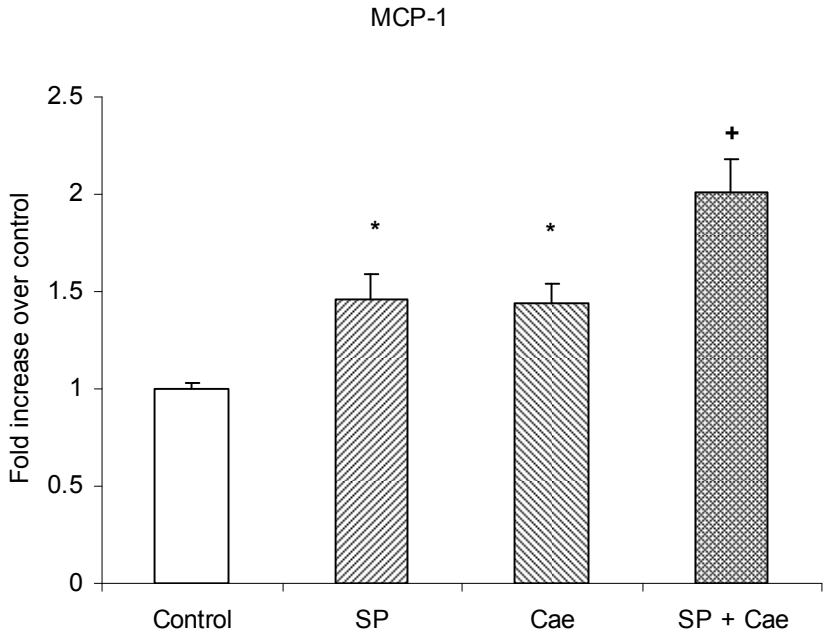


Figure 2.4 (b)

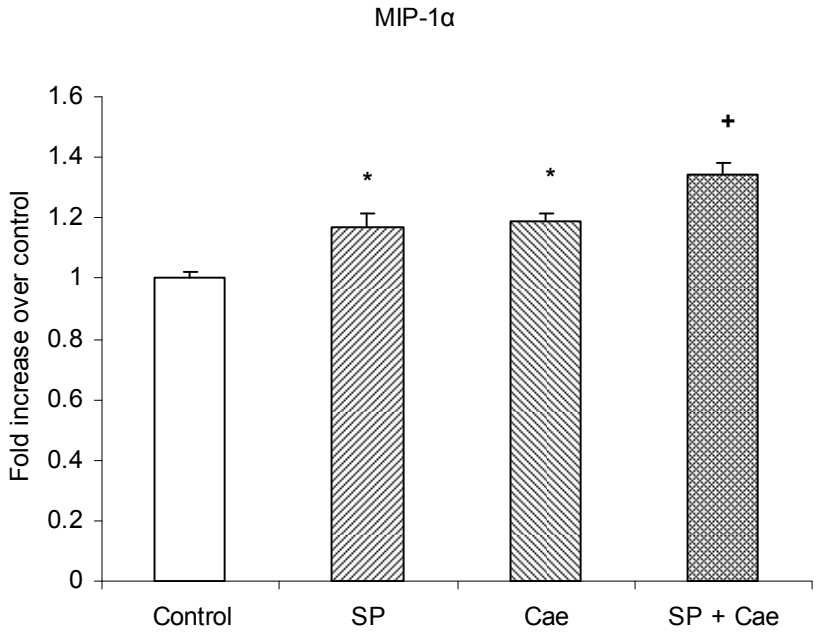


Figure 2.4 (c)

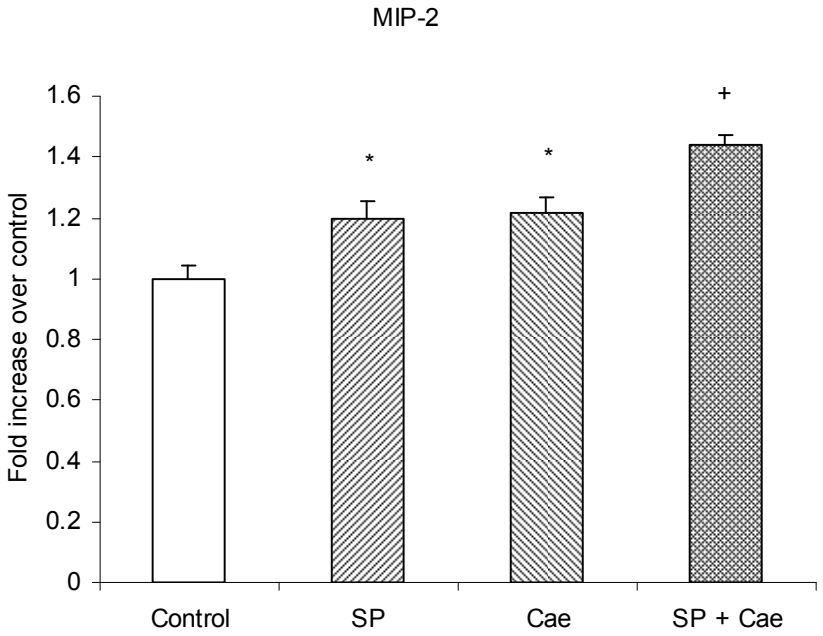


Figure 2.4 Substance P (SP) and caerulein (Cae) act independently in inducing chemokine synthesis. The effects of combined treatment, SP and Cae, on (a) MCP-1, (b) MIP-1α and (c) MIP-2 production. Freshly isolated mouse acini, obtained from three mice, were incubated with either 1 μM SP alone or 0.1 μM Cae alone or both SP and Cae for 45 minutes at 37˚C. (a) MCP-1, (b) MIP-1α and (c) MIP-2 levels in conditioned media were measured by ELISA. Results shown are the means + SE. **P* < 0.05 when SP or Cae-treated acini were compared with vehicle-treated acini. +*P* < 0.05 when acini treated with both SP and Cae were compared to either SP or Cae-treated acini.

Figure 2.5

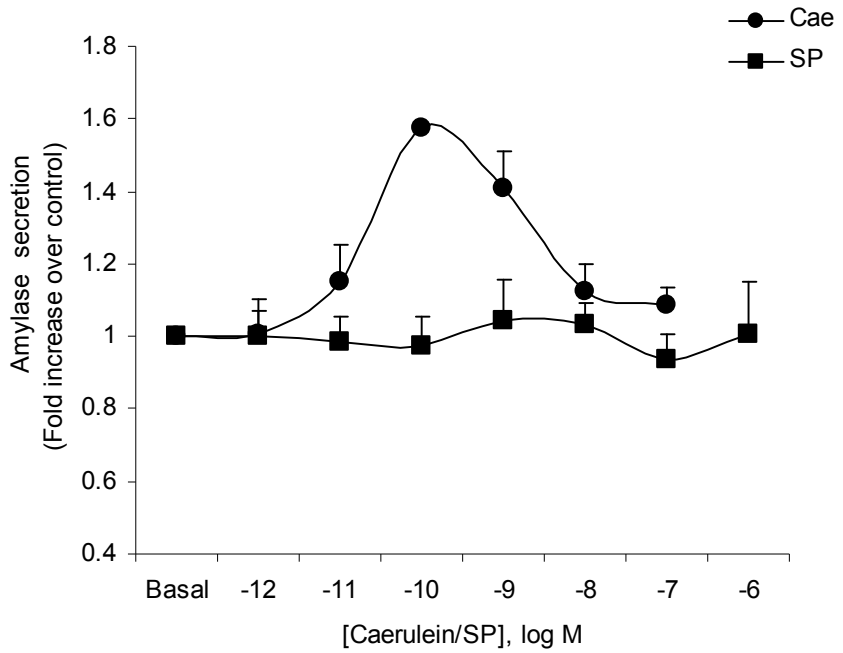


Figure 2.5 Amylase secretion. Freshly isolated mouse pancreatic acini, obtained from three mice, were treated with substance P (SP) ranging from 10^{-12} to 10^{-6} M or caerulein (Cae) ranging from 10^{-12} to 10^{-7} M for 45 minutes at 37˚C. Amylase secretion was determined in response to SP or Cae treatment as described earlier. The results are representative of three independent experiments. Results shown are the means + SE.

CHAPTER 3

EFFECT OF MITOGEN-ACTIVATED PROTEIN KINASES ON CHEMOKINE SYNTHESIS INDUCED BY SUBSTANCE P IN MOUSE PANCREATIC ACINAR CELLS

3.1 INTRODUCTION

Substance P, acting via NK1R, plays an important pro-inflammatory role in acute pancreatitis. During acute pancreatitis, both pancreatic levels of substance P and the expression of its receptor NK1 are elevated (Bhatia, Saluja *et al.* 1998). Genetic deletion of NK1R as well as knockout mice deficient in the PPTA gene, which encodes for substance P, reduces the severity of pancreatitis and pancreatitis-associated lung injury (Bhatia, Saluja *et al.* 1998; Bhatia, Slavin *et al.* 2003). Furthermore, knockout mice deficient in neutral endopeptidase, the enzyme that hydrolyzes substance P, thereby terminating its action are more susceptible to acute pancreatitis and associated lung injury (Bhatia, Saluja *et al.* 1997; Maa, Grady *et al.* 2000). In addition, blockade of substance P receptor with its potent and selective antagonist, CP96345, protected mice against acute pancreatitis and associated lung injury (Lau, Wong *et al.* 2005). The exact mechanism by which substance P contributes to the pro-inflammatory signaling in acute pancreatitis is

not completely understood. However, its interaction with chemokines has been revealed to play a crucial role in the pathogenesis of acute pancreatitis.

In chapter 2, it was shown that substance P stimulated the release of CC chemokines MCP-1, MIP-1α and CXC chemokine MIP-2 production in pancreatic acinar cells. Substance P-induced chemokine production was mediated through the activation of NFκB in pancreatic acinar cells. It has been demonstrated that treatment with CP96345 attenuated the increase in MCP-1, MIP-1α and MIP-2 production in both pancreas and lungs in mice induced with acute pancreatitis (Sun and Bhatia 2007). Hence, substance P plays an important role in the pathogenesis of acute pancreatitis by inducing chemokine production via the NFκB dependent pathway. In parallel with my study, research conducted by various groups has shown that substance P induces the synthesis of chemokines. It has been found to induce IL-8 secretion from human dental pulp cells (Koon, Zhao *et al.* 2005; Park, Hsiao *et al.* 2004). Moreover, it stimulated the production of IL-8 through pro-inflammatory transcription factor NFκB and MAP kinases in lung epithelial cells (Williams, Zou *et al.* 2007).

MAPKs are known to regulate the production of pro-inflammatory cytokines/chemokines and the downstream signaling events leading to inflammation. It is known that substance P receptor (NK1) activates the MAP Kinase families (ERK, JNK, and p38) (Tansky, Pothoulakis *et al.* 2007). Activation of substance P receptor in human glioblastoma cells led to the phosphorylation of ERK1/2 (Yamaguchi, Richardson *et al.* 2005) and blockade of NK1R inhibited phosphorylation of MAPK ERK in sensory neurons (Donnerer and Liebmann 2006).

The signal transduction pathway through which substance P/NK1R interaction induces chemokine MCP-1, MIP-1α, and MIP-2 production in mouse pancreatic acini has not been elucidated yet. Whether MAPKs are involved in substance P-induced pro-inflammatory signaling in pancreatic acinar cells is yet to be investigated. To that end, I examined the participation of MAPKs in substance P-induced synthesis of chemokines MCP-1, MIP-1α and MIP-2 in pancreatic acini and determined the pro-inflammatory signaling pathway involved in acute pancreatitis.

3.2 MATERIALS AND METHODS

3.2.1 Animal Ethics

Please refer to section 2.2.1.

3.2.2 Preparation of mouse pancreatic acini

Mouse pancreatic acinar cells were prepared, as previously described in section 2.2.2.

3.2.3 Viability of mouse pancreatic acinar cells

The viability of mouse pancreatic acinar cells was assessed, as previously described in section 2.2.3.

3.2.4 Cell signaling experiments

Pancreatic acini were treated with substance P (Sigma-Aldrich) at a concentration of 1 μM for 0, 3, 5, 10, 15, 45, 60 and 120 min at 37˚C. After which the cells were subjected to either nuclear extract for NFκB (p65) and AP-1 (c-Jun) detection or cell lysis to detect for MAPKs activation by Western blot analysis. In some experiments, cells were also pre-treated with MAPK kinase (MEK1) inhibitor PD98059 at 10 μM and 30 μM (Calbiochem) for 1 h and then stimulated with 1 μM substance P or vehicle (saline) for 45 min at 37°C. In other experiments, cells were pre-incubated with JNK inhibitor SP600125 at 10 μM and 25 μM (Calbiochem) for 1 h followed by treatment with 1 μM substance P or vehicle (saline) for 45 min at 37°C. In yet another experiment, cells were pre-incubated with the selective NK1R antagonist, CP96345, at 1 μM (Pfizer Diagnostics) for 30 min followed by treatment with 1 μM substance P or vehicle (saline)

for 45 min at 37°C. Subsequently, the supernatant was used for chemokine detection and the pellet was used for either nuclear extract, to detect NFκB (p65) and AP-1 (c-Jun) activation, or cell lysis for Western blot analysis. PD98059 or SP600125 stock solutions were prepared by dissolving 5 mg of PD98059 or SP600125 into 100 µl of DMSO. The final concentration of the vehicle was ≤ 0.1% DMSO. CP96345 stock solution was prepared by dissolving 1 mg of CP96345 into 2 ml of saline. PD98059, SP600125 and CP96345 had no effect on mouse pancreatic acinar cell viability. The negative control in which pancreatic acini were pre-treated with 10 µM (the concentration sufficient to block substance P-mediated activation) of PD98059 or SP600125 for 1 h followed by stimulation with vehicle (saline) for 45 min had no significant effect on MAPKs ERK and JNK, NFκB (p65), AP-1 (c-Jun) and chemokine production when compared to unstimulated controls.

3.2.5 Preparation of cell lysates for Western blot analysis

Pancreatic acini were treated with substance P at a concentration of 1 µM for 0, 3, 5, 10, 15, 45, 60 and 120 min at 37˚C. After treatment, pancreatic acinar cells were homogenized on ice in RIPA buffer supplemented with 1 mM PMSF and the protease inhibitor cocktail containing pepstatin, leupeptin, chymostatin, antipain and aprotinin (5 µg/ml of each), and centrifuged at 4 °C for 15 min at 14,000 *g*. The supernatants were collected and stored at -80°C until use. Protein concentrations were determined by using Bio-Rad protein assay. 5 µl of sample was added to 250 µl of Bradford reagent (Bio-Rad Laboratories, Hercules, CA) and read at 595 nm after a short incubation (5 min) at room temperature.

3.2.6 Western blot analysis

Cell lysates (50 μg of protein) were separated on 12% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes. Non-specific binding was blocked by 1 h incubation of the membranes in 5% non-fat dry milk in PBST (0.05% Tween 20 in PBS). The blots were then incubated overnight with the primary antibodies phospho-ERK1/2, ERK 1/2, phospho-SAPK/JNK, SAPK/JNK, IκBα (Cell Signaling Technology) and HPRT (Santa Cruz Biotechnology) at 1:1000 dilutions in the buffer containing 2.5% non-fat dry milk in PBST. After which they were washed four times with PBST, and finally incubated for 1 h with goat anti-rabbit HRP-conjugated secondary antibody (Santa Cruz Biotechnology) at 1:2000 dilutions in the buffer containing 2.5% non-fat dry milk in PBST. The blots were developed for visualization using enhanced chemiluminescence (ECL) detection kit (Pierce, Rockford, IL).

3.2.7 Preparation of nuclear cell extract

Nuclear cell extract was prepared, as previously described in section 2.2.6.

3.2.8 NFκB DNA-binding actitvity

The binding of NFκB to DNA was measured in nuclear extracts, as previously described in section 2.2.7.

3.2.9 AP-1 DNA-binding activity

TransAM AP-1 kits are designed specifically to detect and quantify AP-1 activation. Similar to TransAM NFκB p65, TransAM AP-1 c-Jun kits contain a 96-well plate on

which has been immobilized an oligonucleotide that contains a TPA- responsive element TRE (5'-TGAGTCA-3'). AP-1 dimers contained in nuclear extract (5 μg of protein) specifically binds to this oligonucleotide. The primary antibodies used recognize accessible epitopes on c-Jun proteins upon DNA binding. Secondary antibody conjugated to HRP generates the colorimetric reaction. Absorbance was read at 450 nm within 5 min.

3.2.10 Chemokine detection

Pancreatic acinar cell supernatants were assayed for MCP-1, MIP-1α and MIP-2 using a sandwich ELISA, as previously described in section 2.2.5.

3.2.11 Statistical analysis

Results are presented as means + SE with 6 replicates for each condition. Each experiment was repeated at least three times. The significance of changes was evaluated by using ANOVA and Tukey's method was used as a post hoc test for the difference between groups. A P value < 0.05 was taken as the level of significance.

3.3 RESULTS

3.3.1 Substance P stimulates ERK1/2 phosphorylation and NFκB activation in a time-dependent manner

To examine if substance P causes ERK1/2 phosphorylation in pancreatic acini, mouse pancreatic acinar cells were treated with 1 μM substance P for 0, 3, 5, 10, 15, 45, 60, 120 min. Cells were then lysed, and cell proteins were subjected to Western blot analysis using antibodies against both phospho-ERK1/2 and total ERK1/2. As shown in Figure 3.1 (a), substance P induced a time-dependent increase in the phosphorylation of ERK1/2 in pancreatic acini. In Figure 3.1 (b), densitometric analysis of Western blot experiments revealed a significant increase in phosphorylation of ERK1/2 when compared to 0 min control. The time-dependent increase in phosphorylation of ERK1/2 was in line with the time-dependent degradation of total IκBα as shown in Figure 3.1 (c). In a similar experiment nuclear extract was used, instead of cell lysate, to detect NFκB (p65) activation by ELISA. As shown in Figure 3.1 (d), treatment with 1 μM substance P caused a time-dependent increase in NFκB (p65) activation. The increase became significant at 10 min after substance P treatment.

3.3.2 ERK1/2-mediated NFκB activation is involved in substance P-induced chemokine synthesis

To determine if the MEK1 inhibitor PD98059 blocks phosphorylation of ERK1/2 in substance P-treated cells, mouse pancreatic acinar cells were pre-incubated with PD98059 for 1 h followed by stimulation with 1 μM substance P for 45 min. Cells were

then lysed, and cell proteins were subjected to Western blot analysis. As shown in Figure 3.2 (a), PD98059 attenuated substance P-induced phosphorylation of ERK1/2. In Figure 3.2 (b), densitometry showed that the two different doses of PD98059 namely 10 μM and 30 μM significantly blocked phosphorylation of ERK1/2 when compared to substance P only treated group. To confirm the role of ERK1/2 in substance P-induced NFκB (p65) activation and chemokine production, I pre-treated the pancreatic acini with PD98059 for 1 h followed by stimulation with 1 μM substance P for 45 min. The results, in Figure 3.3 (a), showed that pre-treatment with PD98059 significantly inhibited substance P-induced NFκB (p65) activation, which was followed by a decrease in CC chemokines (3.3b) MCP-1, (3.3c) MIP-1α and CXC chemokine (3.3d) MIP-2. These results indicate that substance P induces the production of chemokines MCP-1, MIP-1α and MIP-2 via the ERK1/2-mediated NFκB signaling pathway.

3.3.3 Substance P induces phosphorylation of JNK and AP-1 activation in a time dependent manner

To examine if substance P stimulates JNK phosphorylation in pancreatic acini, mouse pancreatic acinar cells were treated with 1 μM substance P for 0, 3, 5, 10, 15, 45, 60, 120 min. Cells were then lysed, and cell proteins were subjected to Western blot analysis using antibodies against both phospho-p54 and -p46 JNK and total JNK. As shown in Figure 3.4 (a), substance P induced phosphorylation of phospho-p54 and -p46 JNK in pancreatic acini in a time-dependent manner. In Figure 3.4 (b), densitometric analysis of Western blot experiments revealed a significant increase in phospho-p54 from 10 min when compared to 0 min control. Densitometric analysis of phospho-p46 could not be carried out as the control for phospho-p46 was undetectable. In Figure 3.4 (c), the time-

dependent increase in phosphorylation of phospho-p54 and -p46 JNK was in accord with the time-dependent increase in AP-1 (c-Jun) activation; with a significant maximal intensity ranged from 45 to 120 min when compared to the 0 min control.

3.3.4 Involvement of JNK in substance P-induced AP-1 activation and chemokine synthesis

SP600125 is an inhibitor of JNK. It prevents the phosphorylation of JNK substrates by blocking the ATP-binding domain of JNKs. To determine if SP600125 inhibits phosphorylation of JNK in substance P-treated cells, mouse pancreatic acinar cells were pre-incubated with SP600125 for 1 h followed by stimulation with 1 μM substance P for 45 min. Cells were then lysed, and cell proteins were subjected to Western blot analysis. As shown in Figure 3.5 (a), SP600125 attenuated substance P-induced phosphorylation of JNK. In Figure 3.5 (b), densitometry showed that 10 μM and 25 μM of SP600125 significantly blocked phosphorylation of JNK when compared to substance P only treated group. To determine if substance P-induced synthesis of chemokines MCP-1, MIP-1α and MIP-2 and AP-1 (c-Jun) activation are mediated through JNK, pancreatic acini were pre-treated with SP600125 for 1 h followed by stimulation with 1 μM substance P for 45 min. The data in Figure 3.6 (a) shows that SP600125 inhibited substance P-induced AP-1 (c-Jun) activation. As shown in Figure 3.6 (b, c, d), pre-treatment with SP600125 caused an attenuation of substance P-induced production of MCP-1, MIP-1α and MIP-2. These results demonstrate that substance P-induced synthesis of MCP-1, MIP-1α and MIP-2 is mediated by JNK/AP-1 (c-Jun) signaling pathway.

3.3.5 Substance P-induced ERK1/2 and JNK cross activate NFκB and AP-1

Pancreatic acini were pre-treated with either PD98059 or SP600125 followed by stimulation with 1 μM substance P for 45 min. As shown in Figure 3.7 (a), PD98059 given at concentrations of 10 μM and 30 μM significantly blocked AP-1 (c-Jun) activation. As shown in Figure 3.7 (b), SP600125 attenuated NFκB (p65) activation. The data suggest that substance P-induced chemokine production takes place through ERK1/2 mediated AP-1 (c-Jun) activation and JNK mediated NFκB (p65) activation. These results imply that there is a cross-talk between the two classical pathways, ERK1/2-NFκB (p65) and JNK-AP-1 (c-Jun), to induce synthesis of chemokines MCP-1, MIP-1α and MIP-2 in pancreatic acini.

3.3.6 Substance P/NK1R interaction is involved in ERK 1/2 and JNK activation

The data show that substance P-induced ERK1/2 and JNK activation were mediated through NK1R. I pre-treated the pancreatic acini with 1 μM of CP96345, a selective NK1R antagonist, for 30 min followed by stimulation with 1 μM of substance P for 45 min. Cells were then lysed, and cell proteins were subjected to Western blot analysis. The results, in Figure 3.8, demonstrate that CP96345 significantly reduced substance P-induced ERK1/2 and JNK activation in pancreatic acinar cells when compared to substance P only treated cells.

3.3.7 Substance P-induced NFκB and AP-1 activation as well as chemokine production are mediated through NK1R

The role of NK1R in substance P-induced NFκB (p65) and AP-1 (c-Jun) activation and chemokine production was confirmed by pre-treating the cells with 1 μM of CP96345 followed by stimulation with 1 μM of substance P for 45 min. The cells were used for nuclear extraction to determine NFκB (p65) and AP-1 (c-Jun) activation whereas the

supernatant obtained was used for detection of chemokines MCP-1, MIP-1α, and MIP-2 by ELISA. The results, in Figure 3.9 (a, b, c, d, e), show that pre-treatment with selective antagonist CP96345 significantly inhibited substance P-induced activation of NFκB (p65) and AP-1 (c-Jun) as well as production of MCP-1, MIP-1α, and MIP-2 in pancreatic acinar cells when compared to substance P only treated cells.

3.4 DISCUSSION

In chapter 2, it was shown that substance P induces production of chemokines MCP-1, MIP-1α and MIP-2 in mouse pancreatic acinar cells. In the present study, I have investigated the signaling cascades through which substance P/NKIR stimulate production of these chemokines in mouse pancreatic acini. Substance P has been shown to stimulate a number of intracellular signaling molecules such as MAPK members. Ligand binding to NK1R activates MAPKs (Luo, Sharif *et al.* 1996). In the present study, I have focussed on the MAPKs ERK1/2 and JNK, as p38 was not activated upon substance P stimulation in my model of isolated acinar cells.

In this study, I have shown that substance P induced JNK phosphorylation which was in accord with AP-1 (c-Jun) activation. It was established that phosphorylation of AP-1 family members by kinases (for example JNK) is required for transactivation activity. Substance P-induced JNK/AP-1(c-Jun) activation led to increase synthesis of MCP-1, MIP-1α and MIP-2. To confirm that production of these chemokines was mediated through the JNK/AP-1(c-Jun) signaling pathway, pancreatic acini were pre-treated with SP600125 followed by stimulation with substance P. SP600125 is a selective inhibitor of JNK. In cells, it dose-dependently inhibits the phosphorylation of c-Jun. It competitively and reversibly inhibits JNK1, 2 and 3 and has been shown to have less inhibitory potency on ERK2, p38 and a range of other kinases (Bennett, Sasaki *et al.* 2001; Shin, Yan *et al.* 2002). My data shows that SP600125 attenuated the activation of AP-1 (c-Jun) as well as the production of MCP-1, MIP-1α and MIP-2. Moreover, I found that SP600125 attenuated NFκB activation. I also confirmed with the use of Western blot analysis that SP600125 indeed blocked phosphorylation of JNK in pancreatic acini. Total JNK was

used as a control. My data suggests that substance P-induced chemokine production occurs not only through the classic JNK/AP-1 (c-Jun) pathway but also through JNK mediated NFκB activation.

Previously, it has been established that MEKK1 induces activation of both IKK-α and IKK-β leading to NFκB activation (Barnes and Karin 1997; Ghosh, May *et al.* 1998). Furthermore, ERK1/2 activity and phosphorylation have been associated with degradation of IκB protein leading to NFκB activation. Moreover, it has been shown that MEK-1 and ERK-1 act as intermediates in the cascade of events that regulate AP-1 and NFκB activation. NFκB activates several genes involved in the pro-inflammatory response, encoding various immunoreceptors, cell adhesion molecules, cytokines and chemokines (Akira and Kishimoto 1997; Baeuerle and Baichwal 1997; Grimm and Baeuerle 1993).

In the present study, I investigated the possible involvement of ERK1/2 in mediating substance P-induced chemokine synthesis in mouse pancreatic acinar cells, in particular their role in increasing NFκB (p65) and AP-1 (c-Jun) activity. I demonstrated that substance P induced ERK1/2 activation. This was in line with NFκB activation and an increased synthesis of chemokines MCP-1, MIP-1α and MIP-2. The ERK pathway-specific inhibitor PD98059 inhibited NFκB activation and chemokine production. PD98059 is a potent, selective and cell-permeable inhibitor of MAP kinase kinase. It selectively inhibits the MAPK-activating enzyme, (MEK), without significant inhibitory activity of MAPK itself. Inhibition of MEK by PD98059 prevents activation of MAPK and subsequent phosphorylation of MAPK substrates both *in vitro* and in intact cells (Alessi, Cuenda *et al.* 1995; Dudley, Pang *et al.* 1995). Here I found that PD98059 also

blocked AP-1(c-Jun) activation. This is in agreement with other studies that reported the N-terminal sites of c-Jun were phosphorylated *in vitro* by ERK1 and ERK2 (Pulverer, Hughes *et al.* 1993; Pulverer, Kyriakis *et al.* 1991). Furthermore, using Western blot analysis I confirmed that PD98059 effectively blocked phosphorylation of ERK1/2 in pancreatic acinar cells.

In chapter 2, I have demonstrated that pre-treatment of pancreatic acini with NEMO-binding domain peptide (NBD), an NFκB inhibitor, completely attenuated the chemokine synthesis induced by substance P. This shows that the increase in chemokine synthesis induced by substance P was specifically dependent on NFκB activation. Taken together, these results demonstrate that substance P-induced production of MCP-1, MIP-1α and MIP-2 is mediated not only through the classic signaling pathways namely ERK1/2-NFκB and JNK/AP-1 (c-Jun) but also through a cross-talk between the two classic signaling pathways.

To further understand the molecular mechanism and to show that chemokine production was indeed mediated by substance P, pancreatic acinar cells were pre-treated with the selective NK1R antagonist, CP96345. In the present study CP96345 decreased the activation of ERK1/2, JNK, NFκB and AP-1 mediated chemokine production; hence showing that substance P-induced chemokine production is dependent on NK1R in pancreatic acinar cells.

To my knowledge, this is the first study that shows the involvement of ERK1/2, JNK, AP-1(c-Jun) and NFκB (p65) in substance P-induced chemokine production in pancreatic acini. In this chapter, I provide evidence that activation of p42/p44 MAPK pathways by substance P is necessary for production of MCP-1, MIP-1α and MIP-2. Furthermore,

JNK seems to be the other MAPK required for substance P-induced chemokine production in these cells. These results show that substance P-induced activation of both ERK1/2 and JNK cascades are essential for NFκB and AP-1 activation, resulting in increased production of chemokines MCP-1, MIP-1α and MIP-2 in mouse pancreatic acini. The study presented in this chapter gives us an insight into the mechanism by which substance P contributes to the inflammatory responses in acute pancreatitis.

Figure 3.1 (a)

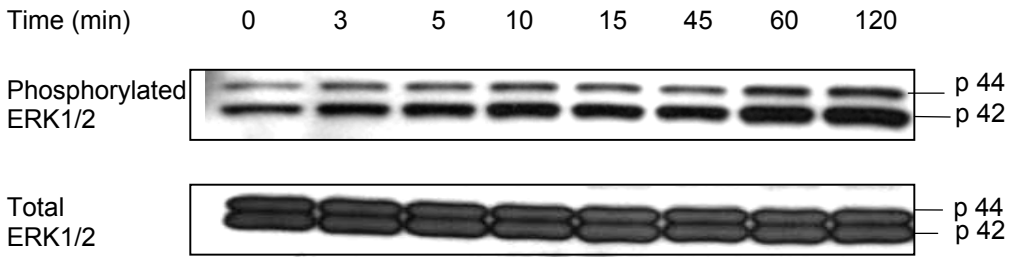


Figure 3.1 (b)

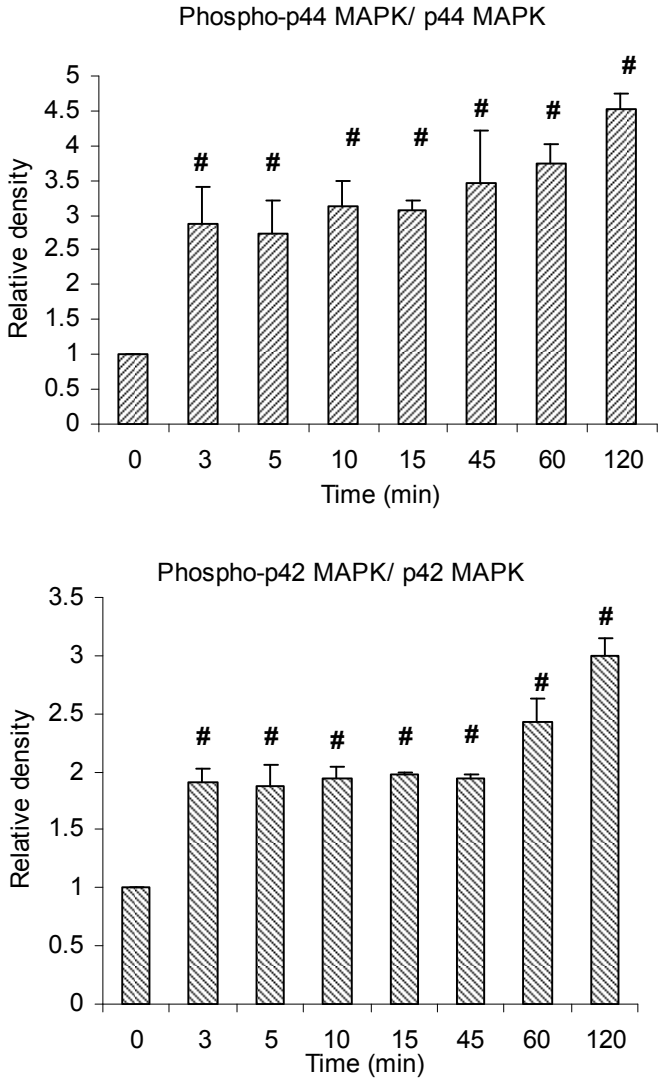


Figure 3.1 (c)

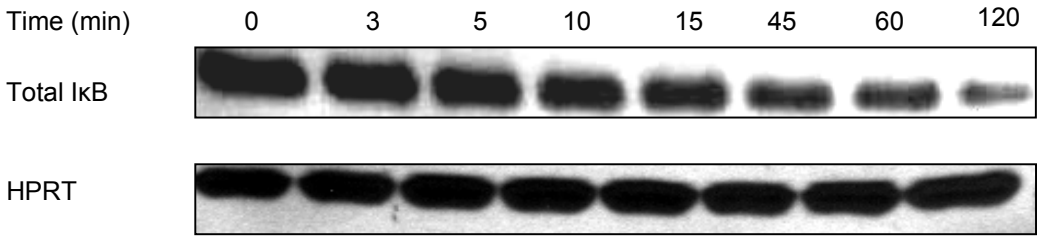


Figure 3.1 (d)

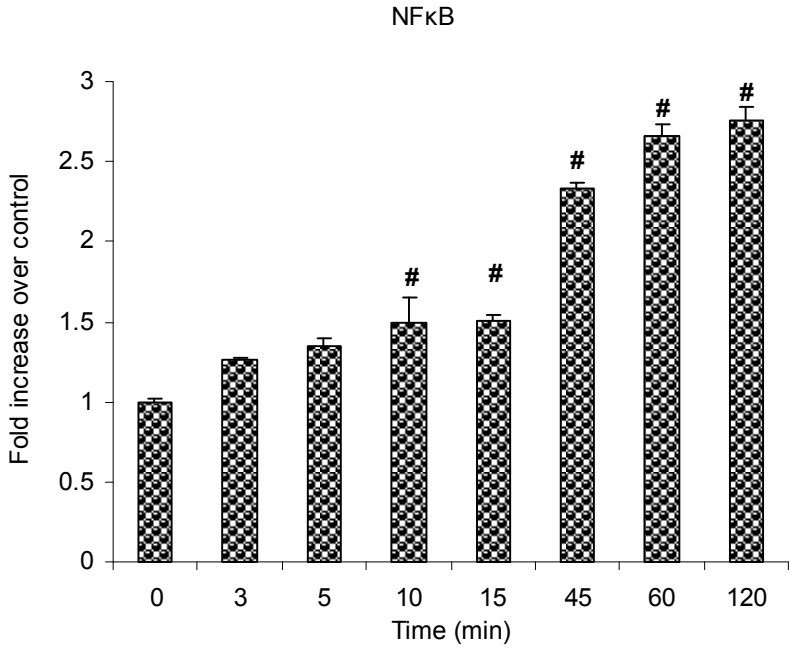


Figure 3.1 Substance P (SP) stimulates ERK1/2 phosphorylation and NFκB activation in a time-dependent manner. SP induces a time-dependent phosphorylation of ERK1/2 which coincides with a time dependent activation of NFκB (p65). Freshly isolated pancreatic acini, obtained from three mice, were incubated with 1 μM SP for 0, 3, 5, 10, 15, 45, 60, 120 min at 37˚C. In some experiments, cells were lysed, and cell proteins were subjected to Western blot analysis using antibodies against (a) phospho-ERK1/2, total ERK1/2 and (c) IκBα. (b) Densitometric analysis of Western blot experiments from pancreatic acini. In another experiment, the nuclear extract was used to isolate NFκB and ELISA was carried out to detect activation of (d) NFκB (p65). MW in (kDa) for IκBα is 39, ERK1/2 is 44/42 and HPRT is 24. The results are representative of three independent experiments. Results shown are the means + SE. #$P < 0.05$ when compared to 0 min control.

Figure 3.2 (a)

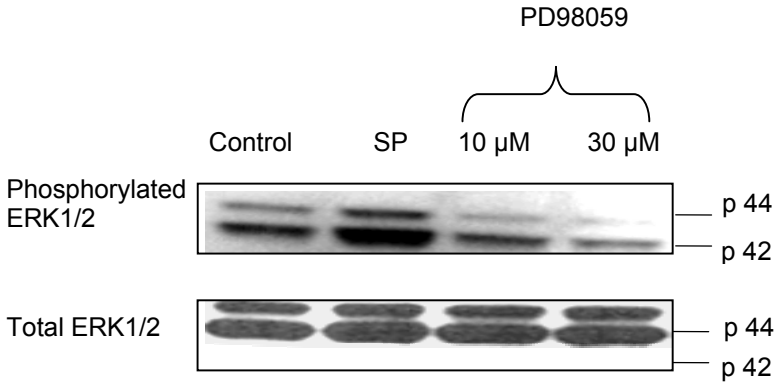
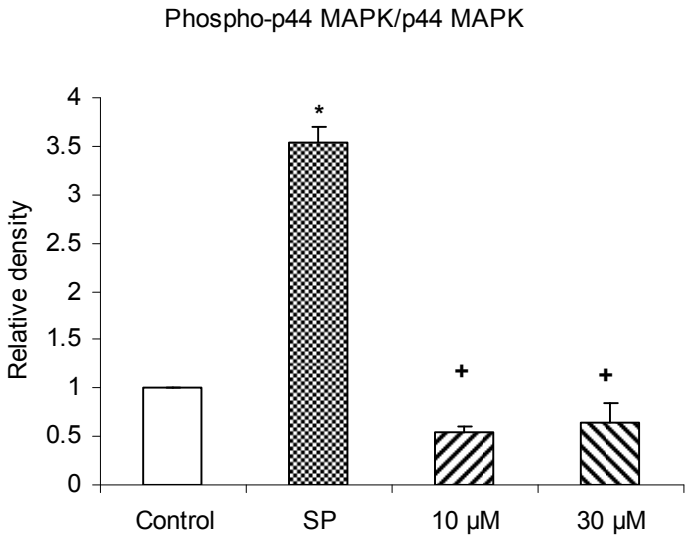


Figure 3.2 (b)



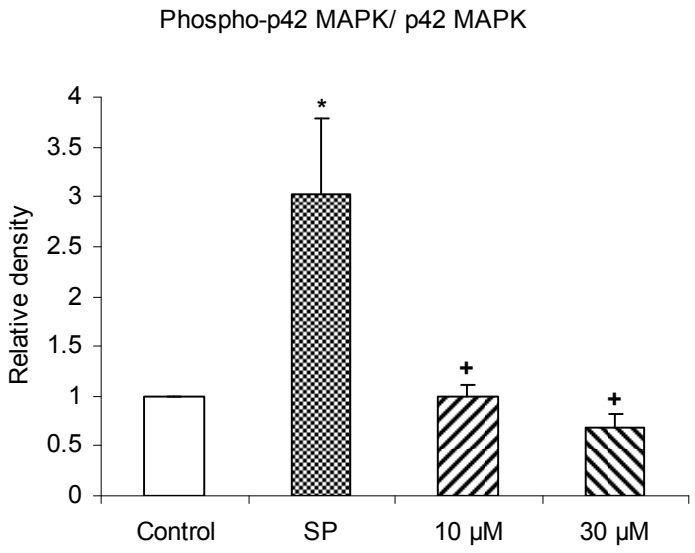


Figure 3.2 PD98059 decreases phosphorylation of ERK1/2 in pancreatic acini. PD98059 (an inhibitor of MEK1/2) effectively blocked phosphorylation of ERK1/2. Freshly isolated pancreatic acini, obtained from three mice, were pre-incubated with PD98059 at doses of 10 µM and 30 µM for 1 h at 37˚C followed by stimulation with 1 µM substance P (SP) for 45 min at 37˚C. Cells were subsequently lysed, and cell proteins were subjected to Western blot analysis using antibodies against (a) phospho-ERK1/2 and total ERK1/2. (b) Densitometric analysis of Western blot experiments from pancreatic acini. MW in (kDa) for ERK1/2 is 44/42. The results are representative of three independent experiments. Results shown are the means + SE. $*P < 0.05$ when compared to control, $+P < 0.05$ when compared to SP.

Figure 3.3 (a)

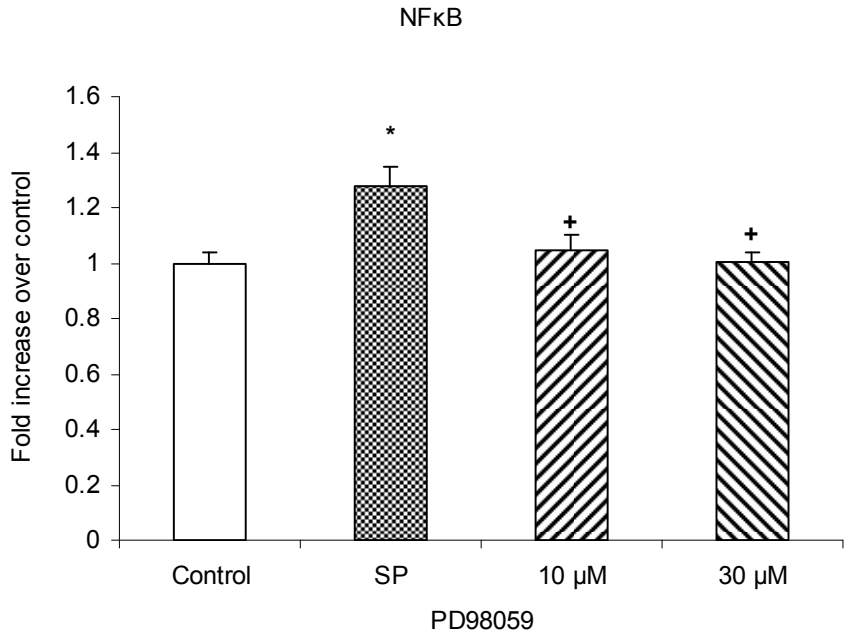


Figure 3.3 (b)

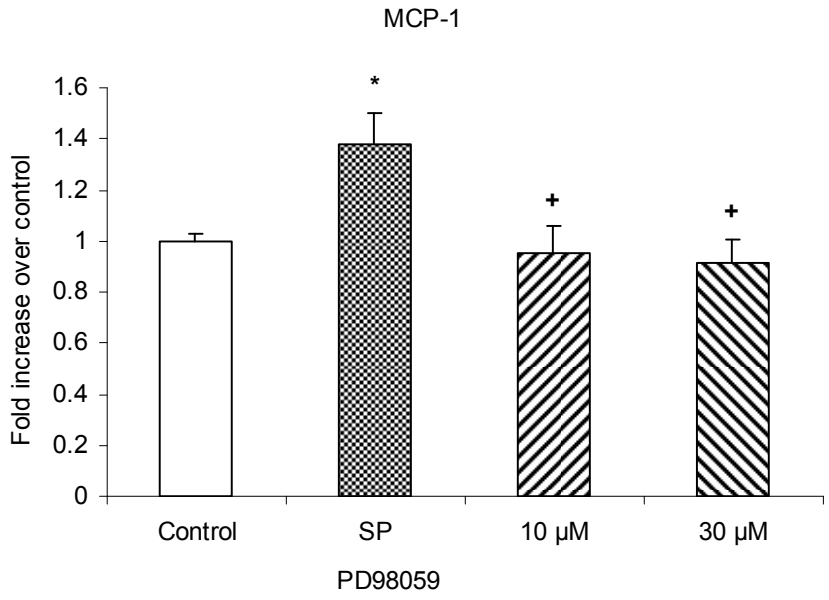


Figure 3.3 (c)

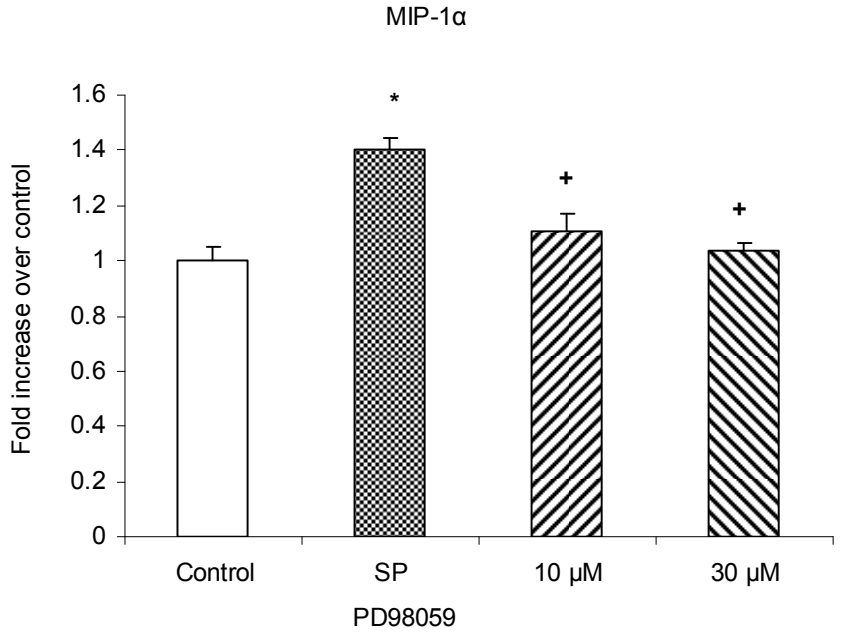


Figure 3.3 (d)

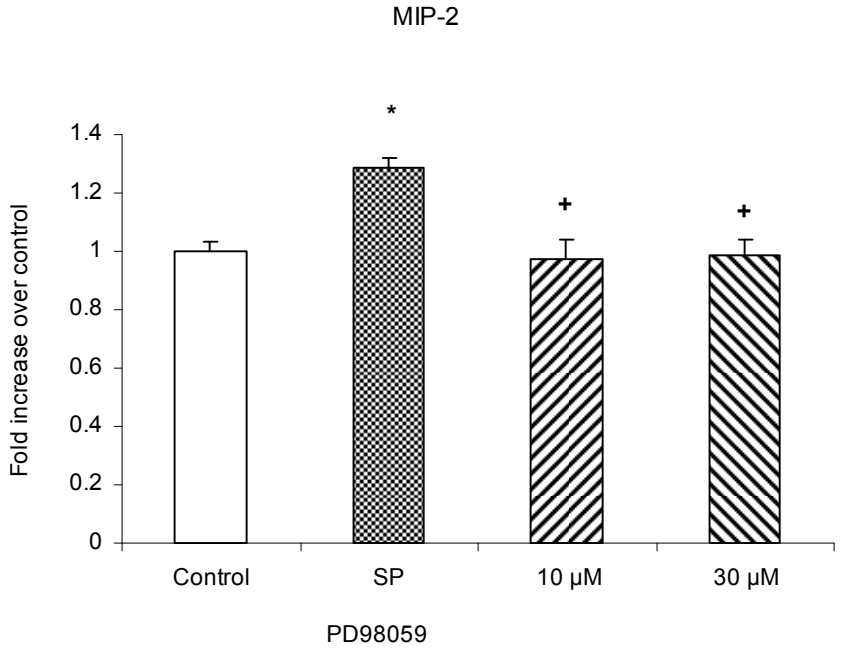


Figure 3.3 ERK1/2-mediated NFκB activation is involved in substance P (SP)-induced chemokine synthesis. SP-induced ERK1/2 phosphorylation and activation mediate NFκB (p65) activation and chemokine production. Freshly isolated pancreatic acini, obtained from three mice, were pre-incubated with MEK1 inhibitor PD98059 for 1 h followed by stimulation with 1 μM SP for 45 min. Acini were separated from incubation medium by centrifugation. (a) The pellet (acini) was used for NFκB (p65) extraction and detection whereas the supernatant was used to measure (b) MCP-1, (c) MIP-1α and (d) MIP-2 levels by ELISA. The results are representative of three independent experiments. Results shown are the means + SE. *****$P < 0.05$ when compared to control, +$P < 0.05$ when compared to SP.

Figure 3.4 (a)

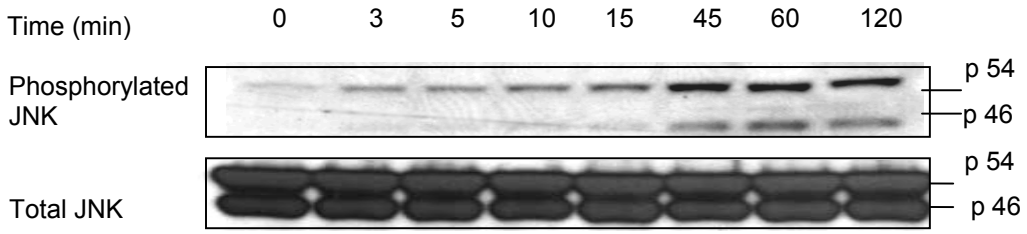


Figure 3.4 (b)

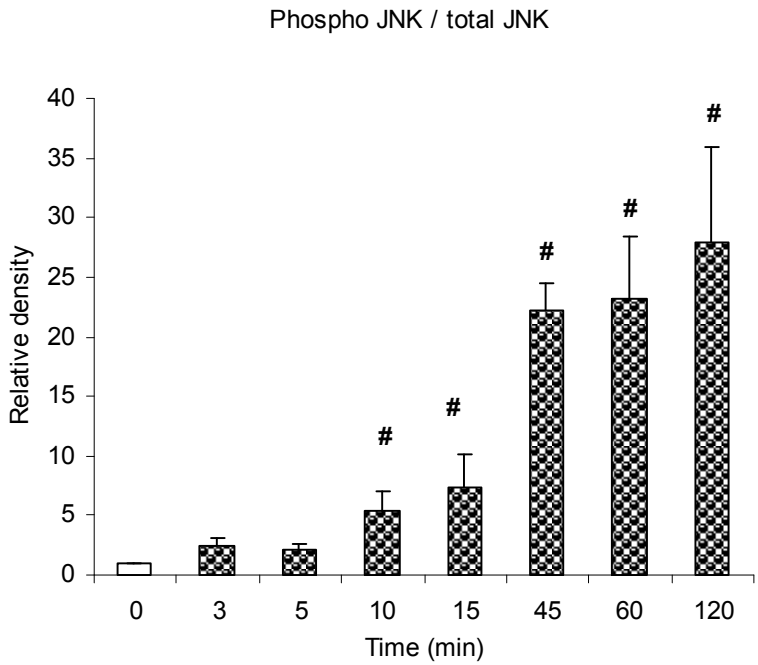


Figure 3.4 (c)

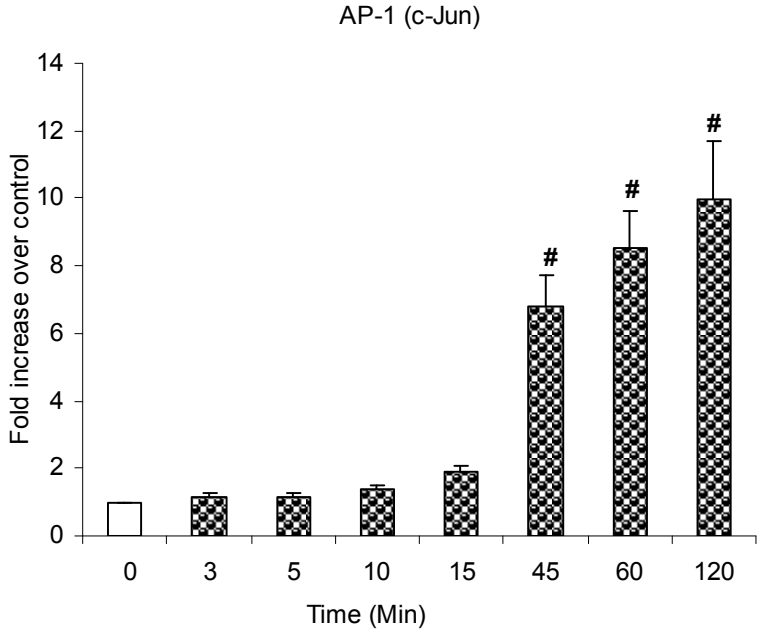


Figure 3.4 Substance P (SP) induces phosphorylation of JNK and AP-1 activation in a time-dependent manner. SP induces a time-dependent phosphorylation of JNK which is in line with a time-dependent activation of AP-1 (c-Jun). Freshly isolated pancreatic acini, obtained from three mice, were incubated with 1 μM SP for 0, 3, 5, 10, 15, 45, 60, 120 min at 37˚C. In some experiments, cells were lysed, and cell proteins were subjected to Western blot analysis using antibodies against (a) phospho-JNK, total JNK. In another experiment, the nuclear extract was used to isolate AP-1 (c-Jun) and ELISA was carried out to detect activation of (c) AP-1 (c-Jun). (b) Densitometric analysis of Western blot experiments from pancreatic acini. MW in (kDa) for JNK 2/1 is 54/46. The results are representative of three independent experiments. Results shown are the means + SE. #$P < 0.05$ when compared to control (0 min).

Figure 3.5 (a)

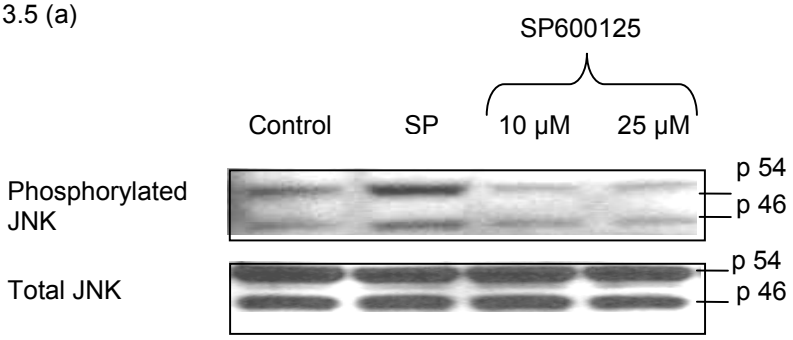
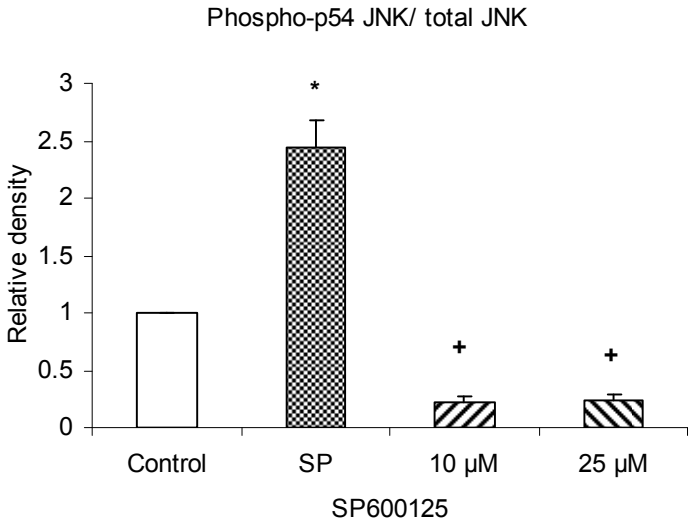


Figure 3.5 (b)



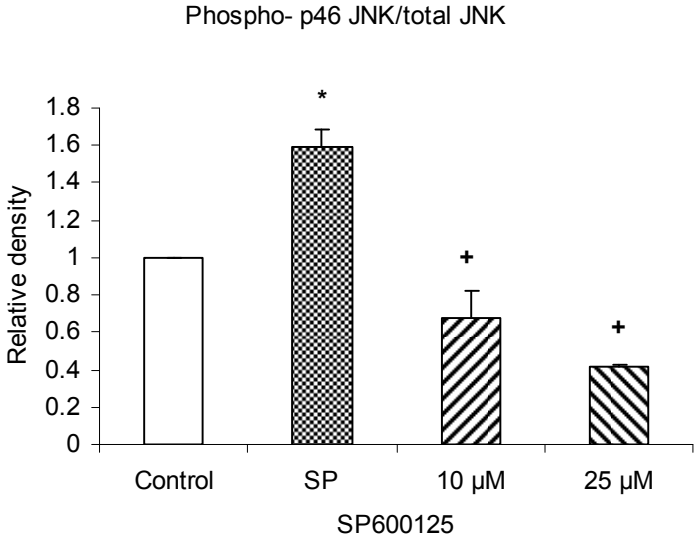
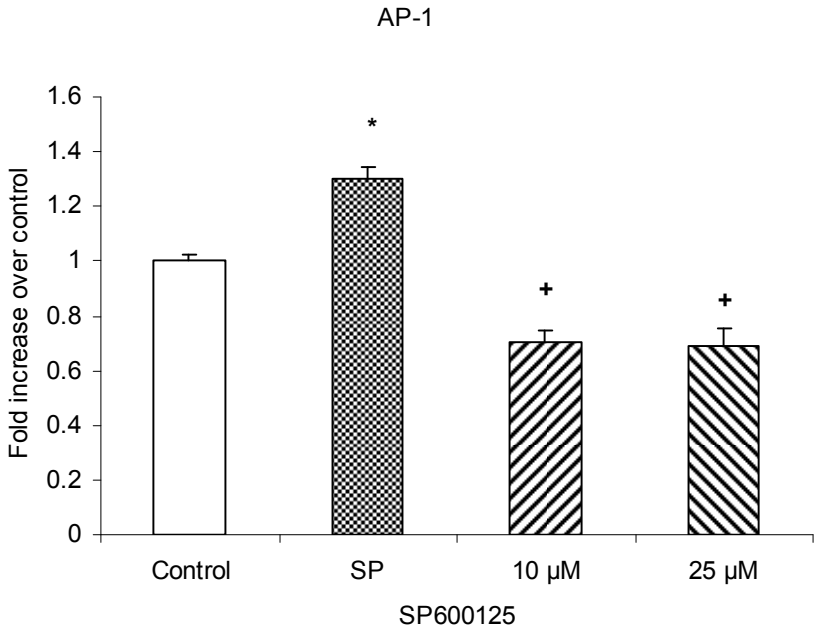


Figure 3.5 SP600125 decreases phosphorylation of JNK in pancreatic acini. SP600125 (JNK inhibitor) effectively blocked phosphorylation of JNK. Freshly isolated pancreatic acini, obtained from three mice, were pre-incubated with SP600125 at different doses of 10 µM and 25 µM, for 1 h at 37˚C followed by stimulation with 1 µM substance P (SP) for 45 min at 37˚C. Cells were subsequently lysed, and cell proteins were subjected to Western blot analysis using antibodies against (a) phospho-JNK and total JNK. (b) Densitometric analysis of Western blot experiments from pancreatic acini. The results are representative of three independent experiments. MW in (kDa) for JNK 2/1 is 54/46. Results shown are the means + SE. *****$P < 0.05$ when compared to control, +$P < 0.05$ when compared to SP.

Figure 3.6 (a)



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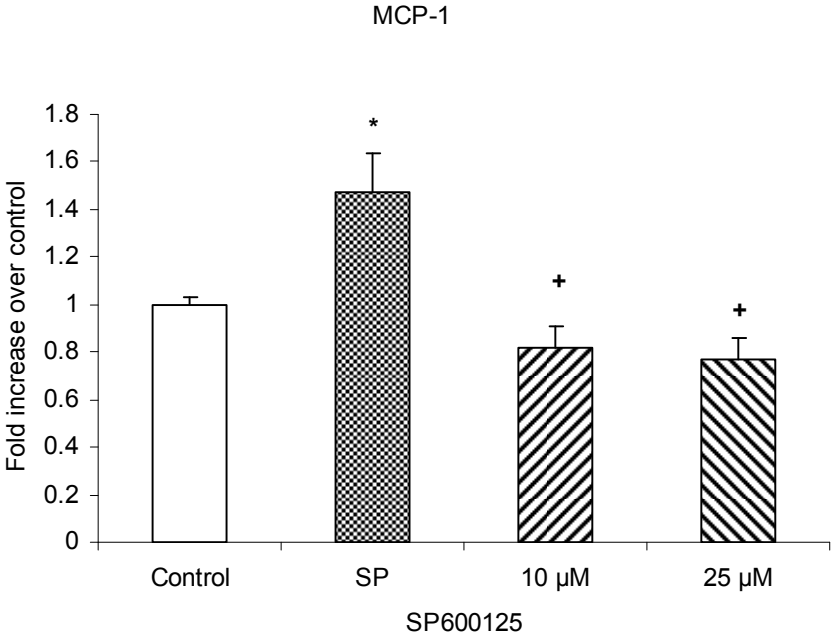
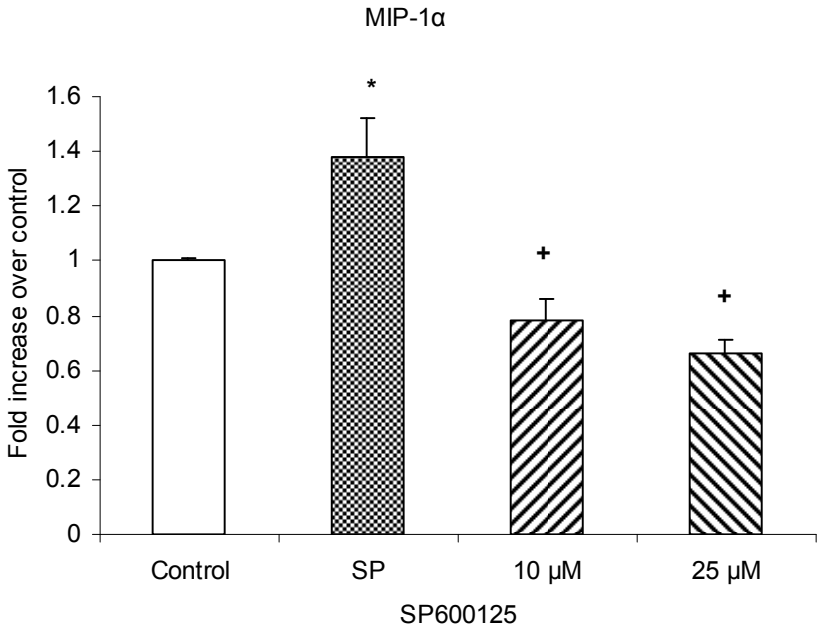


Figure 3.6 (c)



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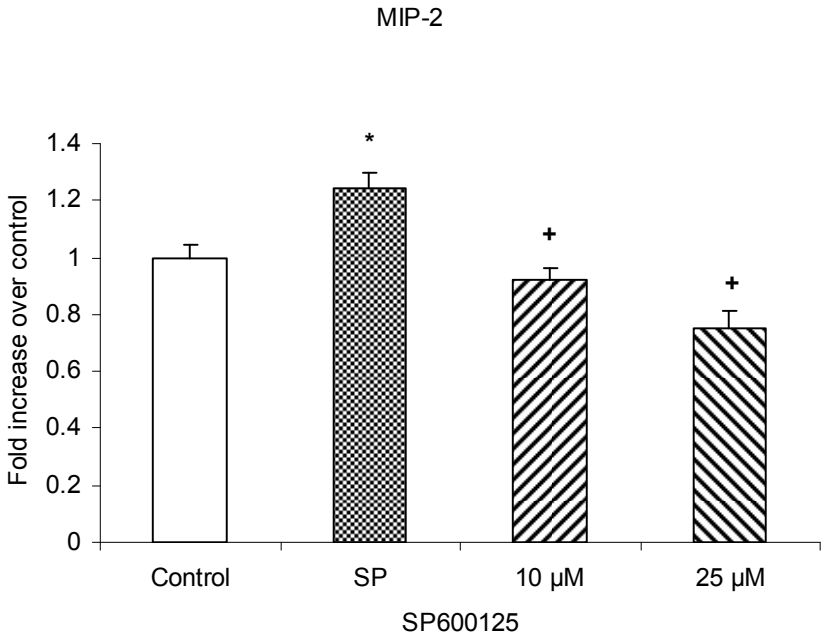


Figure 3.6 JNK is involved in substance P (SP)-induced AP-1 activation and chemokine synthesis. SP-induced JNK phosphorylation and activation mediate AP-1 (c-Jun) activation and chemokine production. Freshly isolated pancreatic acini, obtained from three mice, were pre-incubated with JNK inhibitor SP600125 for 1 h followed by stimulation with 1 μM SP for 45 min. Acini were separated from incubation medium by centrifugation. (a) The pellet (acini) was used for AP-1 (c-Jun) extraction and detection whereas the supernatant was used to measure (b) MCP-1, (c) MIP-1α and (d) MIP-2 levels by ELISA. The results are representative of three independent experiments. Results shown are the means + SE. $*P < 0.05$ when compared to control, $+P < 0.05$ when compared to SP.

Figure 3.7 (a)

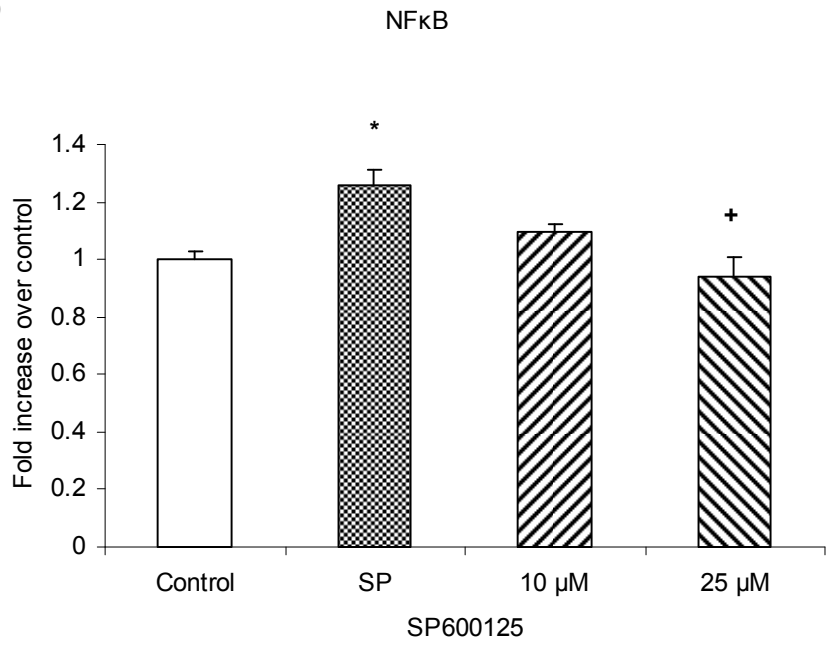


Figure 3.7 (b)

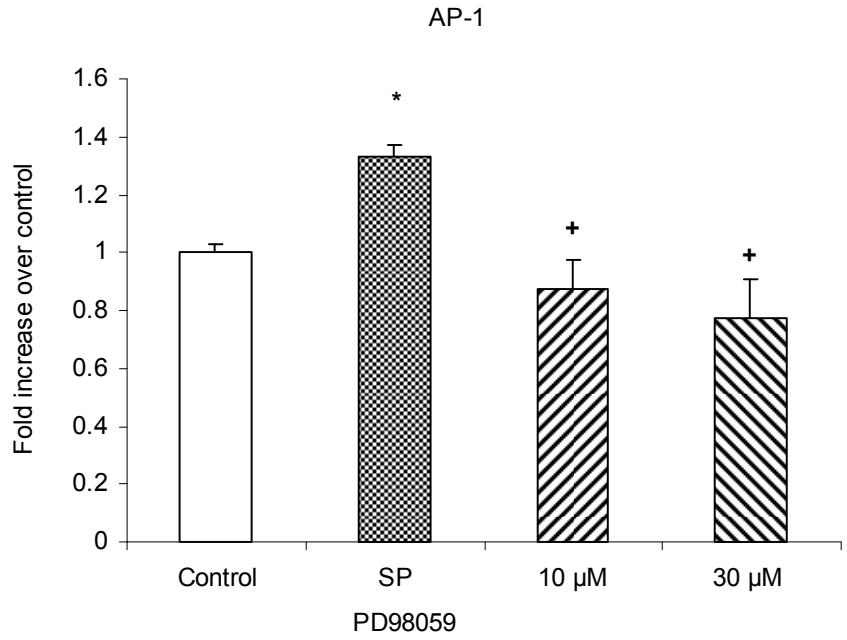


Figure 3.7 Substance P (SP)-induced ERK1/2 and JNK cross activate NFκB and AP-1. Freshly isolated pancreatic acini were obtained from three mice. The acini were then pre-incubated with either MEK1 inhibitor PD98059 or JNK inhibitor SP600125 for 1 h followed by stimulation with 1 μM SP for 45 min. The acini were separated from incubation medium by centrifugation. The pellet (acini) was used for extraction and detection of (a) NFκB (p65) activation and (b) AP-1 (c-Jun) activation. The results are representative of three independent experiments. Results shown are the means + SE. ***P* < 0.05 when compared to control, +*P* < 0.05 when compared to SP.

Figure 3.8 (a)

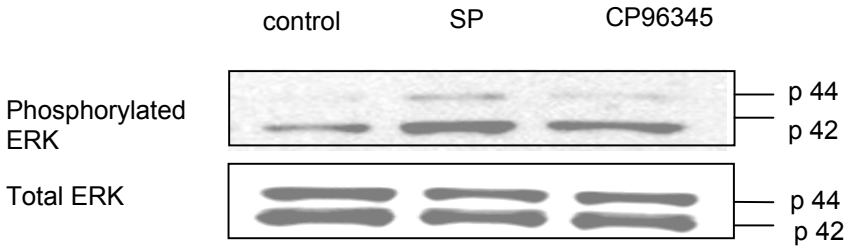
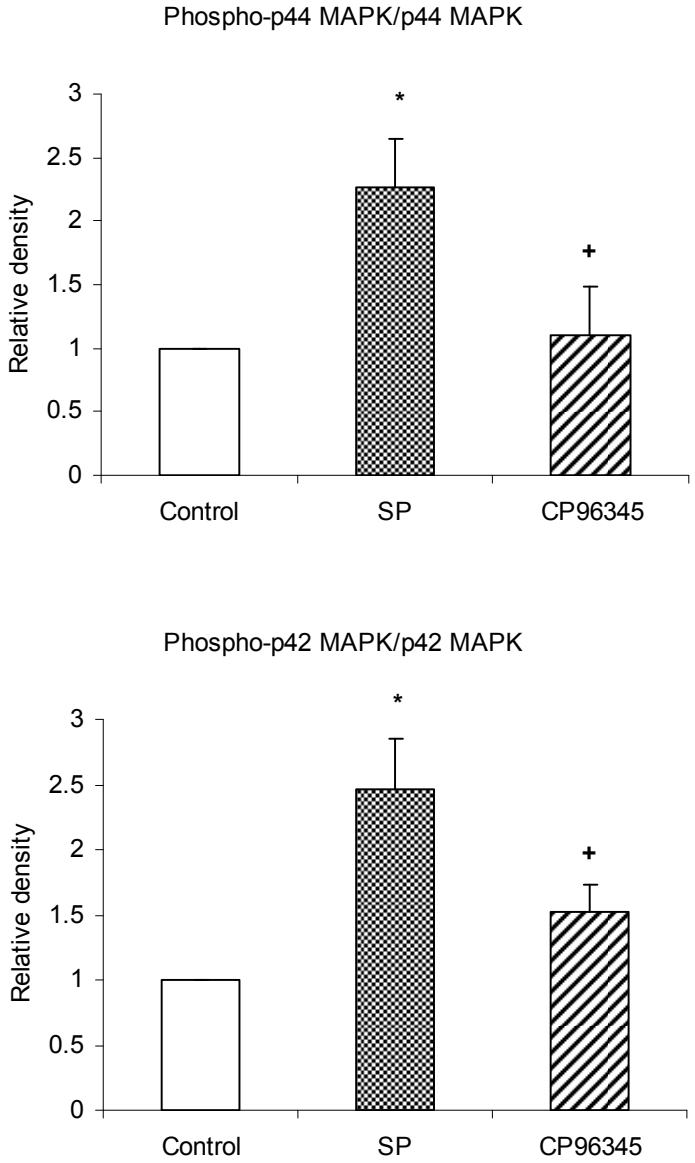


Figure 3.8 (b)



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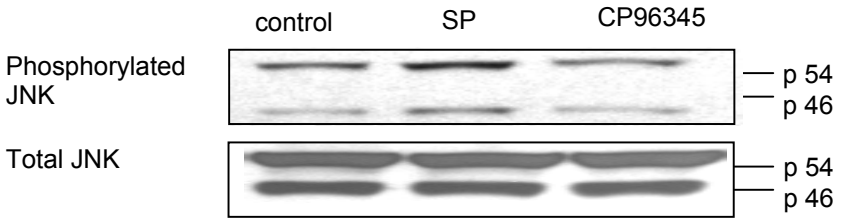
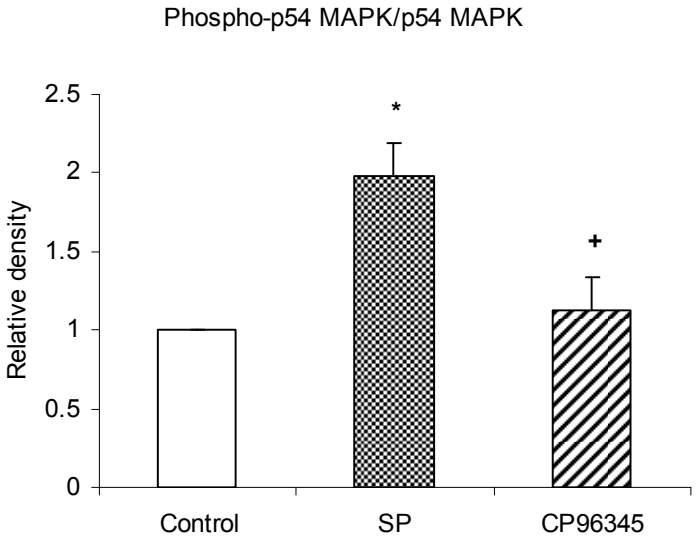


Figure 3.8 (d)



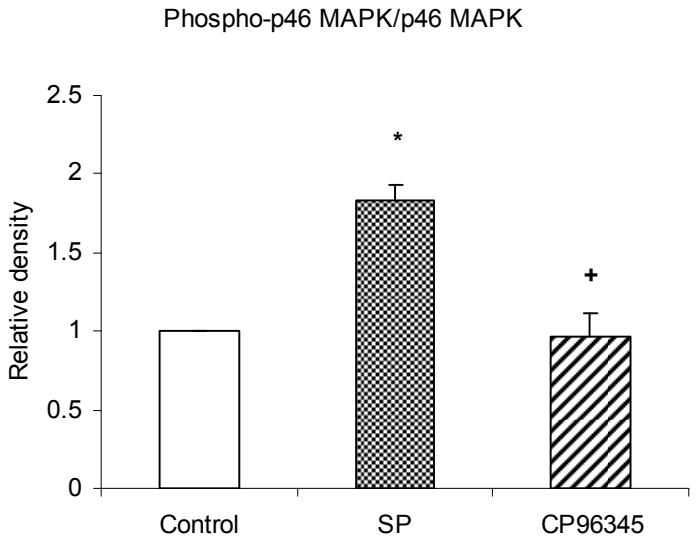


Figure 3.8 Substance P (SP)/NK1R interaction is involved in ERK1/2 and JNK activation. Freshly isolated pancreatic acini, obtained from three mice, were pre-incubated with 1 μM CP96345 for 30 min at 37˚C followed by stimulation with 1 μM substance P for 45 min at 37˚C. Cells were subsequently lysed, and cell proteins were subjected to Western blot analysis using antibodies against (a) phospho-ERK, total ERK1/2 (b) phospho-JNK, total JNK. Corresponding densitometric analysis of Western blot experiments from pancreatic acini. The results are representative of three independent experiments. MW in (kDa) for JNK 2/1 is 54/46 and ERK1/2 is 44/42. Results shown are the means + SE. $*P < 0.05$ when compared to control, $+P < 0.05$ when compared to SP.

Figure 3.9 (a)

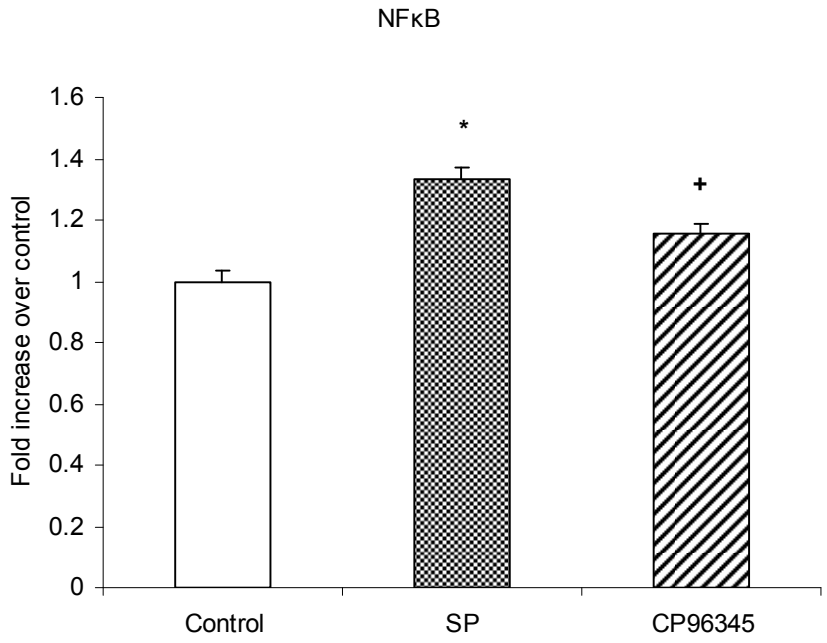
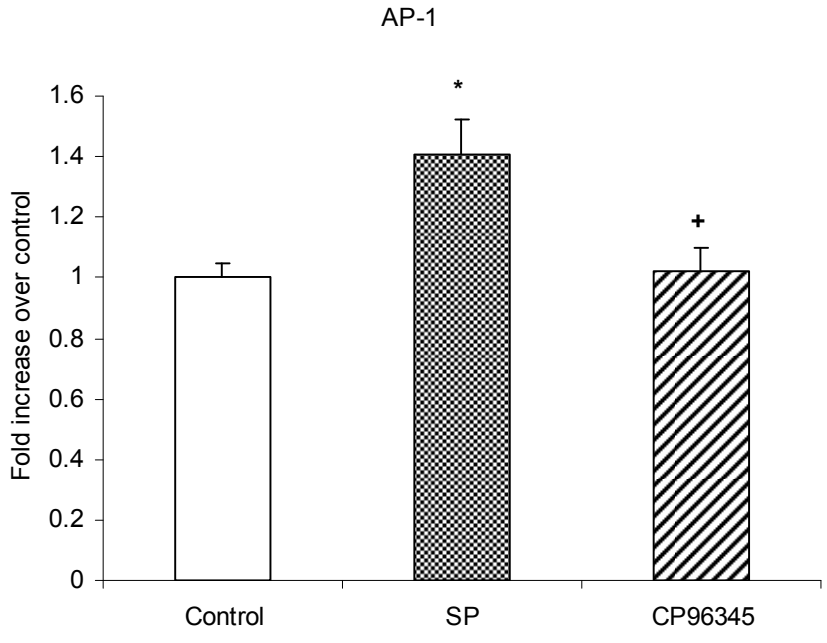


Figure 3.9 (b)



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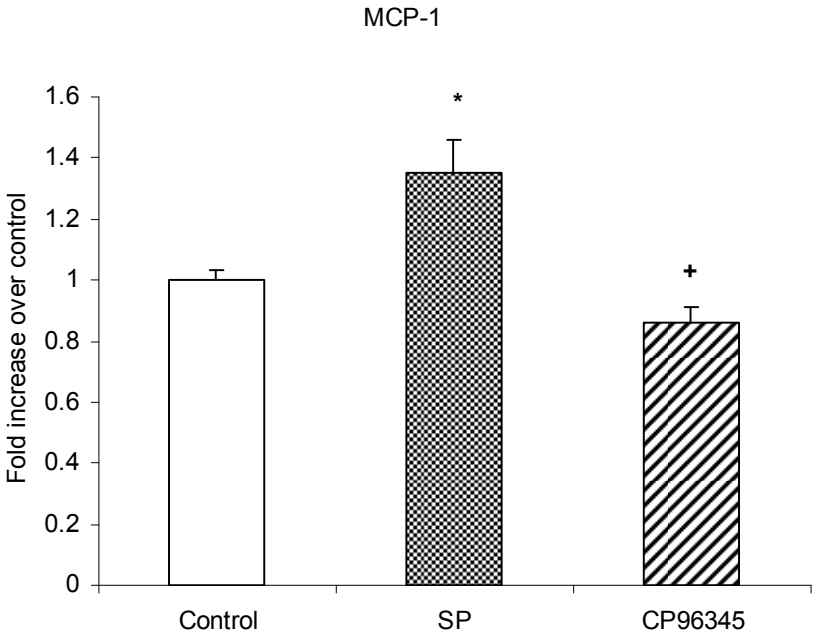

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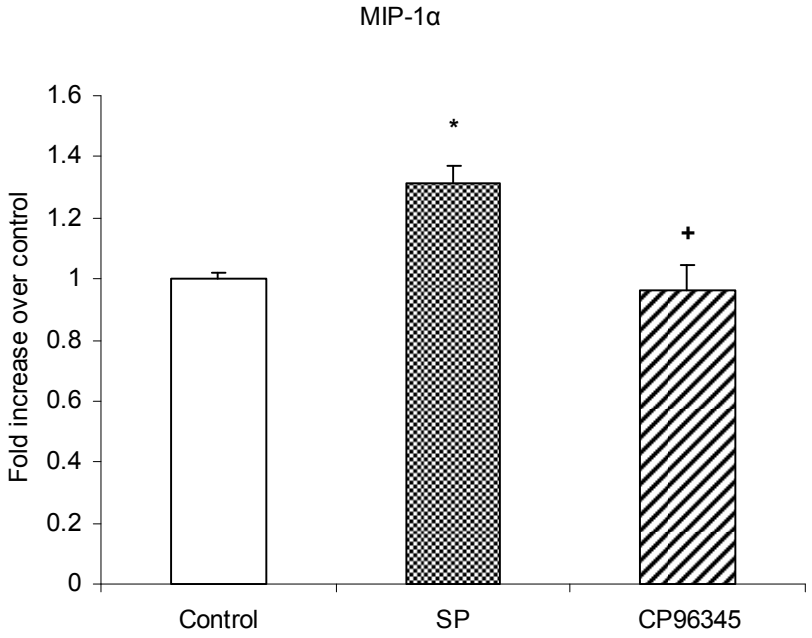

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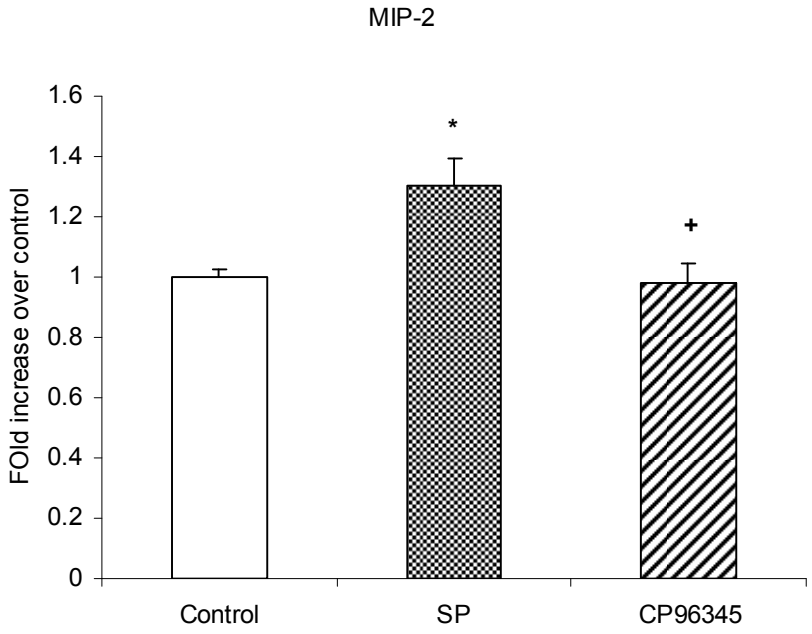


Figure 3.9 NK1R is involved in substance P (SP)-induced NFκB and AP-1 activation as well as MCP-1, MIP-1α and MIP-2 production. Freshly isolated pancreatic acini, obtained from three mice, were pre-incubated with 1 μM CP96345 for 30 min followed by stimulation with 1 μM SP for 45 min. Acini were separated from incubation medium by centrifugation. The pellet was used for (a) NFκB and (b) AP-1 extraction and NFκB (p65) and AP-1 (c-Jun) DNA-binding assays were carried out. The supernatant was used to measure (c) MCP-1, (d) MIP-1α and (e) MIP-2 levels by ELISA. The results are representative of three independent experiments. Results shown are the means + SE. ***P* < 0.05 when compared to control, +*P* < 0.05 when compared to SP.

CHAPTER 4

ROLE OF PROTEIN KINASE C δ ON SUBSTANCE P-INDUCED CHEMOKINE SYNTHESIS IN MOUSE PANCREATIC ACINAR CELLS

4.1 INTRODUCTION

The critical initiating events in acute pancreatitis occur in the pancreatic acinar cells. Some of the key early acinar cell events include activation of zymogens, decrease apical secretion of digestive zymogens, edema, release of inflammatory mediators and cell death. Activation of selective signaling molecules in pancreatic acinar cells mediates and contributes to the development of acute pancreatitis.

The protein kinase C (PKC) family plays a prominent role in critical stages of acute pancreatitis. It mediates physiological as well as pathophysiological responses in the pancreatic acinar cells. PKC α, δ, ε, and ζ are present in rodent acinar cells (Gorelick, Pandol *et al.* 2008). The role of PKC isoforms has been determined by using cellular and *in vivo* experimental models of acute pancreatitis. These models often use supramaximal concentrations of the hormone CCK or its analog caerulein to induce acute pancreatitis (Satoh, Gukovskaya *et al.* 2006; Tapia JA, Garcia-Marin *et al.* 2003). Studies have shown that CCK at a supraphysiological concentration of 100 nM resulted in activation of PKC δ, ε, and ζ whereas CCK at a physiological concentration of 100 pM induced activation of only PKCδ in acinar cells (Satoh, Gukovskaya *et al.* 2004). CCK-induced PKC activation

has been shown to stimulate NFκB activation, an upstream mediator of inflammatory responses (Gukovskaya, Hosseini *et al.* 2004).

It has been earlier demonstrated that PKCδ modulates inflammatory responses, as evidenced by its capability to induce matrix metalloproteinase and chemokine expression *in vitro* (Jedrzkiewicz, Nakamura *et al.* 2000; Liu, Crepin *et al.* 2002). Various studies have reported that PKCδ regulates the expression of inflammatory mediators (Cosen-Binker, Lam *et al.* 2007; Satoh, Gukovskaya *et al.* 2006; Tapia JA, Garcia-Marin *et al.* 2003). PKC is one of the downstream effectors of the NK1 activation. Studies have shown that upon stimulation of the NK1 receptor by substance P, PKC in its active form translocates from the cytoplasm to the plasma membrane (Monastyrskaya, Hostettler *et al.* 2005).

A limited number of studies have examined substance P-induced activation of PKCδ signaling pathways. It has been reported that substance P-stimulated IL-8 expression in human colonic epithelial cells involves PKCδ activation and Rho family small GTPases (Koon, Zhao *et al.* 2005; Zhao, Kuhnt-Moore *et al.* 2002). Moreover, activation of NK1R by substance P or GR73632 (a potent NK1R agonist) triggered activation of PKC and MAP Kinases in cultured adult rat dorsal root ganglion neurons (Tang, Li *et al.* 2007). However, little is known of the ability of substance P/NK1R interaction to activate PKCδ and its effect on the pro-inflammatory mediator chemokine in pancreatic acinar cells. In this study, I sought to investigate whether substance P activates PKCδ in pancreatic acinar cells and also to determine the underlying signaling mechanism in the involvement of substance P/NK1R-PKCδ in chemokine production in pancreatic acinar cells.

4.2 MATERIALS AND METHODS

4.2.1 Animal Ethics

Please refer to section 2.2.1.

4.2.2 Preparation of mouse pancreatic acini

Mouse pancreatic acinar cells were prepared, as previously described in section 2.2.2.

4.2.3 Viability of mouse pancreatic acinar cells

The viability of mouse pancreatic acinar cells was assessed, as previously described in section 2.2.3.

4.2.4 Acinar experimental protocol

Pancreatic acini were treated with substance P (Sigma-Aldrich) at a concentration of 1 μM for 0, 3, 5, 10, 15, 30, 45, 60 and 120 min at 37˚C. After which the cells were lysed to detect for PKCδ or MEKK1 activation by Western blot analysis. In some experiments, cells were also pre-treated with the selective PKCδ inhibitor rottlerin at 1 μM, 5 μM and 10 μM (Calbiochem) for 1 h and then stimulated with 1 μM substance P or vehicle (saline) for 10 or 45 min at 37°C. A specific PKCδ translocation inhibitor (δV1–1: S-F-N-S-Y-E-L-G-S-L) (Chen, Hahn *et al.* 2001) was synthesized (SIGMA Genosys). The peptide was conjugated to a *Drosophila antennapedia* peptide (R-Q-I-K-I-W-F-Q-N-R-R-M-K-W-K-K) to make it cell permeable. This peptide corresponds to specific sequences in the V1 regions that are responsible for anchoring PKCδ to its translocation site. Thus the peptide competitively inhibits the binding of PKCδ to its anchoring protein. Hence, this prevents activation of this particular PKC isoform (Satoh, Gukovskaya *et al.* 2004; Satoh, Gukovskaya *et al.* 2006). Pancreatic acinar cells were pre-treated with 5 μM

and 10 μM of the specific PKCδ translocation inhibitor for 3 h followed by stimulation with 1 μM substance P or vehicle for 10 or 45 min at 37°C.

In other experiments, cells were pre-incubated with the selective NK1R antagonist, CP96345, at 1 μM (Pfizer Diagnostics) for 30 min followed by treatment with 1 μM substance P or vehicle for 10 min at 37°C. Subsequently, the supernatant was used for chemokine detection and the pellet was used for either nuclear extract, to detect NFκB (p65) and AP-1(c-Jun) activation, or cell lysis for Western blot analysis to detect PKCδ, MEKK1, ERK and JNK.

4.2.5 Immunofluorescence

After treatment with substance P, pancreatic acinar cells were fixed in 3.7% formaldehyde and placed on microscope slides using CytoFuge 2 cytocentrifuge (StatSpin, Westwood, MA, USA). Then, the fixed cells were blocked for non-specific binding with 1% BSA for 30 min at room temperature. Fluorescent labeling was performed by incubating the cells with a 1:50 dilution of PKCδ mAb [p-PKCδ Tyr 523 (Konishi, Tanaka *et al.* 1997), Santa Cruz Biotechnology] for 90 min at room temperature, followed by secondary detection for 40 min in the dark with a 1:200 dilution of FITC-conjugated, donkey anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Staining was observed with a fluorescence microscope (Carl Zeiss, Oberkochen, Germany) and captured by a digital camera (Carl Zeiss).

4.2.6 Preparation of total cell lysates for Western blot analysis

After treatment, pancreatic acinar cells were homogenized on ice in RIPA buffer supplemented with 1 mM PMSF and the protease inhibitor cocktail containing pepstatin, leupeptin, chymostatin, antipain and aprotinin (5 μg/ml of each), and centrifuged at 4 °C

for 15 min at 14,000 *g*. The supernatants were collected and stored at -80°C until use. Protein concentrations were determined by using Bio-Rad protein assay. 5 µl of sample was added to 250 µl of Bradford reagent (Bio-Rad Laboratories, Hercules, CA) and read at 595 nm after a 5 min incubation at room temperature.

4.2.7 Western blot analysis

Cell lysates (50 µg of protein) were separated on 12% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes. Non-specific binding was blocked by 1 h incubation of the membranes in 5% nonfat dry milk in PBST (0.05% Tween 20 in PBS). The blots were then incubated overnight with the primary antibodies phospho-PKCδ Thr505 (Koon, Zhao *et al.* 2005; Parekh, Ziegler *et al.* 1999; Tan, Xu *et al.* 2003**)**, phospho-ERK1/2, phospho-SAPK/JNK (Cell Signaling Technology), MEKK1 and HPRT were purchased from Santa Cruz Biotechnology. HPRT was used as the housekeeping protein. The above antibodies were used at a 1:1000 dilutions in the buffer containing 2.5% nonfat dry milk in PBST. After which they were washed four times with PBST, and finally incubated for 1 h with goat anti-rabbit HRP-conjugated secondary antibody (Santa Cruz Biotechnology) at 1:2000 dilutions in the buffer containing 2.5% nonfat dry milk in PBST. The blots were developed for visualization using enhanced chemiluminescence (ECL) detection kit (Pierce, Rockford, IL).

4.2.8 Nuclear cell extract preparation

Nuclear cell extract was prepared, as previously described in section 2.2.6.

4.2.9 NFκB DNA-binding activity

The binding of NFκB to DNA was measured in nuclear extracts, as previously described in section 2.2.7.

4.2.10 AP-1 DNA-binding activity

The binding of AP-1 to DNA was measured in nuclear extracts, as previously described in section 3.2.9.

4.2.11 Total RNA isolation

Total RNA was extracted from pancreatic acinar cells by using TRIzol reagent (Invitrogen) following the manufacturer's instructions with some modifications. Briefly, isolated cells were homogenized in TRIzol reagent. Chloroform was then added to the homogenates, and samples were shaken, incubated for 5 min at 4°C, and centrifuged for 15 min at 12,000 g at 4°C. The aqueous phase was separated and RNA was precipitated by addition of isopropyl alcohol. After RNA was pelleted by centrifugation (12,000 *g* for 10 min at 4°C), the pellet was washed twice in 70% ethanol, air-dried, and dissolved in RNase-free water. RNA was quantitated spectrophotometrically by absorbance at 260 nm. The purity of RNA was assessed by a 260/280 ratio between 1.6 and 2.0. The integrity of RNA was verified by the presence of distinct 28S and 18S rRNA bands on a denaturing agarose gel.

4.2.12 Semiquantitative RT-PCR

Semiquantitative RT-PCR was performed to analyze mRNA expression levels of MCP-1, MIP-1α, and MIP-2 in the acini. Total RNA (1 μg) was reverse transcribed by use of the iScript cDNA synthesis kit (Bio-Rad). The cDNA synthesized was used as the template for PCR amplification by iQ Supermix (Bio-Rad). The PCR protocol consisted of optimized 36 cycles of denaturation at 95°C for 30 s, annealing for 30 s (at 55, 58 and

55°C, for MCP-1, MIP-1α, and MIP-2 respectively), and extension at 72°C for 30 s performed in MyCycler (Bio-Rad). The following specific primer pairs (Proligo, Singapore) of chemokines and were used: MCP-1 sense 5'-GGAAAAATGGATCCACACCTTGC-3'and antisense 5'-TCTCTTCCTCCACCACCA TGCAG-3' resulting in a 582-bp product; MIP-1α sense 5'-ACTGCCCTTGCTGTTCTTCTCT-3' and antisense 5'-GCATTCAGTTCCAGGTCAGTGA-3' resulting in a 261-bp product; MIP-2 sense 5'-TGCCTGAAGACCCTGCCAAGG-3'and anti sense 5'-GTTAGCCTTGCCTTTGTTCAG-3' resulting in a 189-bp product; 18S sense 5'-GTAACCCGTTGAACCCCATT-3' and antisense 5'-CCATCCAATCGGTAGTAGCG-3' resulting in a 150-bp product. All PCR products were analyzed on 1.5% wt/vol agarose gels containing 0.05 mg/100 ml ethidium bromide and image captured by use of Gel Doc-It Imaging System (UVP). Product sizes were identified by comparison with DNA size standards included in the gels. Densitometry results from PCR products were normalized to 18S internal controls.

4.2.13 Chemokine detection

Pancreatic acinar cell supernatants were assayed for MCP-1, MIP-1α and MIP-2 using a sandwich ELISA, as previously described in section 2.2.5.

4.2.14 Statistical analysis

Results are presented as means + SE with 6 replicates for each condition. Each experiment was repeated at least three times. The significance of changes was evaluated by using ANOVA and Tukey's method was used as a post hoc test for the difference between groups. A P value < 0.05 was taken as the level of significance.

4.3 RESULTS

4.3.1 Substance P induces phosphorylation of PKCδ in a time dependent manner.

In order to investigate if substance P causes phosphorylation of PKCδ, pancreatic acinar cells were stimulated with 1 μM substance P for 0, 3, 5, 10, 15, 30 and 45 min. Cells were then lysed, and cell proteins were subjected to Western blot analysis using antibodies against phospho-PKCδ and HPRT. As shown in Figure 4.1 (a, b), substance P induced phosphorylation of PKCδ in pancreatic acini which was evident from 3 min and increased in a time-dependent manner up to 10 min, followed by a time-dependent decrease until 45 min. Densitometric analysis of Western blot experiments revealed a significant increase in phosphorylation of PKCδ at all the above mentioned time points when compared to 0 min control.

The specific effect of substance P on PKCδ activation in pancreatic acinar cells was further confirmed by immunostaining. As shown in Figure 4.1 (c), in unstimulated pancreatic acini, the staining of PKCδ was present in the cytosolic area of the pancreatic acinar cell. Treatment of the cells with 1 μM substance P decreases the presence of PKCδ in the cytosol and increases its presence near the membrane, indicating translocation of PKCδ from the cytosol to the cell membranes as a measure of PKCδ activation.

4.3.2 Substance P stimulates activation of MEKK1 in a time dependent manner.

To find out if MEKK1 is activated upon substance P stimulation, pancreatic acinar cells were stimulated with 1 μM substance P for 0, 3, 5, 10, 30, 45, 60 and 120 min. Cells were then lysed, and cell proteins were subjected to Western blot analysis using antibodies against MEKK1 and HPRT. As shown in Figure 4.2 (a), substance P induced activation of MEKK1 in pancreatic acini which was evident from 3 min and increased in a time-

dependent manner up to 45 min, followed by a time-dependent decrease until 120 min. Densitometric analysis of Western blot experiments, Figure 4.2 (b), revealed a significant increase in MEKK1 at 10, 30 and 45 min when compared to 0 min control.

4.3.3 Substance P-induced PKCδ is involved in activation of MEKK1, ERK and JNK.

To determine if PKCδ is involved in the activation of MEKK1, ERK and JNK, pancreatic acinar cells were pre-treated with either rottlerin, a selective PKCδ inhibitor, ranging from 1 to 10 μM or with 5 μM and 10 μM of the specific PKCδ translocation inhibitor peptide (δV1–1) followed by stimulation with 1 μM of substance P. Cells were then lysed, and cell proteins were subjected to Western blot analysis. Both rottlerin and δV1–1 concentration-dependently decreased phosphorylation of PKCδ, Figure 4.3, hence confirming their inhibitory effect on substance P-induced activation of PKCδ. Rottlerin as well as δV1–1 attenuated substance P-induced activation of MEKK1 and phosphorylation of ERK and JNK in a concentration-dependent manner. The data presented in Figure 4.3 (e) is qualitative.

4.3.4 PKCδ is involved in substance P-induced NFκB and AP-1 activation.

To determine the role of PKCδ in substance P-induced NFκB and AP-1 activation, pancreatic acinar cells were pre-incubated with either rottlerin, ranging from 1 to 10 μM or with 5 μM and 10 μM of the specific PKCδ translocation inhibitor peptide (δV1–1) followed by stimulation with 1 μM of substance P. After which nuclear fractions from the cells were extracted. NFκB and AP-1 DNA-binding assay revealed that treatment with substance P led to a notable increase in the activity of NFκB p65 and AP-1 c-jun, indicating that NFκB and AP-1 play an important role in substance P triggered signaling

pathway in acinar cells as shown in chapter 3. As shown in Figure 4.4 (a, b), pre-treatment with either rottlerin or δV1–1 attenuated substance P-induced DNA-binding activity of NFκB p65 and AP-1 c-jun in a concentration-dependent manner.

4.3.5 Effect of PKCδ inhibitors on the gene expression and secretion of several pro-inflammatory chemokines in pancreatic acinar cells.

I have also determined the role of PKCδ on substance P-induced chemokine production both at the mRNA and protein levels. Treatment of pancreatic acini with 1 μM substance P resulted in enhanced expression of pro-inflammatory chemokines MCP-1, MIP-1α, and MIP-2. Pancreatic acinar cells were pre-treated with either rottlerin, ranging from 1 to 10 μM, or with 5 μM and 10 μM of the specific PKCδ translocation inhibitor peptide (δV1–1) followed by stimulation with 1 μM of substance P. Total RNA from cells was extracted, and RT-PCR for MCP-1, MIP-1α, and MIP-2 was performed. I have also determined the protein levels of these chemokines by ELISA. The results, in Figure 4.5 and 4.6, showed that pre-treatment with rottlerin markedly decreased MCP-1, MIP-1α, and MIP-2 mRNA levels as well as their protein levels in a concentration-dependent manner. Similarly, pre-treatment with δV1–1 significantly attenuated substance P-induced MCP-1, MIP-1α, and MIP-2 production in pancreatic acinar cells.

4.3.6 Substance P/NK1R interaction is involved in PKCδ and MEKK1 activation.

To show that substance P-induced PKCδ and MEKK1 activation was indeed mediated by substance P, I pre-treated the pancreatic acini with 1 μM of CP96345, a selective NK1R antagonist, followed by stimulation with 1 μM of substance P. Cells were then lysed, and cell proteins were subjected to Western blot analysis. The results, in Figure 4.7 (a, b), demonstrated that CP96345 significantly reduced substance P-induced PKCδ activation

in pancreatic acinar cells when compared to substance P only treated cells. Moreover, substance P-induced MEKK1 activation was significantly downregulated in the presence of CP96345 when compared to substance P only treated cells.

4.4 DISCUSSION

Although the mechanism of inflammation in acute pancreatitis is still not fully understood, a substantial body of evidence suggests that inflammatory mediators such as substance P and chemokines (MCP-1, MIP-1α and MIP-2) play a critical role in the pathogenesis of acute pancreatitis (Bhatia, Brady *et al.* 2000; Bhatia, Brady *et al.* 2002; Bhatia, Ramnath *et al.* 2005; Bhatia, Saluja *et al.* 1998; Bhatia, Slavin *et al.* 2003). In chapters 2 and 3, I have demonstrated that substance P stimulated the production of chemokines MCP-1, MIP-1α and MIP-2 in pancreatic acinar cells via activation of NFκB, AP-1 and MAP Kinases. However, the signaling mechanisms by which the interaction between substance P and the G protein-coupled receptor, NK1R, mediates chemokine production remain unclear. Previous studies have demonstrated that substance P induced the phosphorylation of PKCδ in rat parotid acinar cells. Moreover, it has been reported that PKCδ plays an important role in substance P-induced pro-inflammatory signaling in human colonocytes (Koon, Zhao *et al.* 2005; Soltoff and Toker 1995); however to my knowledge no studies have explored the involvement of PKCδ in substance P/NK1R-induced chemokine production in pancreatic acinar cells.

In this chapter, I treated isolated pancreatic acinar cells, which is known to express NK1R (Jensen, Jones *et al.* 1984; Patto, Vinayek *et al.* 1992; Sjodin and Gylfe 1992), to the neuropeptide substance P and determine the role of PKCδ in substance P-triggered signaling pathway. I report here that substance P induced a rapid increase in phosphorylation of PKCδ. Substance P also caused the translocation of PKCδ from the cytosol to the cell membranes, as a measure of PKCδ activation which was confirmed by the immunofluorescence visualization. I determined the role of PKCδ in substance P-

induced MCP-1, MIP-1α, and MIP-2 production by demonstrating that rottlerin, a selective PKCδ inhibitor, decreased the gene expression and secretion of these pro-inflammatory chemokines in pancreatic acinar cells. To further confirm the critical role of PKCδ in chemokine production, I have shown that pre-treatment of the cells with the specific PKCδ translocation inhibitor peptide attenuated substance P-induced chemokine production. Satoh et al. has previously shown that PKCδ translocation inhibitor peptide has high potency and specificity in pancreatic acini (Satoh, Gukovskaya *et al.* 2004; Satoh, Gukovskaya *et al.* 2006).

It is generally known that NFκB plays a key role in the regulation of inflammation because of its ability to control the expression of numerous inflammatory mediators. However, despite its crucial role in inflammation, it is unlikely that the mere activation of NFκB is sufficient to trigger any single target gene that is involved in the initiation of inflammatory responses (Karin 2005). Therefore, NFκB requires the support from other transcription factors, for instance AP-1. Our group, as well as others, has shown that substance P stimulates the activation of not only NFκB but AP-1 as well (Christian, Gilbert *et al.* 1994). In chapter 3, I have shown that substance P activates NFκB and AP-1 in a time dependent manner and that both transcription factors are required for substance P-induced chemokine production in pancreatic acinar cells. The study presented in this chapter demonstrates that rottlerin as well as PKCδ translocation inhibitor attenuated substance P-induced NFκB and AP-1 activation in a concentration-dependent manner, consequently leading to a concentration-dependent decrease in chemokine production in pancreatic acinar cells. Studies conducted by Satoh et al (Satoh, Gukovskaya *et al.* 2004) have shown that both PKCδ and ε are required to activate NFκB when induced by CCK

in pancreatic acinar cells. However, in my study I have found that supraphysiologic concentration of substance P significantly increased PKCδ activation and its activation alone was sufficient to significantly upregulate the activation of not only NFκB but also AP-1 in pancreatic acinar cells. Both PKCδ translocation inhibitor and rottlerin attenuated substance P-induced activation of PKCδ as well as NFκB and AP-1 activation. In chapter 3, I have shown that substance P stimulates ERK and JNK phosphorylation in a time-dependent manner and that these MAPKs mediate NFκB and AP-1 signaling pathways in mouse pancreatic acini. Similarly, various studies have implicated substance P in acute inflammation, and that it activates a number of intracellular signaling molecules such as MAPK members (Castagliuolo, Valenick *et al.* 2000; Koon, Zhao *et al.* 2004; Lallemend, Lefebvre *et al.* 2003; Luo, Sharif *et al.* 1996; Yang, Hsiao *et al.* 2002). A typical MAPK cascade is composed of MAPK (e.g ERK and JNK), the kinase that activates the MAPK by phosphorylation on serine and tyrosine residues is MAPK kinase (e.g MEK) and the kinase that activates the MAPK kinase is MAPKK kinase (e.g MEKK1) (Karin 2005). It is known that once activated, MEKK1 can activate at least three known downstream pathways, thus leading to ERK, JNK and NFκB activation (Garrington and Johnson 1999). In the current study substance P activated MEKK1 in a time dependent manner and induced the phosphorylation of ERK and JNK in pancreatic acinar cells. The activation of these kinases was significantly inhibited by PKCδ translocation inhibitor as well as rottlerin, a specific PKCδ inhibitor but not MAPKs inhibitor (Kim, Lim *et al.* 2007; Koon, Zhao *et al.* 2005; Smyth, Kerr *et al.* 2006). These results indicate that the activation of the above kinases is mediated through PKCδ. Moreover, 10 μM of PD98059 [selective MEK inhibitor (Alessi, Cuenda *et al.* 1995;

Dudley, Pang *et al.* 1995)] or 10 μM of SP600125 [selective JNK inhibitor (Bennett, Sasaki *et al.* 2001; Shin, Yan *et al.* 2002)] had no significant effect on substance P/NK1R-induced phosphorylation of PKCδ in pancreatic acinar cells, suggesting that PKCδ is upstream of MAP Kinases ERK and JNK in substance P-induced chemokine synthesis. Zhou et al (Zhou, Yang *et al.* 2006) has shown that activation of MEKK1 and NFκB were mediated by PKCβ in LPS-stimulated rat peritoneal macrophages. It is highly likely that substance P is activating conventional as well as other novel PKC isoforms besides PKCδ and thus contributing to the activation of NFκB and ultimately to chemokine production.

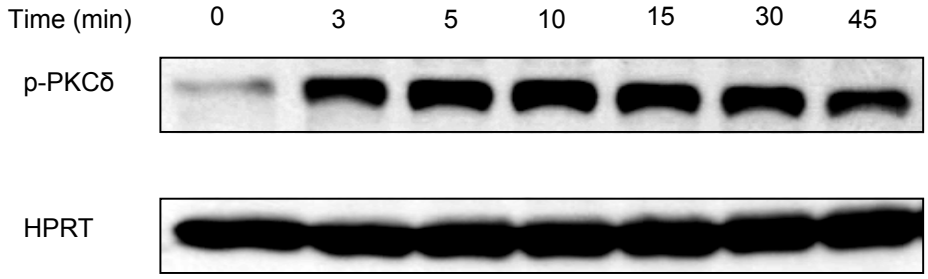
To further understand the molecular mechanism and to show that activation of PKCδ was indeed mediated by substance P, and not by some non-specific effects, I pre-treated the cells with the selective NK1R antagonist, CP96345. In the present study, CP96345 decreased substance P-induced PKCδ activation. Moreover, CP96345 attenuated substance P-induced MEKK1 activation in pancreatic acinar cells. In chapter 3, I have shown that substance P-induced activation of ERK, JNK, NFκB and AP-1 driven chemokine production were attenuated by CP96345 in pancreatic acinar cells; hence showing that substance P-induced PKCδ activation and its downstream signaling pathway are dependent on NK1R.

My findings indicate that PKCδ acts as an important pro-inflammatory signal transducer in substance P/ NK1R-induced production of pro-inflammatory mediators MCP-1, MIP-1α and MIP-2 in pancreatic acinar cells. The secretion of these pro-inflammatory mediators is mediated through the signaling cascade of PKCδ-MEKK1-(ERK & JNK)-

(NFκB & AP-1), thereby contributing to local inflammation and consequently leading to systemic inflammation in AP.

Figure 4.1

(a)



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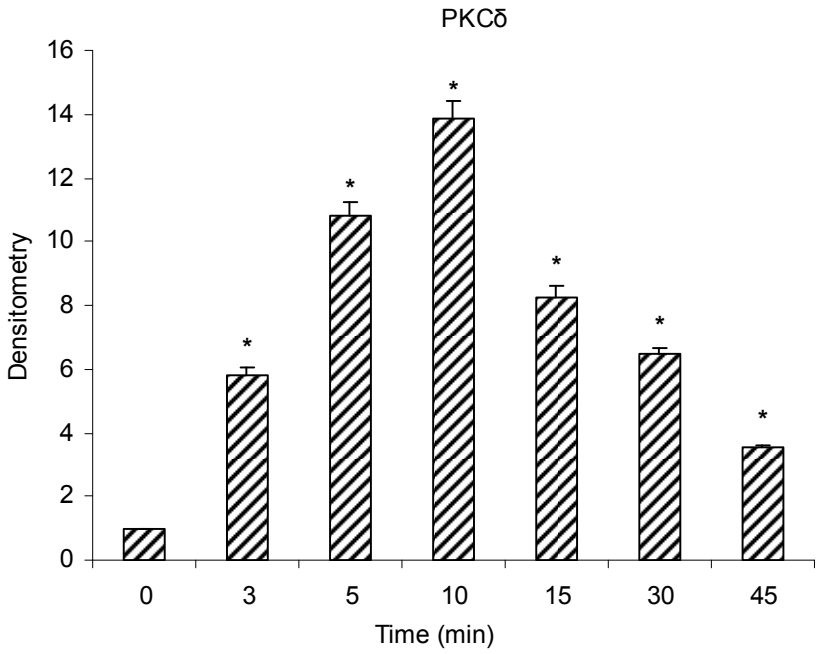
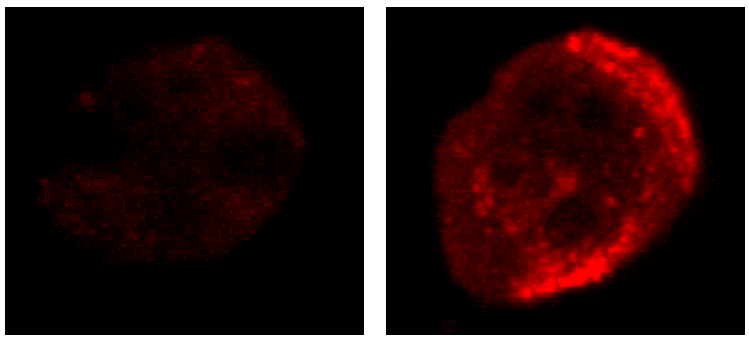


(c)



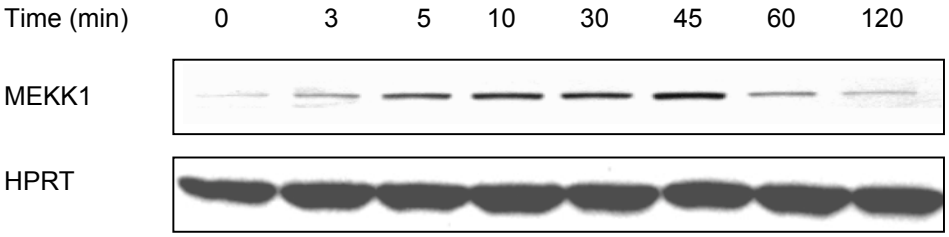
Control, 0 min

SP, 10 min

Figure 4.1 Substance P (SP) induces phosphorylation of PKCδ in a time dependent manner. Freshly isolated pancreatic acini, obtained from three mice, were incubated with 1 μM SP for 0, 3, 5, 10, 15, 30 and 45 min at 37˚C. The cells were lysed, and cell proteins were subjected to Western blot analysis using antibodies against (a) phospho-PKCδ and HPRT. (b) Densitometric analysis of Western blot experiments from pancreatic acini. (c) The expression of PKCδ on the cell surface was visualized by fluorescence microscopy. Original magnification is ×400. MW in (kDa) for PKCδ is 78, and HPRT is 24. The results are representative of three independent experiments. Results shown are the means + SE. **P* < 0.05 when compared to 0 min control.

Figure 4.2

(a)



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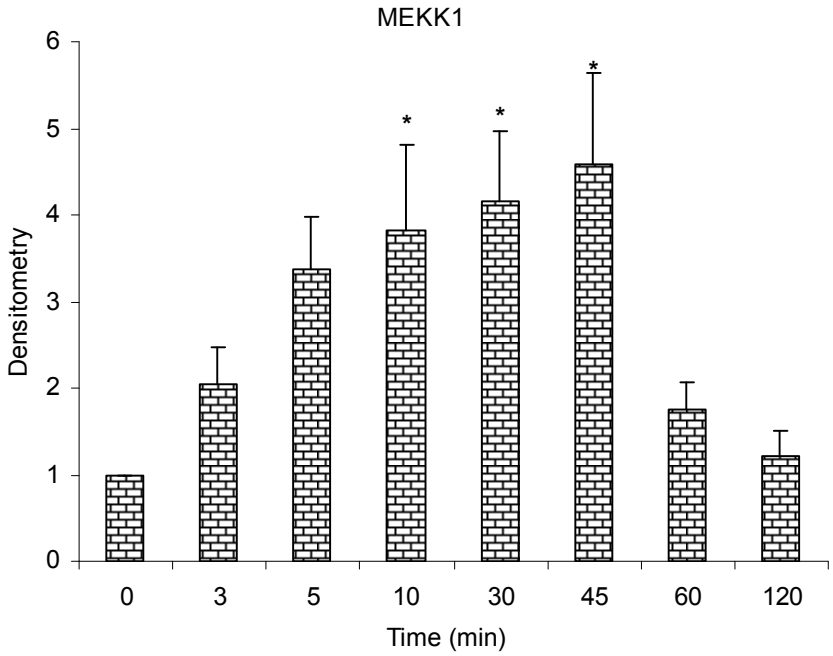


Figure 4.2 Substance P (SP) stimulates activation of MEKK1 in a time dependent manner. Freshly isolated pancreatic acini, obtained from three mice, were incubated with 1 μM SP for 0, 3, 5, 10, 30, 45, 60 and 120 min at 37˚C. The cells were lysed, and cell proteins were subjected to Western blot analysis using antibodies against (a) MEKK1 and HPRT. (b) Densitometric analysis of Western blot experiments from pancreatic acini. MW in (kDa) for MEKK1 is 190, and HPRT is 24.Results shown are the means + SE. **P* < 0.05 when compared to 0 min control.

Figure 4.3

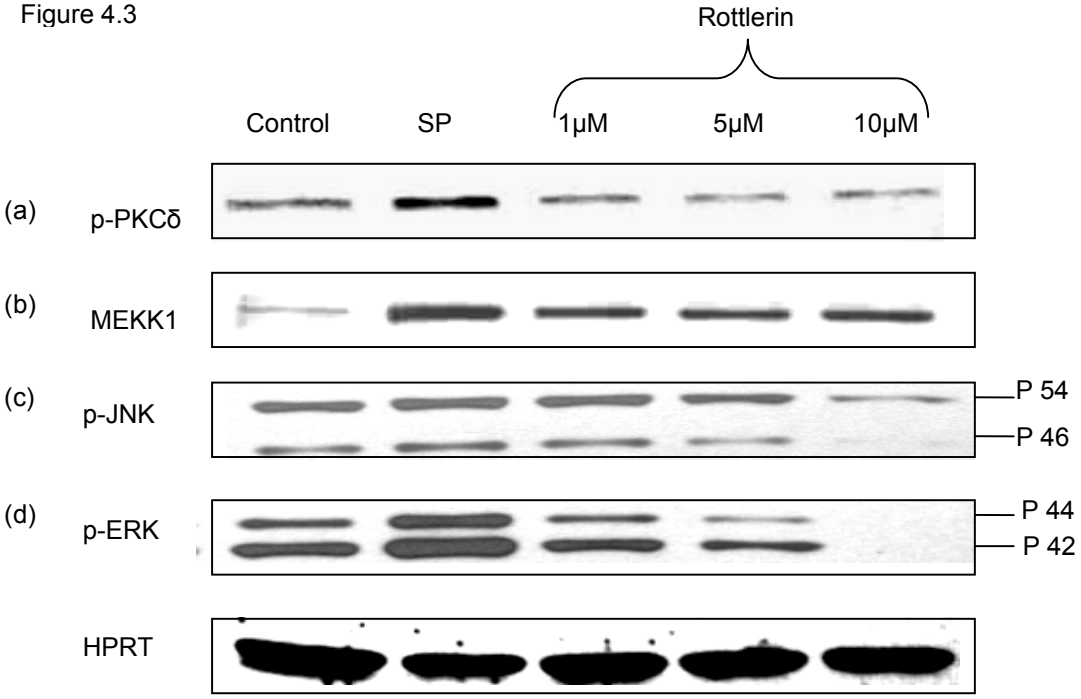
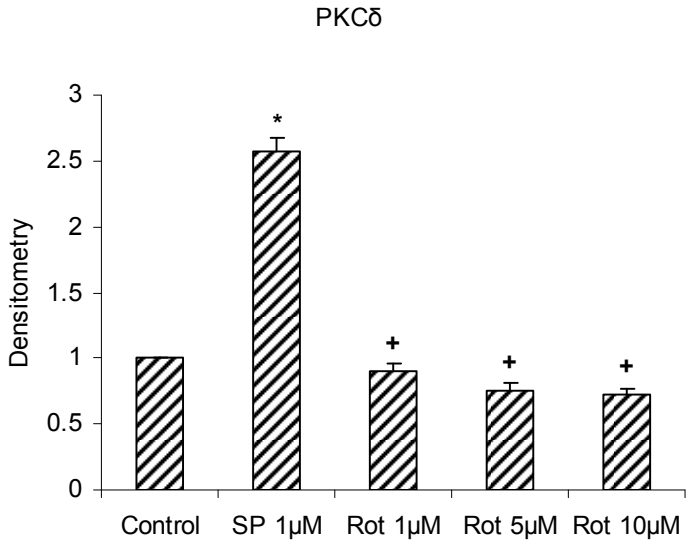


Figure 4.3 (a)



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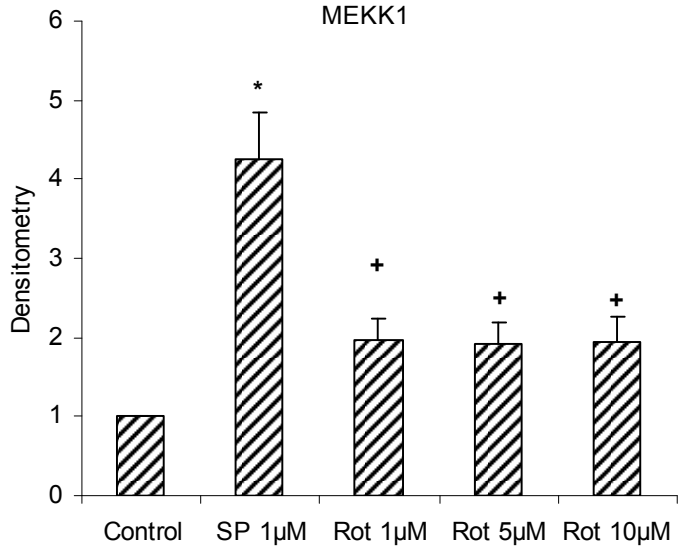
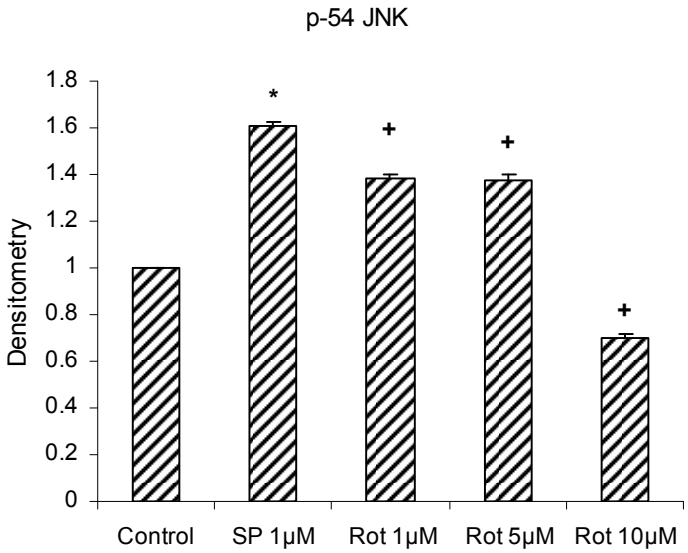


Figure 4.3 (c)



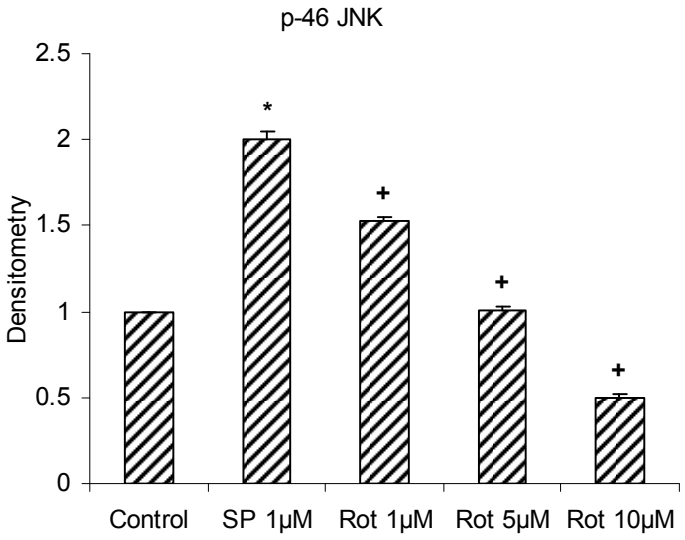
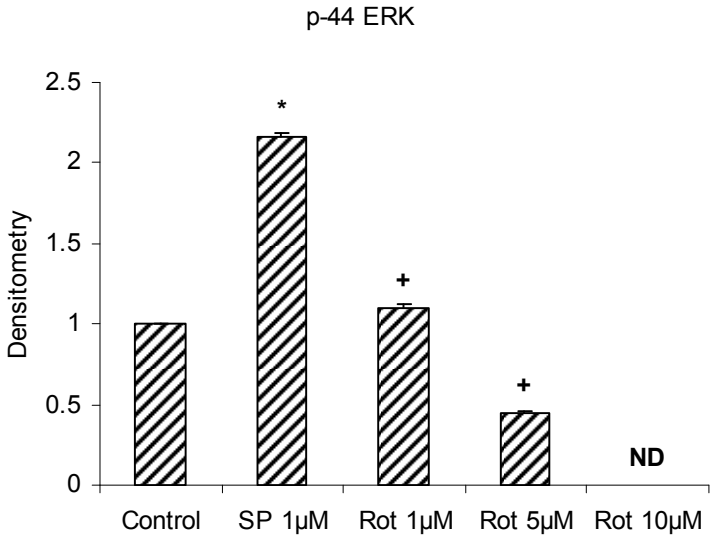

p-46 JNK
Densitometry
2.5
2
1.5
1
0.5
0
*
+
+
+
Control
SP 1μM
Rot 1μM
Rot 5μM
Rot 10μM

Figure 4.3 (d)


p-44 ERK
Densitometry
2.5
2
1.5
1
0.5
0
*
+
+
ND
Control
SP 1μM
Rot 1μM
Rot 5μM
Rot 10μM

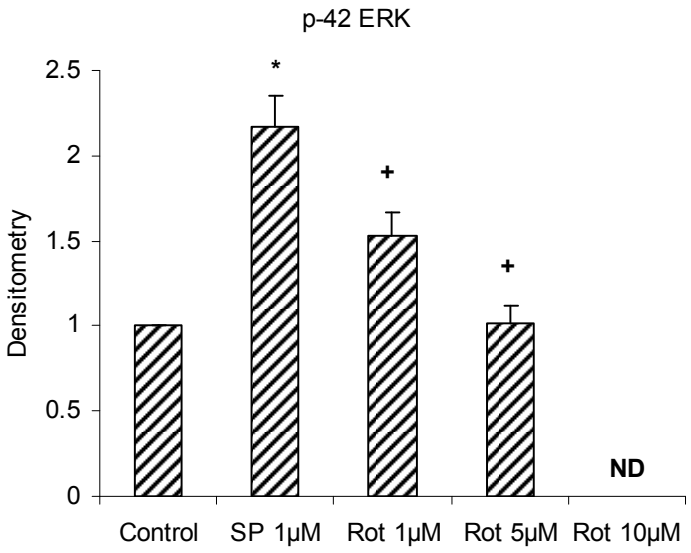

p-42 ERK
Densitometry
2.5
2
1.5
1
0.5
0
*
+
+
ND
Control
SP 1μM
Rot 1μM
Rot 5μM
Rot 10μM

Figure 4.3 (e)

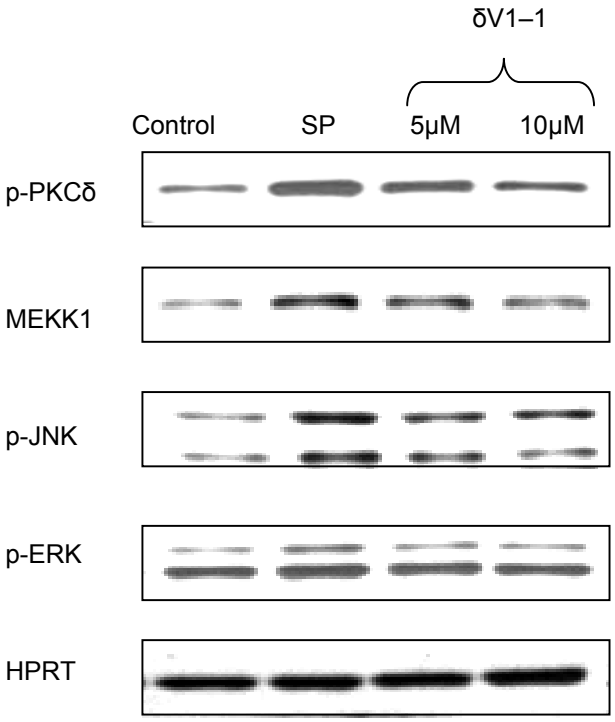
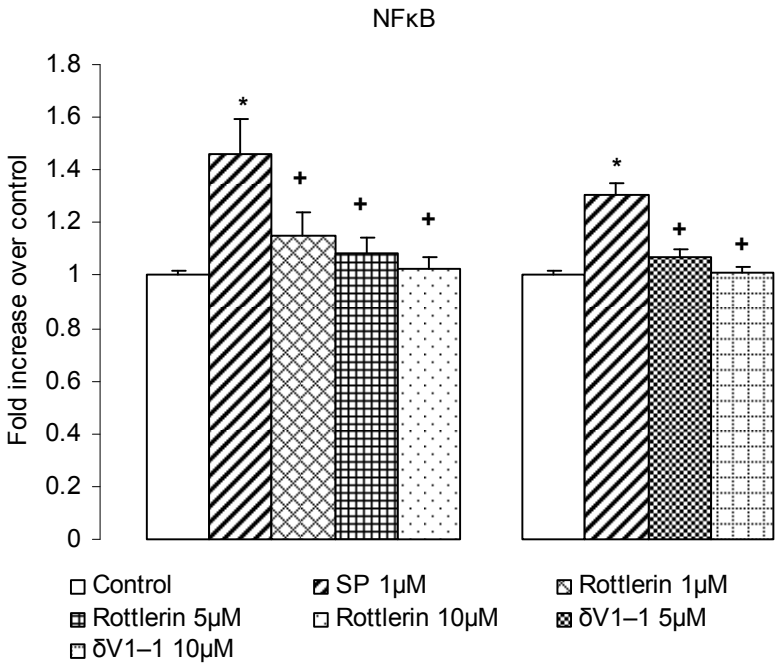

δV1–1
Control
SP
5μM
10μM
p-PKCδ
MEKK1
p-JNK
p-ERK
HPRT

Figure 4.3 Substance P (SP)-induced PKCδ is involved in activation of MEKK1, ERK and JNK. Freshly isolated pancreatic acini, obtained from three mice, were pre-incubated with either rottlerin at different doses of 1 μM, 5 μM and 10 μM for 1h at 37˚C or with 5 μM and 10 μM of the specific PKCδ translocation inhibitor peptide (δV1–1) for 3h at 37˚C followed by stimulation with 1 μM SP for 10 min at 37˚C. Cells were subsequently lysed, and cell proteins were subjected to Western blot analysis using antibodies against (a) phospho-PKCδ, (b) MEKK1, (c) phospho-JNK (d) phospho-ERK, and HPRT. The corresponding densitometric analysis of Western blot experiments from pancreatic acini is shown in (a, b, c, d). The data for 4.3 (e) is qualitative. MW in (kDa) for PKCδ is 78, MEKK1 is 190, JNK 2/1 is 54/46, ERK1/2 is 44/42 and HPRT is 24.The results are representative of three independent experiments. ND means not detected. Results shown are the means + SE. $*P < 0.05$ when compared to control, $+P < 0.05$ when compared to SP.

Figure 4.4

(a)


NFκB
Fold increase over control
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
SP 1μM
Rottlerin 1μM
Rottlerin 5μM
Rottlerin 10μM
δV1–1 5μM
δV1–1 10μM

(b)

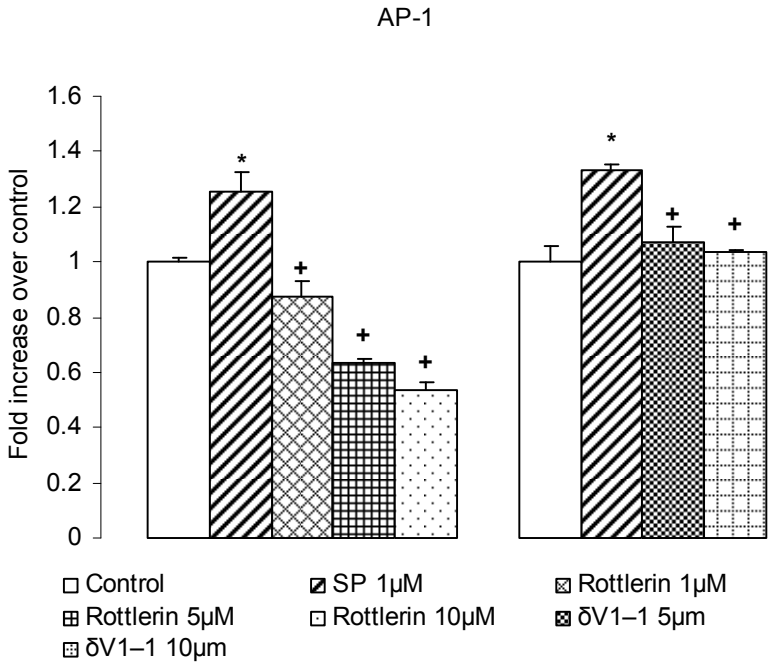
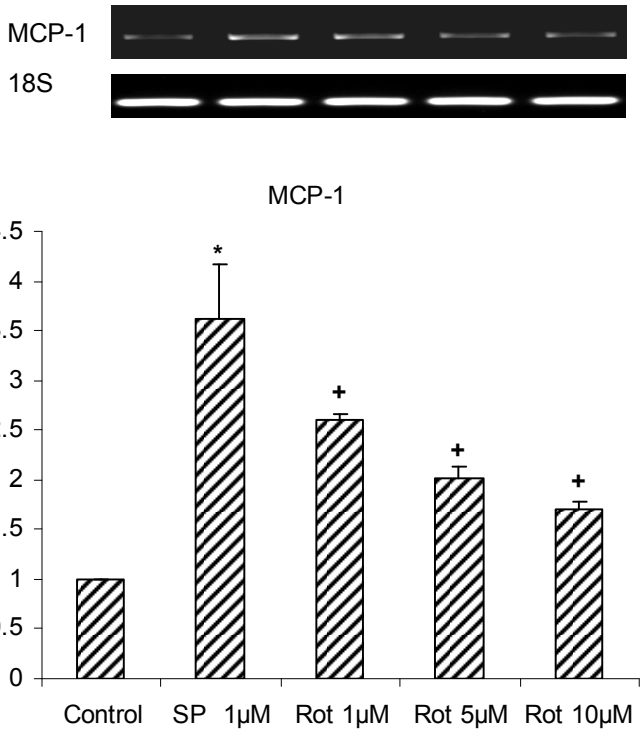

AP-1
Fold increase over control
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
SP 1μM
Rottlerin 1μM
Rottlerin 5μM
Rottlerin 10μM
δV1–1 5μm
δV1–1 10μm

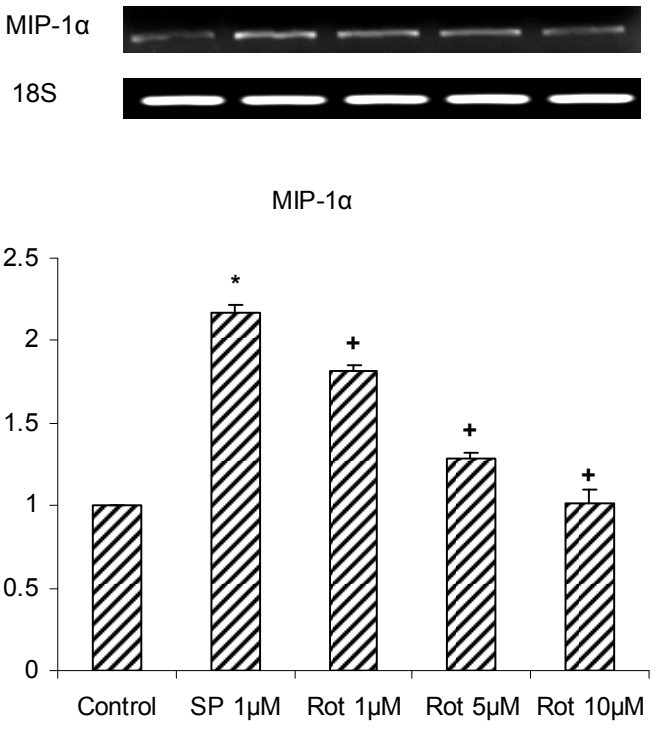
Figure 4.4 PKCδ is involved in substance P (SP)-induced NFκB and AP-1 activation. 1. Freshly isolated pancreatic acini, obtained from three mice, were pre-incubated with rottlerin at different doses of 1 μM, 5 μM and 10 μM for 1h followed by stimulation with 1 μM SP for 45 min. 2. In another experiment, pancreatic acinar cells from three mice were pre-incubated with 5 μM and 10 μM of the specific PKCδ translocation inhibitor peptide (δV1–1) for 3h followed by stimulation with 1 μM SP for 45 min. Acini were separated from incubation medium by centrifugation. The pellet (acini) was used for (a) NFκB and (b) AP-1 nuclear extraction and NFκB (p65) and AP-1 (c-jun) DNA-binding assays were carried out. The results are representative of three independent experiments. Results shown are the means + SE. $*P < 0.05$ when compared to control, $+P < 0.05$ when compared to SP.

Figure 4.5

(a)


MCP-1
18S
MCP-1
Fold increase over control
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
+
+
+
Control
SP 1μM
Rot 1μM
Rot 5μM
Rot 10μM

(b)


MIP-1α
18S
MIP-1α
Fold increase over control
2.5
2
1.5
1
0.5
0
*
+
+
+
Control
SP 1μM
Rot 1μM
Rot 5μM
Rot 10μM

(c)

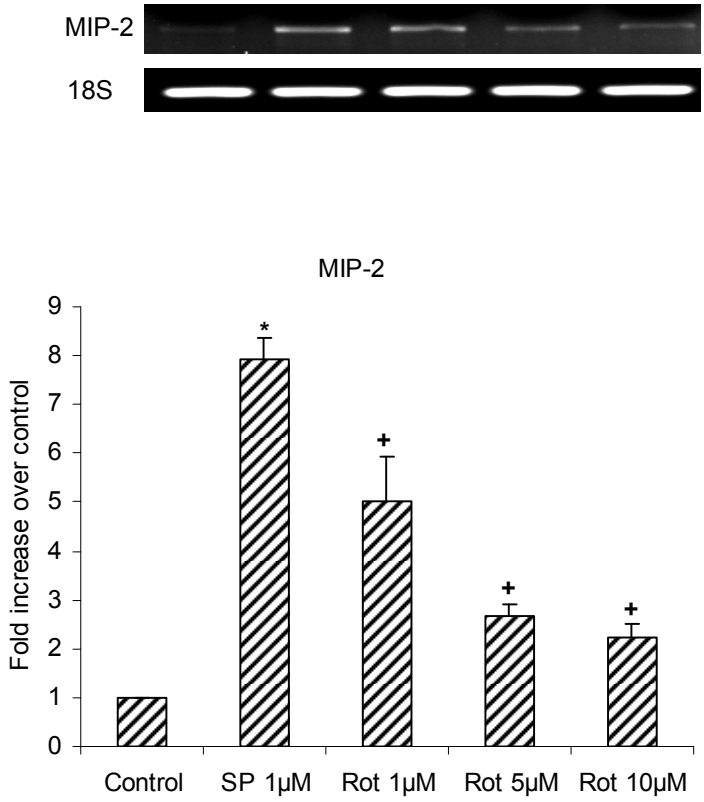
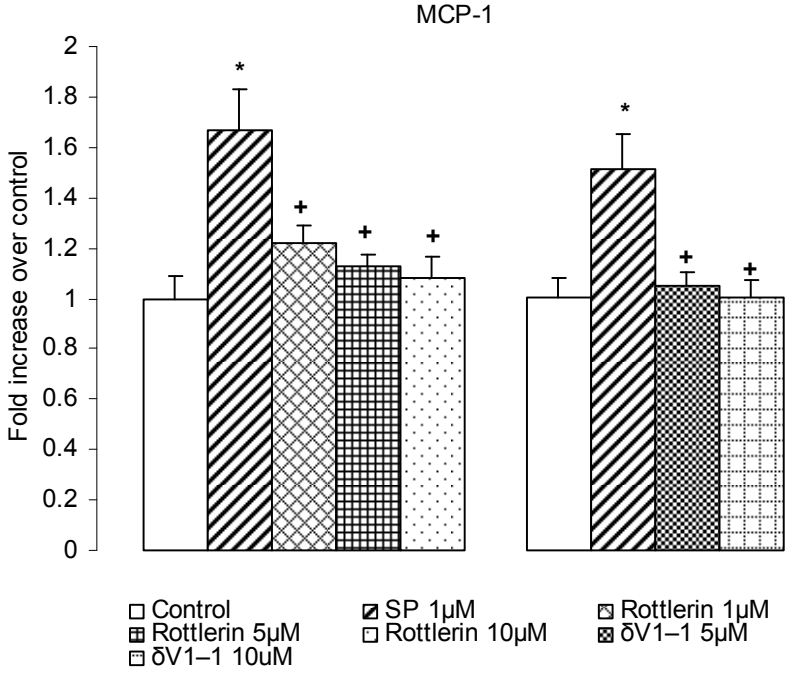


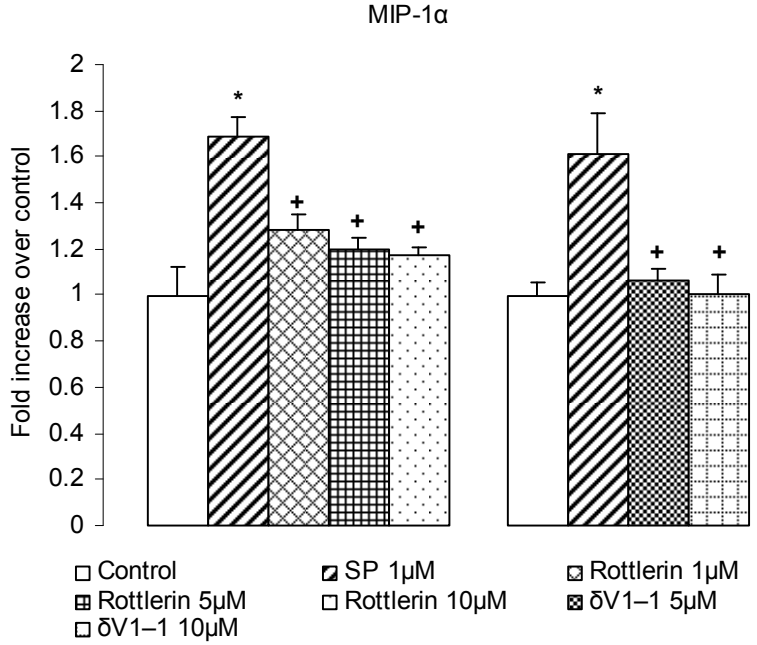
Figure 4.5 PKCδ is involved in substance P (SP)-induced MCP-1, MIP-1α and MIP-2 mRNA expression. Freshly isolated pancreatic acini, obtained from three mice, were pre-incubated with rottlerin at different doses of 1 μM, 5 μM and 10 μM for 1h followed by stimulation with 1 μM SP for 45 min. Acini were separated from incubation medium by centrifugation to carry out total RNA extraction followed by mRNA expression for (a) MCP-1, (b) MIP-1α and (c) MIP-2 by RT-PCR. 18S was used as a loading control. The results are representative of three independent experiments. Results shown are the means + SE. ***P* < 0.05 when compared to control, +*P* < 0.05 when compared to SP.

Figure 4.6

(a)


MCP-1
Fold increase over control
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
+
Control
SP 1μM
Rottlerin 1μM
Rottlerin 5μM
Rottlerin 10μM
δV1–1 5μM
δV1–1 10uM

(b)


MIP-1α
Fold increase over control
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
+
Control
SP 1μM
Rottlerin 1μM
Rottlerin 5μM
Rottlerin 10μM
δV1–1 5μM
δV1–1 10μM

(c)

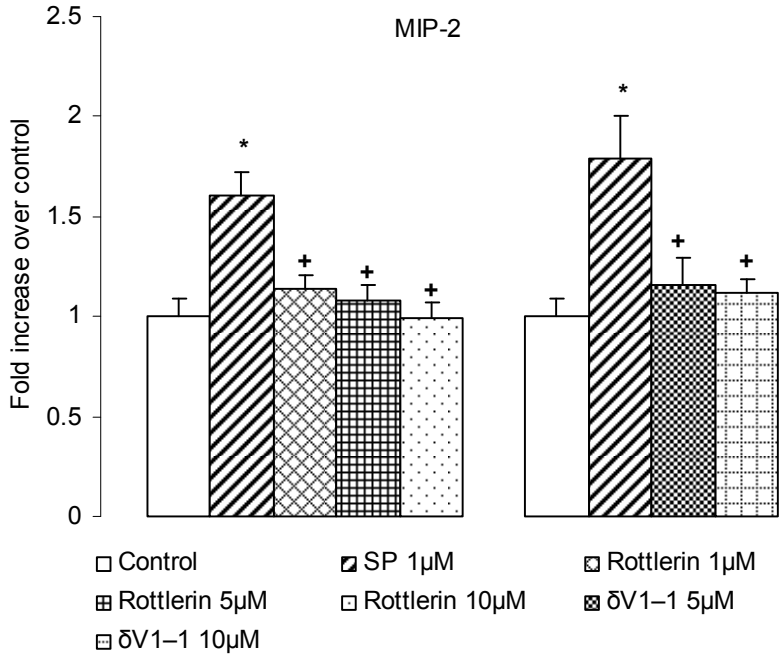
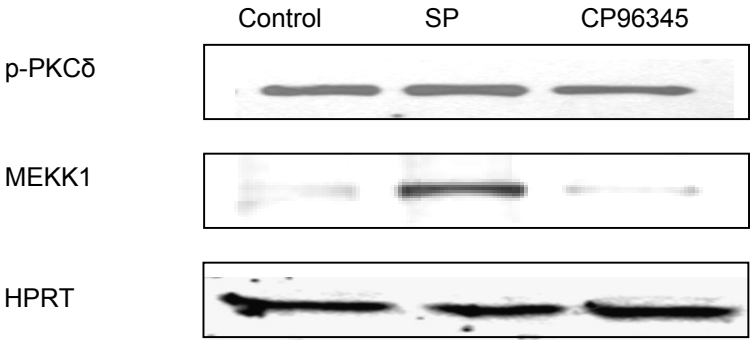


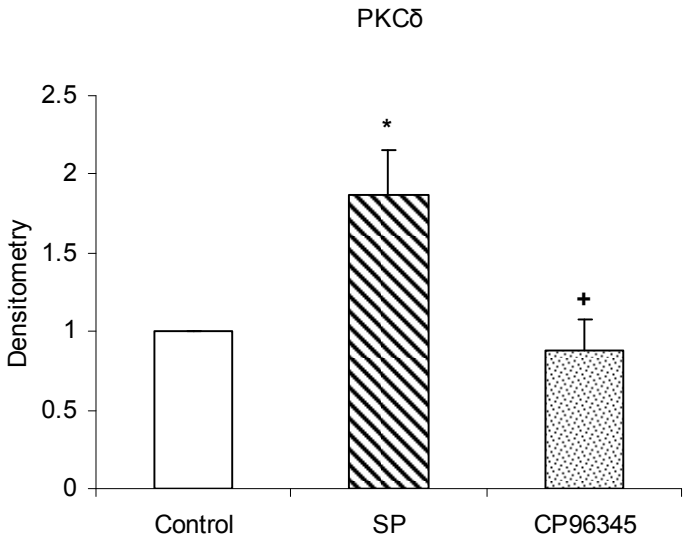
Figure 4.6 PKCδ is involved in substance P (SP)-induced MCP-1, MIP-1α and MIP-2 secretion. 1. Freshly isolated pancreatic acini, obtained from three mice, were pre-incubated with rottlerin at different doses of 1 μM, 5 μM and 10 μM for 1 h followed by stimulation with 1 μM SP for 45 min. 2. In another experiment, pancreatic acinar cells from three mice were pre-incubated with 5 μM and 10 μM of the specific PKCδ translocation inhibitor peptide (δV1–1) for 3 h followed by stimulation with 1 μM SP for 45 min. The supernatant was used to measure (a) MCP-1, (b) MIP-1α and (c) MIP-2 levels by ELISA. The results are representative of three independent experiments. Results shown are the means + SE. *****$P < 0.05$ when compared to control, **+**$P < 0.05$ when compared to SP.

Figure 4.7

(a)


Control
SP
CP96345
p-PKCδ
MEKK1
HPRT

(b)


PKCδ
Densitometry
2.5
2
1.5
1
0.5
0
*
+
Control
SP
CP96345

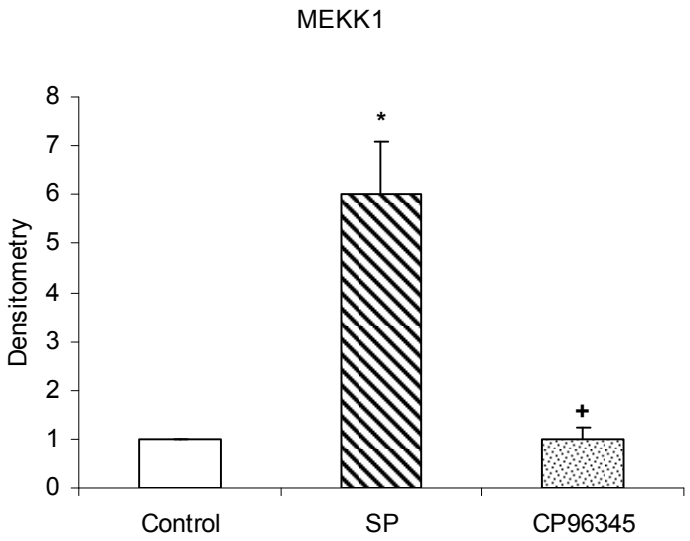


Figure 4.7 Substance P (SP)/NK1R interaction is involved in PKCδ and MEKK1 activation. Freshly isolated pancreatic acini, obtained from three mice, were pre-incubated with 1 μM CP96345 for 30 min at 37˚C followed by stimulation with 1 μM SP for 10 min for PKCδ and MEKK1 at 37˚C. Cells were subsequently lysed, and cell proteins were subjected to Western blot analysis using antibodies against (a) phospho-PKCδ, (b) MEKK1, and (c) HPRT. Corresponding densitometric analysis of Western blot experiments from pancreatic acini. MW in (kDa) for PKCδ is 78, MEKK1 is 190 and HPRT is 24. The results are representative of three independent experiments. Results shown are the means + SE. *****$P < 0.05$ when compared to control, **+**$P < 0.05$ when compared to SP.

CHAPTER 5

ROLE OF CALCIUM AND CONVENTIONAL PROTEIN KINASE C α/βII IN SUBSTANCE P-INDUCED CHEMOKINE SYNTHESIS IN MOUSE PANCREATIC ACINAR CELLS

5.1 INTRODUCTION

Acute pancreatitis is an acute inflammatory process of the pancreas. Inflammatory mediators have been shown to play a key role in the pathogenesis of acute pancreatitis. The interaction between substance P and chemokines is critically involved in mediating acute pancreatitis. There is an increased production of substance P and chemokines during acute pancreatitis. I have previously demonstrated that substance P stimulated the production of inflammatory mediators MCP-1, MIP-1α and MIP-2 in pancreatic acinar cells. Blockade of substance P receptor attenuated chemokine production in pancreatic acinar cells as well as protected mice against acute pancreatitis (Lau, Wong *et al.* 2005; Sun and Bhatia 2007).

Phospholipase C (PLC)-dependent signaling pathways have been implicated in the development of acute and chronic inflammatory responses *in vivo* (Hou, Kirchner *et al.* 2004). Studies have shown that neuropeptide bombesin stimulation of pancreatic acinar cells leads to activation of PLC. PLC then hydrolyzes membrane phospholipid, PIP2 to generate two intracellular messengers, DAG and IP3, which in turn, mediate the

activation of PKC and intracellular calcium [Ca^{2+}]i release, respectively (Hou, Kirchner *et al.* 2004; Schulz, Krause *et al.* 1999; Wu, Huang *et al.* 2000).

It has been shown that substance P induced elevation of [Ca^{2+}]i, in Chinese hamster ovary cells expressing the substance P receptor (Mochizuki-Oda, Nakajima *et al.* 1993), in dorsal horn neurons (Ansel, Kaynard *et al.* 1996) and in rat pancreatic acinar cell line (Gallacher, Hanley *et al.* 1990). The [Ca^{2+}]i elevation induced by substance P was blocked by the NK1 receptor antagonist CP96345 (Mochizuki-Oda, Nakajima *et al.* 1994). Another NK1 receptor antagonist, GR 82334, completely inhibited the increase in [Ca^{2+}]i that was recorded in polymorphonuclear leukocytes when exposed to substance P (Tanabe, Otani *et al.* 1996). There is evidence suggesting that elevated Ca^{2+} in pancreatic acinar cells is involved in the development of acute pancreatitis (Frick, Fernandez-del Castillo *et al.* 1997).

Substance P, chemokines and Ca^{2+} play critical roles in the pathogenesis of acute pancreatitis. As demonstrated in chapter 2, substance P stimulated the production of chemokines in pancreatic acinar cells via NFκB. Moreover, as shown in chapters 3 and 4, PKCδ, MAPKs ERK and JNK as well as transcription factor AP-1 were involved in substance P-induced chemokine production in pancreatic acinar cells. To my knowledge, no study has explored the interplay between substance P and Ca^{2+} in inducing the production of pro-inflammatory mediators in pancreatic acinar cells. Therefore, the aim is to investigate the role of Ca^{2+} in substance P-induced chemokine production in an *in vitro* model of isolated pancreatic acinar cells and also, to study the underlying signaling mechanisms involved.

5.2 MATERIALS AND METHODS

5.2.1 Animal Ethics

Please refer to section 2.2.1.

5.2.2 Test system used

Mouse pancreatic acinar cells were prepared, as previously described in section 2.2.2.

5.2.3 Viability of mouse pancreatic acinar cells

The viability of mouse pancreatic acinar cells was assessed, as previously described in section 2.2.3.

5.2.4 Experimental design

Pancreatic acinar cells (500 µl of cell suspension) were treated with substance P (Sigma-Aldrich) at a concentration of 1 µM for 0, 3, 5, 10, 15, 30, 45 and 60 min at 37˚C. Following treatment with substance P, the cells were lysed to detect for PKCα/βII activation by Western blot analysis. In some experiments, cells were either pre-treated with the specific Ca^{2+} chelator 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis-acetoxymethyl ester (BAPTA-AM), Sigma-Aldrich**,** at 1 µM, 5 µM and 10 µM, or the selective PLC inhibitor, 1-[6-((17b-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione (U73122), Calbiochem/Merck, at 1 µM, 2.5 µM or the PKC inhibitor (chelerythrine chloride), Calbiochem/Merck, at 10 µM for 30 min and then stimulated with 1 µM substance P or vehicle for 10 or 45 min at 37°C. Subsequently, the supernatant was used for chemokine detection and the pellet was used for either nuclear extract, to detect NFκB (p65) and AP-1(c-Jun) activation, or cell lysis for Western blot analysis to detect PKCα/βII, ERK and JNK. The negative control in

which pancreatic acini were pre-treated with 1 μM BAPTA-AM or 1 μM U73122 or 10 μM chelerythrine chloride for 30 min followed by stimulation with vehicle (saline) for 45 min had no significant effect on chemokine production when compared to unstimulated controls.

5.2.5 Preparation of total cell lysates for Western blot analysis

Total cell lysates for Western blot analysis were prepared, as previously described in section 3.2.5.

5.2.6 Western blot analysis

Cell lysates (50 μg of protein) were separated on 12% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Non-specific binding was blocked by 1 h incubation of the membranes, at room temperature, in 5% nonfat dry milk in phosphate buffered saline Tween 20 (PBST) (0.05% Tween 20 in phosphate buffered saline). The blots were then incubated overnight at 4°C with the primary antibodies (at a 1:1000 dilutions in the buffer containing 2.5% nonfat dry milk in PBST) phospho-PKCα/βII, phospho-ERK1/2, total ERK1/2, phospho-SAPK/JNK, total SAPK/JNK (Cell Signaling Technology) and HPRT purchased from Santa Cruz Biotechnology. HPRT was used as the housekeeping protein. PKCα/βII antibody detects PKCα only when phosphorylated at threonine 638 and PKC βII only when phosphorylated at threonine 641. It reacts weakly with phosphorylated PKC βI and γ. After which the nitrocellulose membranes were washed four times with PBST, and finally incubated for 1 h at room temperature with goat anti-rabbit HRP-conjugated secondary antibody (Santa Cruz Biotechnology) at 1:2000 dilutions in the buffer containing 2.5% nonfat dry milk in PBST. The blots were developed for visualization

using enhanced chemiluminescence (ECL) detection kit (Pierce, Rockford, IL). The densities of the bands were quantified using a UVP GelDoc-It Imaging Systems (Scimed, Asia).

5.2.7 Nuclear cell extract preparation

Nuclear cell extract was prepared, as previously described in section 2.2.6.

5.2.8 NFκB DNA-binding activity

The binding of NFκB to DNA was measured in nuclear extracts, as previously described in section 2.2.7.

5.2.9 AP-1 DNA-binding activity

The binding of AP-1 to DNA was measured in nuclear extracts, as previously described in section 3.2.9.

5.2.10 Chemokine detection

Pancreatic acinar cell supernatants were assayed for MCP-1, MIP-1α and MIP-2 using a sandwich ELISA, as previously described in section 2.2.5.

5.2.11 Cytosolic calcium measurement

Cytosolic calcium was measured as described previously (Melendez and Ibrahim 2004; Zhi, Leung *et al.* 2006). Briefly, cells were loaded with 1 mg/ml Fura2-AM (Invitrogen) in calcium assay buffer (in mM: 145 NaCl, 5 KCl, 1 $MgSO_4$, 1 $CaCl_2$, 10 HEPES, pH 7.4) containing 1.8 mg/ml glucose and 2 mg/ml BSA. After removal of excess reagents by dilution and centrifugation at 4°C, the cells were re-suspended in calcium assay buffer and warmed to 37°C in the cuvette; after a baseline measurement was obtained, the cells were stimulated by the addition of 1 μM substance P. Fluorescence was measured at 340 and 380 nm.

5.2.12 Statistical analysis

Results are presented as means + SE with 6 replicates for each condition. 3 independent experiments (n = 3) were carried out. The significance of changes was evaluated by using ANOVA and Tukey's method was used as a post hoc test for the difference between groups. A P value < 0.05 was taken as the level of significance.

5.3 RESULTS

5.3.1 Substance P induces phosphorylation of PKCα/βII in a time dependent manner.

Pancreatic acinar cells were treated with 1 μM substance P or vehicle (saline) for 0, 3, 5, 10, 15, 30, 45 and 60 min at 37˚C. Cells were then lysed, and cell proteins were subjected to Western blot analysis. As shown in Figure 5.1 (a, b), substance P induced phosphorylation of PKCα/βII in pancreatic acini which was evident from 3 min and increased in a time-dependent manner up to 10 min, followed by a time-dependent decrease until 60 min. Densitometric analysis of Western blot experiments revealed a significant increase in phosphorylation of PKCα/βII at 10 min when compared to 0 min control. No significant change was detected in the housekeeping proteins HPRT.

5.3.2 Substance P-induced $[Ca^{2+}]i$ is involved in activation of PKCα/βII, ERK and JNK.

The data in Figure 5.2 (a, b) shows that substance P stimulation rapidly triggered calcium release from internal stores in pancreatic acinar cells. However, when these cells were pre-treated for 30 min with 1 μM BAPTA-AM, a calcium-specific chelator, the increase in $[Ca^{2+}]i$ was inhibited (Figure 5.2).

Stimulation of pancreatic acinar cells with 1 μM substance P significantly upregulated phosphorylation of PKCα/βII, ERK and JNK. Pancreatic acinar cells were pre-treated for 30 min with different concentration of BAPTA-AM, ranging from 1 to 10 μM, followed by stimulation with 1 μM of substance P for 10 min. Cells were then lysed, and cell proteins were subjected to Western blot analysis. As shown in Figure 5.3, BAPTA-AM

concentration-dependently decreased phosphorylation of PKCα/βII, ERK and JNK in pancreatic acinar cells when compared to substance P only stimulated cells. No significant change was detected in the housekeeping proteins HPRT, total ERK and total JNK.

5.3.3 Role of PLC in substance P-induced activation of PKCα/βII, ERK and JNK in pancreatic acinar cells

Pancreatic acini were pre-treated with the selective PLC inhibitor, U73122, at 1 μM, 2.5 μM for 30 min followed by stimulation with 1 μM of substance P for 10 min. Cells were then lysed, and cell proteins were subjected to Western blot analysis. As shown in Figure 5.4, U73122 decreased phosphorylation of PKCα/βII, ERK and JNK in pancreatic acinar cells when compared to substance P only stimulated cells. No significant change was detected in the housekeeping proteins HPRT, total ERK and total JNK.

5.3.4 PLC and Ca^{2+} are involved in substance P-induced NFκB p65 and AP-1 c-Jun activation in pancreatic acinar cells.

Pancreatic acinar cells were pre-incubated for 30 min with either BAPTA-AM, ranging from 1 to 10 μM, or U73122, at 1 μM, 2.5 μM followed by stimulation with 1 μM of substance P for 45 min. NFκB p65 and AP-1 c-Jun DNA-binding assay revealed that treatment with substance P led to a notable increase in the activity of NFκB p65 and AP-1 c-Jun, in pancreatic acinar cell. As shown in Figure 5.5 (a, b), pre-treatment with either BAPTA-AM or U73122 attenuated substance P-induced DNA-binding activity of NFκB p65 and AP-1 c-Jun when compared to substance P only treated cells.

5.3.5 PLC, Ca^{2+} and PKC are involved in the secretion of several pro-inflammatory chemokines in pancreatic acinar cells.

Treatment of pancreatic acini with 1 μM substance P resulted in enhanced expression of pro-inflammatory chemokines MCP-1, MIP-1α, and MIP-2. Pancreatic acinar cells were pre-treated with BAPTA-AM, ranging from 1 to 10 μM, or U73122, at 1 μM, 2.5 μM or chelerythrine chloride at 10 μM for 30 min followed by stimulation with 1 μM of substance P for 45 min. The protein levels of these chemokines were determined by ELISA. Figure 5.6 (a, b, c) shows that pre-treatment with either BAPTA-AM or U73122 markedly decreased MCP-1, MIP-1α, and MIP-2 production when compared to substance P only treated cells. Furthermore, treatment with PKC inhibitor chelerythrine chloride significantly decreased the secretion of MCP-1, MIP-1α, and MIP-2 in pancreatic acinar cells. These results demonstrated that PLC, Ca^{2+} and PKC were involved in the production of several chemokines in pancreatic acinar cells.

5.4 DISCUSSION

In the present study, I have investigated the role of PLC in substance P/NK1R induced chemokine production in pancreatic acinar cells using U73122. U73122 is a membrane-permeable aminosteroid PLC inhibitor. It was reported to selectively inhibit the PLC-dependent process in human platelets and neutrophils and was thus proven to be useful in evaluating the role of PLC in cell activation (Bleasdale, Thakur *et al.* 1990; Smith, Justen *et al.* 1996; Smith, Sam *et al.* 1990; Stam, Michiels *et al.* 1998). Inhibition of PLC significantly decreased substance P-induced phosphorylation of Ca^{2+}-dependent PKCα/βII, ERK and JNK. Previous studies have shown that in PLC deficient mice, there was a reduction in chemoattractant-induced phosphorylation of JNK and MAPK in murine neutrophils (Li, Jiang *et al.* 2000). Moreover, in my study U73122 significantly inhibited substance P-induced activation of NFκB p65 and AP-1 c-Jun and the secretion of MCP-1, MIP-1α, and MIP-2 in pancreatic acinar cells.

Acute pancreatitis was found to be associated with hypercalcaemia in humans (Frick, Fryd *et al.* 1987) and in experimental animal models, acute pancreatitis was induced by bolus infusions (intravenous injection) of calcium (Mithofer, Fernandez-del Castillo *et al.* 1995). It has been shown that hypercalcaemia induces a secretory block and accumulation of digestive zymogens within the pancreatic acinar cell (Frick, Mithofer *et al.* 1995). It is generally believed that ectopic activation of digestive enzymes, particularly intracellular trypsinogen activation, is an early event in the pathophysiology of acute pancreatitis (Frick, Fernandez-del Castillo *et al.* 1997; Mithofer, Fernandez-del Castillo *et al.* 1995). In the current study, I were interested in elucidating the mechanisms by which substance P-induced $[Ca^{2+}]i$ elevation leads to production of pro-inflammatory

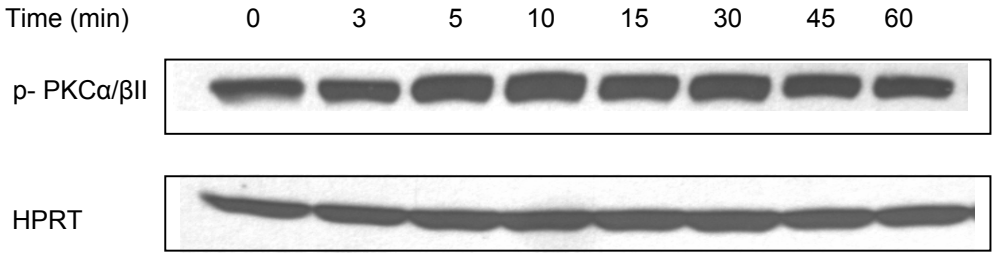
mediators. I have shown that substance P elevates [Ca^{2+}]i in pancreatic acinar cells which is in accordance with previous studies conducted in various cells expressing the substance P receptor (Ansel, Kaynard *et al.* 1996; Gallacher, Hanley *et al.* 1990; Mochizuki-Oda, Nakajima *et al.* 1993). Moreover, treatment with BAPTA-AM significantly attenuated the increase in [Ca^{2+}]i in pancreatic acinar cells. BAPTA-AM, the membrane-permeable form of BAPTA, is hydrolyzed by cytosolic esterases and is trapped intracellularly as the active Ca^{2+} chelator BAPTA and is widely used as an intracellular calcium sponge (Ahluwalia, Topp *et al.* 2001; Harrison and Bers 1987; Kim, Kim *et al.* 2002; Smith, Selak *et al.* 1992). BAPTA-AM significantly attenuated phosphorylation of PKCα/βII, ERK and JNK, subsequently leading to a decrease in activation of NFκB p65, AP-1 c-Jun and production of MCP-1, MIP-1α, and MIP-2 in pancreatic acinar cells.

The PKC superfamily is classified into three subfamilies based on their domain structure and their ability to respond to calcium and diacylglycerol (Newton and Johnson 1998). In this study, I have focused on the Ca^{2+}-dependent conventional PKCα/βII. Substance P induced a time-dependent increase in phosphorylation of PKCα/βII in pancreatic acinar cells. The increase in phosphorylation was significantly blocked by pre-treatment with either U73122 or BAPTA-AM. Moreover, chelerythrine chloride significantly abrogated the increase in substance P-induced production of chemokines MCP-1, MIP-1α, and MIP-2 in pancreatic acinar cells. Studies have shown that chelerythrine chloride specifically blocked PKC phosphorylation and it has been shown to inhibit PKCα and PKCβ translocation from the cytosol to the membrane in isolated ileal synaptosomes (Chao, Chen *et al.* 1998; Herbert, Augereau *et al.* 1990).

In conclusion, substance P stimulates pancreatic acinar cells to release chemokines MCP-1, MIP-1α, and MIP-2 through PLC dependent mechanisms. Substance P induces an increase in $[Ca^{2+}]i$ which results in the phosphorylation of PKCα/βII, ERK and JNK; consequently leading to the activation of NFκB p65, AP-1 c-Jun and ultimately to chemokine production. Apart from being involved in trypsinogen activation (Frick, Fernandez-del Castillo *et al.* 1997; Mithofer, Fernandez-del Castillo *et al.* 1995), an increase in $[Ca^{2+}]i$ also entailed elevated production of chemokines which play a critical role in the pathogenesis of acute pancreatitis. In light of this study, I proposed that drugs targeting the substance P-calcium mediated signaling pathways could prove beneficial in improving the treatment of acute pancreatitis.

Figure 5.1

(a)



(b)

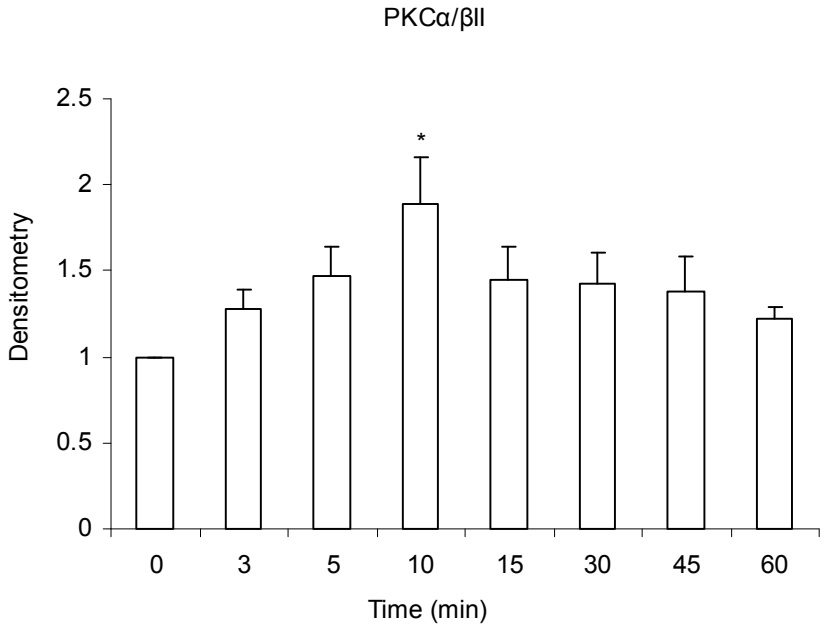
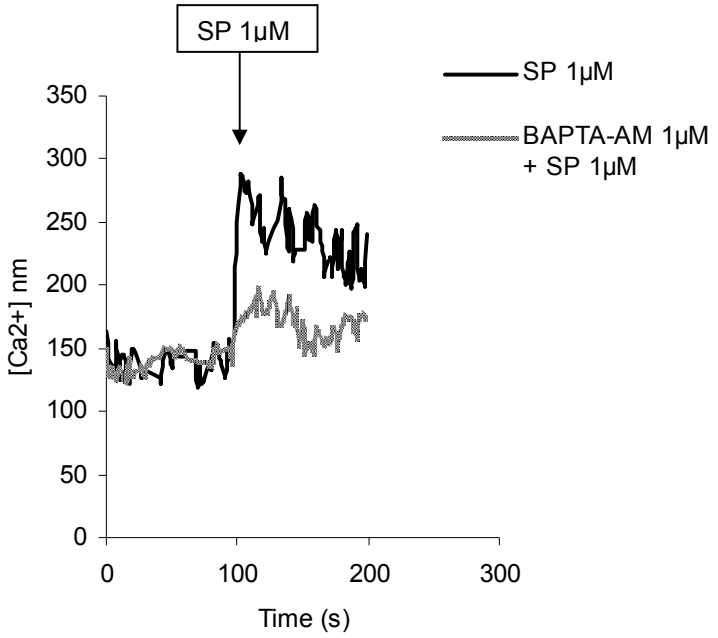


Figure 5.1 Substance P (SP) induces phosphorylation of PKCα/βII in a time dependent manner. Freshly isolated pancreatic acini were incubated with either 1 μM SP or vehicle (saline) for 0, 3, 5, 10, 15, 30, 45 and 60 min at 37˚C. The cells were lysed, and cell proteins were subjected to Western blot analysis using antibodies against (a) phospho-PKCα/βII and HPRT. (b) Densitometric analysis of Western blot experiments from pancreatic acini. MW in (kDa) for PKCα/βII is 80 and HPRT is 24.The results are representative of three independent (n=3) experiments. Results shown are the means + SE. *$P < 0.05$ when compared to 0 min control.

Figure 5.2

(a)


SP 1µM
SP 1µM
BAPTA-AM 1µM
+ SP 1µM
[Ca2+] nm
350
300
250
200
150
100
50
0
0
100
200
300
Time (s)

(b)

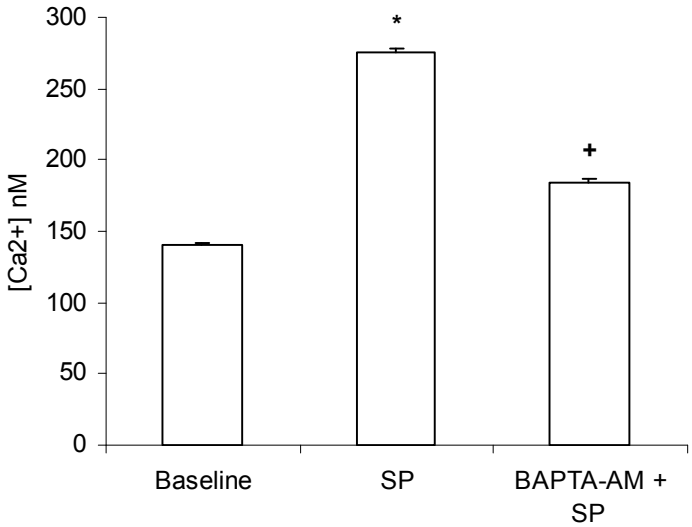
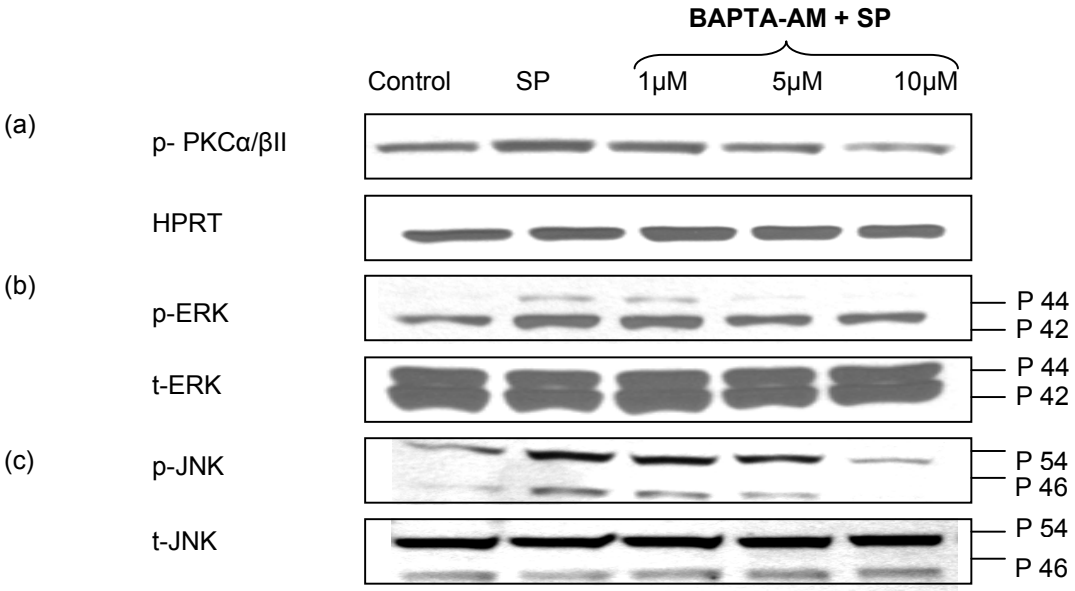
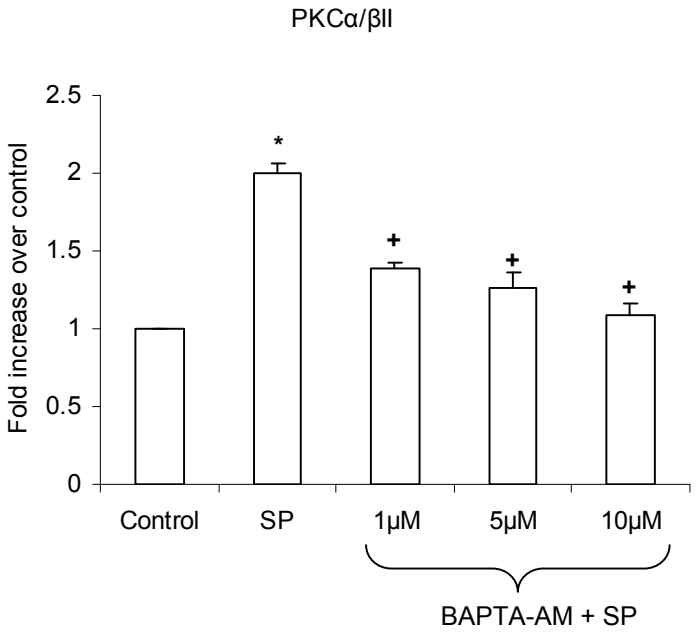

[Ca2+] nM
300
250
200
150
100
50
0
*
+
Baseline
SP
BAPTA-AM +
SP

Figure 5.2 Substance P (SP) induces mobilization of [Ca^{2+}]i in pancreatic acinar cells. Freshly isolated pancreatic acini were pre-incubated with either 1 μM BAPTA-AM or vehicle (DMSO) for 30 min. The cells were then rinsed twice with calcium assay buffer and then loaded with 1 mg/ml Fura2-AM. After removal of excess reagents, the cells were re-suspended in calcium assay buffer and warmed to 37°C in the cuvette; after the baseline reading was obtained, the cells were stimulated by the addition of 1 μM SP. Fluorescence was measured at 340 and 380 nm. Figure 2 (a) is a representative example. The results shown in figure 2 (b) are representative of three independent (n=3) experiments. Results shown are the means + SE. *P < 0.05 when compared to control, +P < 0.05 when compared to SP.

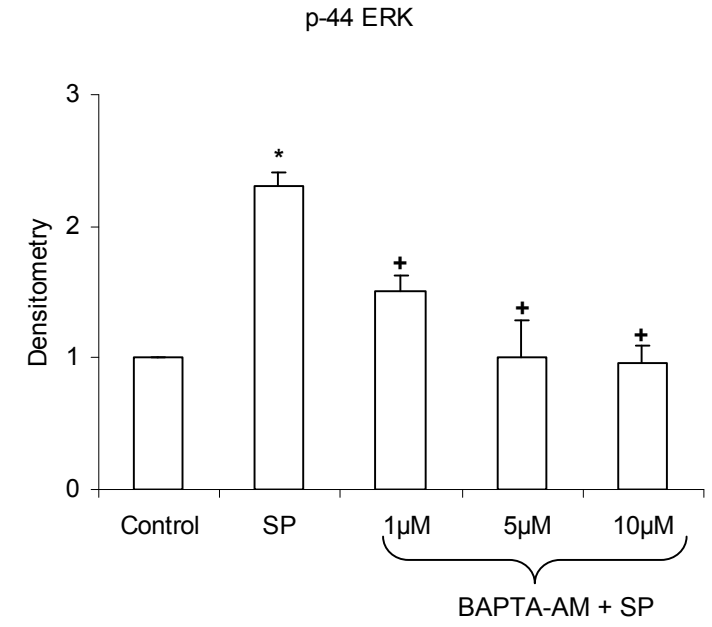
Figure 5.3

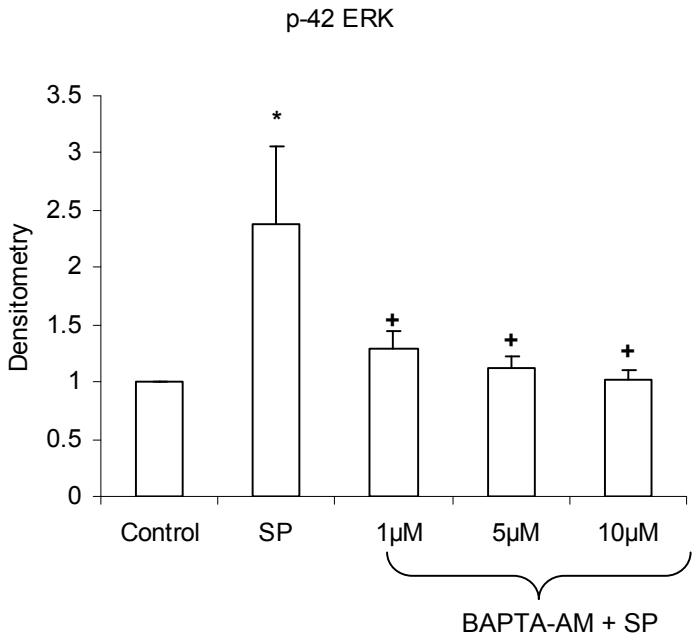

BAPTA-AM + SP
Control
SP
1μM
5μM
10μM
(a)
p- PKCα/βII
HPRT
(b)
p-ERK
P 44
P 42
t-ERK
P 44
P 42
(c)
p-JNK
P 54
P 46
t-JNK
P 54
P 46

(d)

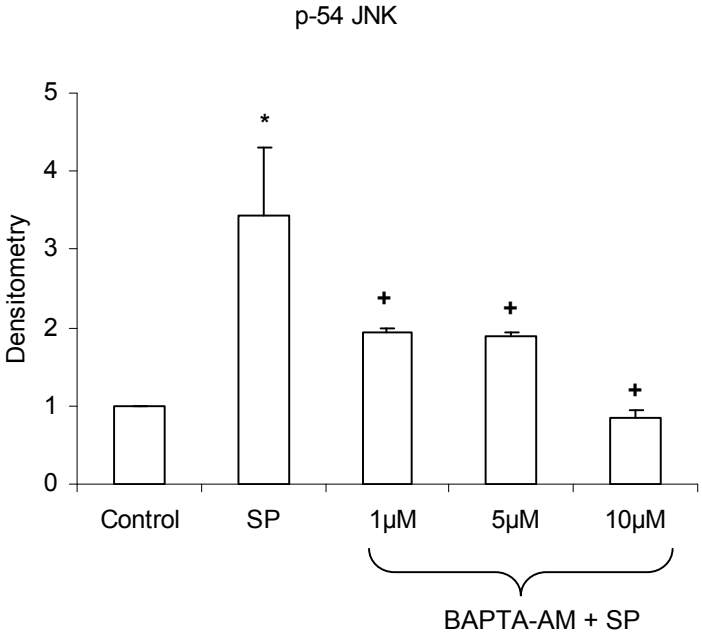

PKCα/βII
Fold increase over control
2.5
2
1.5
1
0.5
0
*
+
+
+
Control
SP
1μM
5μM
10μM
BAPTA-AM + SP

(e)


p-44 ERK
Densitometry
3
2
1
0
*
+
+
+
Control
SP
1μM
5μM
10μM
BAPTA-AM + SP


p-42 ERK
Densitometry
3.5
3
2.5
2
1.5
1
0.5
0
*
+
+
+
Control
SP
1μM
5μM
10μM
BAPTA-AM + SP

(f)


p-54 JNK
Densitometry
0
1
2
3
4
5
*
+
+
+
Control
SP
1μM
5μM
10μM
BAPTA-AM + SP

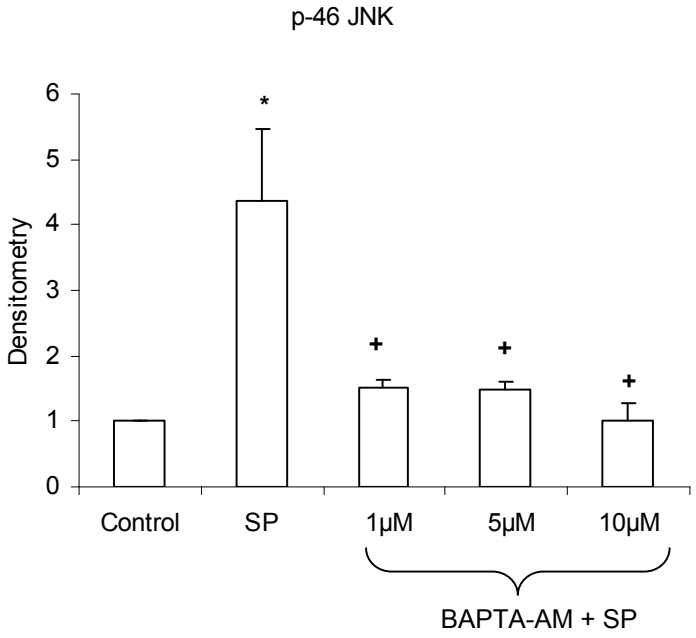
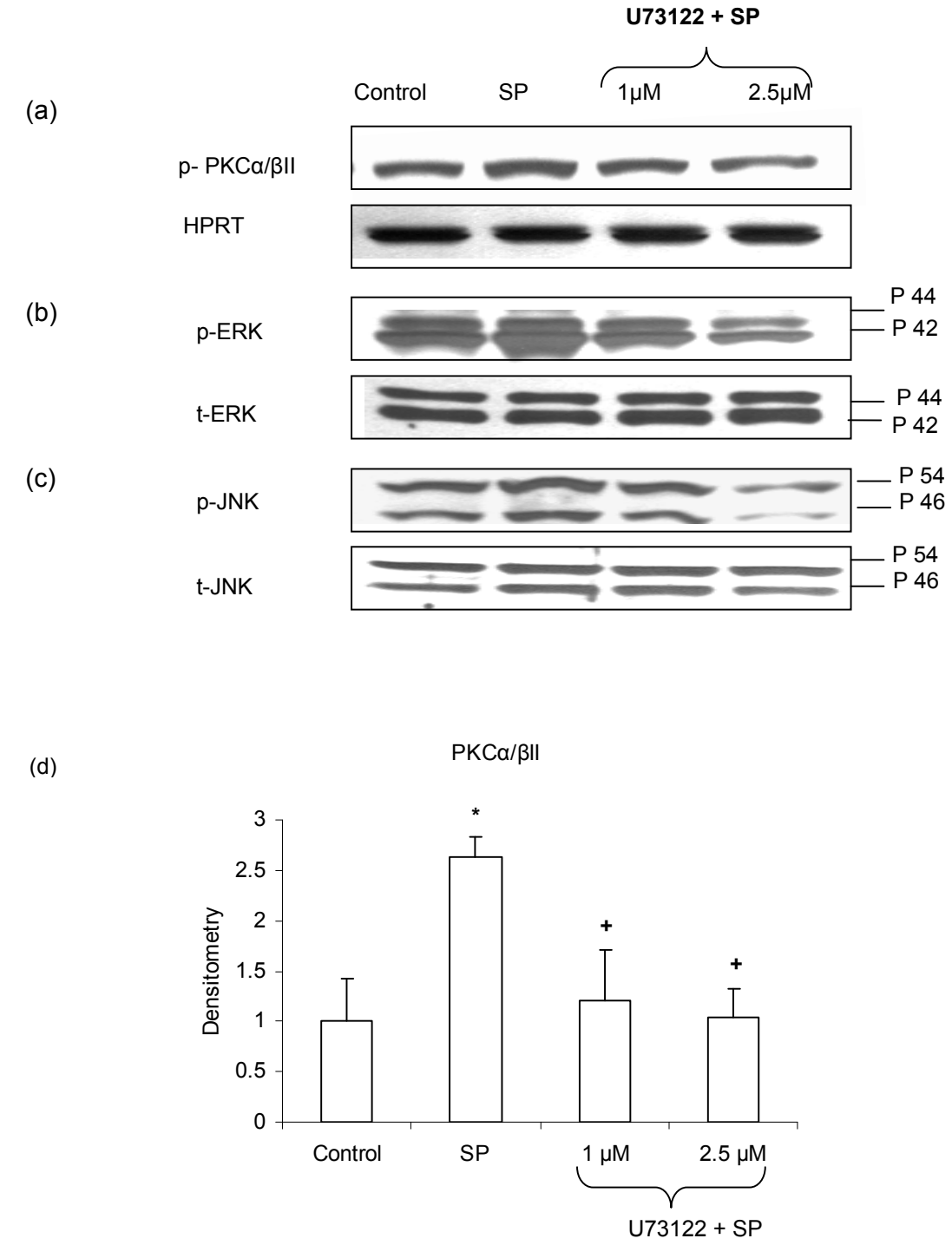
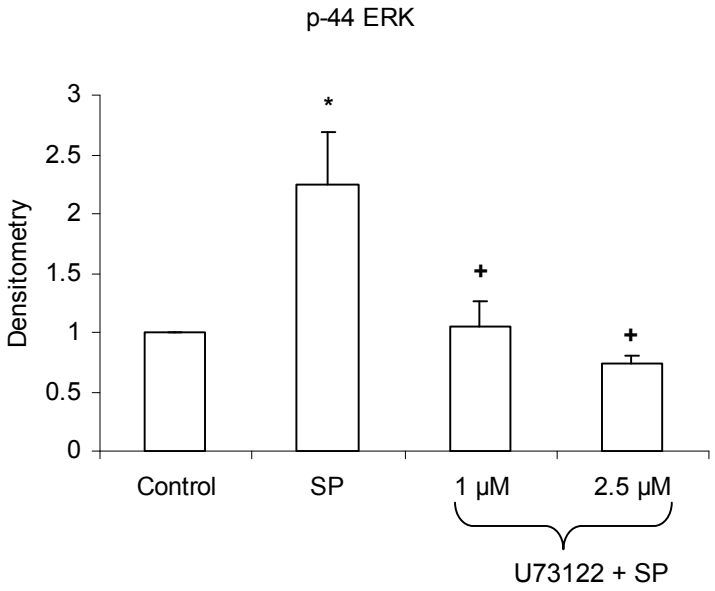

p-46 JNK
Densitometry
0
1
2
3
4
5
6
*
+
+
+
Control
SP
1μM
5μM
10μM
BAPTA-AM + SP

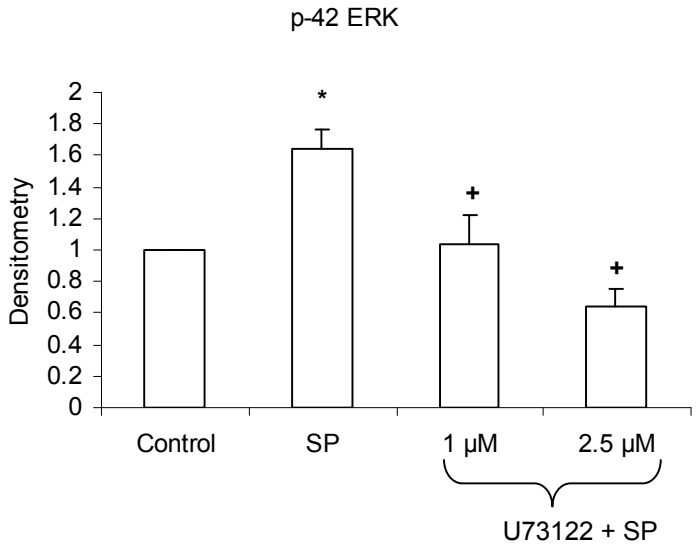
Figure 5.3 Substance P (SP)-induced [Ca^{2+}]i is involved in activation of PKCα/βII, ERK and JNK. Freshly isolated pancreatic acini were pre-incubated with either different concentration of BAPTA-AM, ranging from 1 to 10 μM, or vehicle (DMSO) for 30 min followed by stimulation with 1 μM of SP for 10 min. Cells were then lysed, and cell proteins were subjected to Western blot analysis using antibodies against (a) phospho-PKCα/βII, HPRT (b) phospho-ERK, total ERK (c) phospho-JNK and total JNK. The phosphorylated sub-units such as p-PKCα/βII, p-44 ERK, p-42 ERK, p-54 JNK and p-46 JNK have been quantified. Densitometric analysis of Western blot experiments was performed and the group data from 3 independent preparations (*n*=3) are presented in (d, e, f). MW in (kDa) for PKCα/βII is 80, JNK 2/1 is 54/46, ERK1/2 is 44/42 and HPRT is 24. Results shown are the means + SE. **P* < 0.05 when compared to control, +*P* < 0.05 when compared to SP.

Figure 5.4

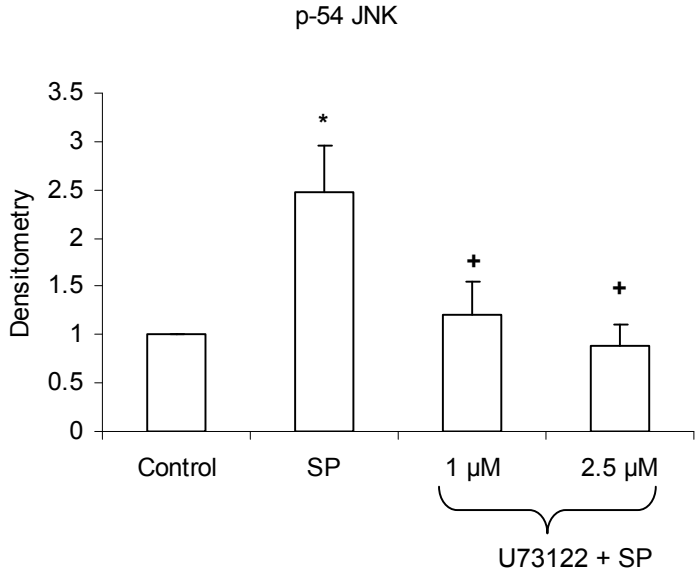

U73122 + SP
Control
SP
1μM
2.5μM
(a)
p- PKCα/βII
HPRT
(b)
p-ERK
t-ERK
P 44
P 42
P 44
P 42
(c)
p-JNK
t-JNK
P 54
P 46
P 54
P 46
(d)
PKCα/βII
Densitometry
3
2.5
2
1.5
1
0.5
0
*
+
+
Control
SP
1 μM
2.5 μM
U73122 + SP

(e)


p-44 ERK
Densitometry
3
2.5
2
1.5
1
0.5
0
*
+
+
Control
SP
1 μM
2.5 μM
U73122 + SP


p-42 ERK
Densitometry
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
+
+
Control
SP
1 μM
2.5 μM
U73122 + SP

(f)


p-54 JNK
Densitometry
3.5
3
2.5
2
1.5
1
0.5
0
*
+
+
Control
SP
1 μM
2.5 μM
U73122 + SP

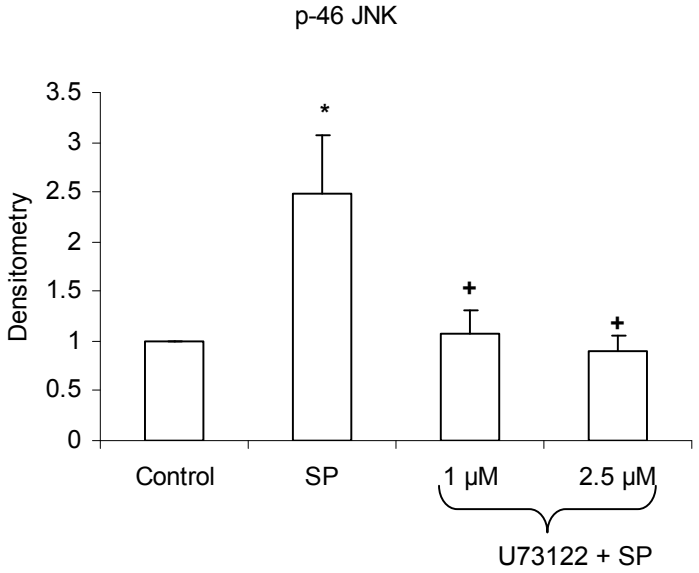
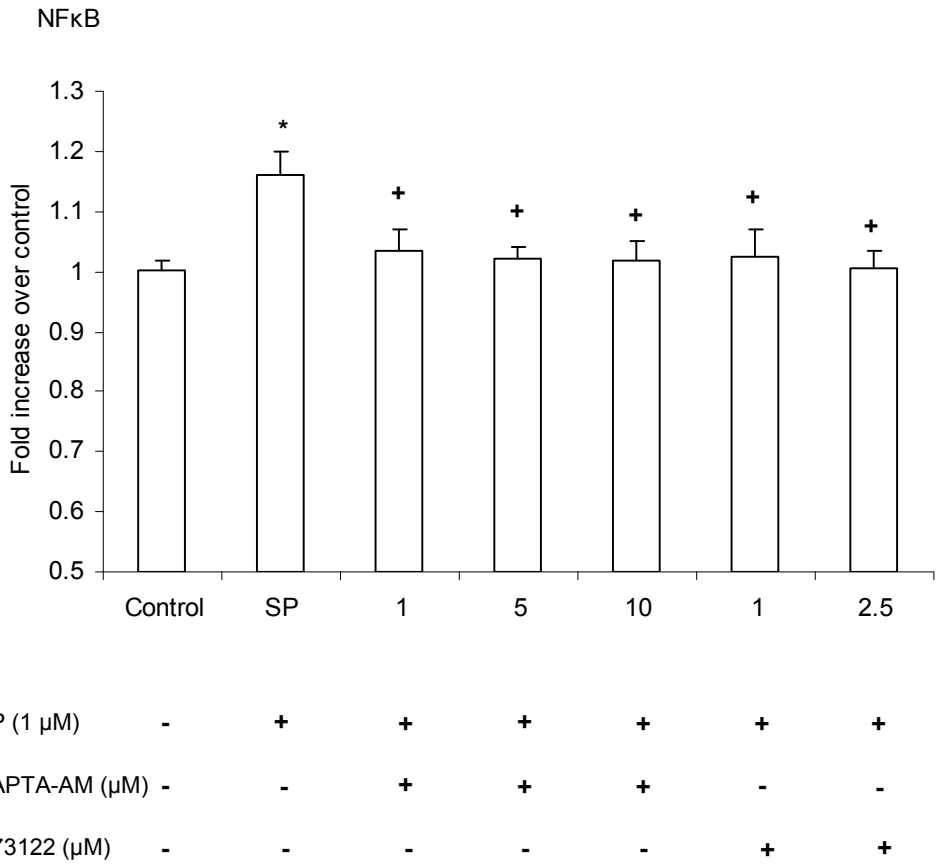

p-46 JNK
Densitometry
3.5
3
2.5
2
1.5
1
0.5
0
*
+
+
Control
SP
1 μM
2.5 μM
U73122 + SP

Figure 5.4 Substance P (SP)-induced activation of PKCα/βII, ERK and JNK is mediated by PLC in pancreatic acinar cells. Freshly isolated pancreatic acini were pre-incubated with either U73122 at 1 μM, 2.5 μM or vehicle (DMSO) for 30 min at 37˚C followed by stimulation with 1 μM SP for 10 min at 37˚C. Cells were subsequently lysed, and cell proteins were subjected to Western blot analysis using antibodies against (a) PKCα/βII, HPRT, (b) phospho-ERK, total ERK, (c) phospho-JNK and total JNK. The phosphorylated sub-units such as p-PKCα/βII, p-44 ERK, p-42 ERK, p-54 JNK and p-46 JNK have been quantified. Densitometric analysis of Western blot experiments were performed and the group data from 3 independent preparations (*n*=3) are presented in (d, e, f). MW in (kDa) for PKCα/βII is 80, JNK 2/1 is 54/46, ERK1/2 is 44/42 and HPRT is 24. Results shown are the means + SE. **P* < 0.05 when compared to control, +*P* < 0.05 when compared to SP.

Figure 5.5

(a)


NFκB
Fold increase over control
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
*
+
+
+
+
+
Control
SP
1
5
10
1
2.5
SP (1 μM) - + + + + + +
BAPTA-AM (μM) - - + + + - -
U73122 (μM) - - - - - + +

(b)

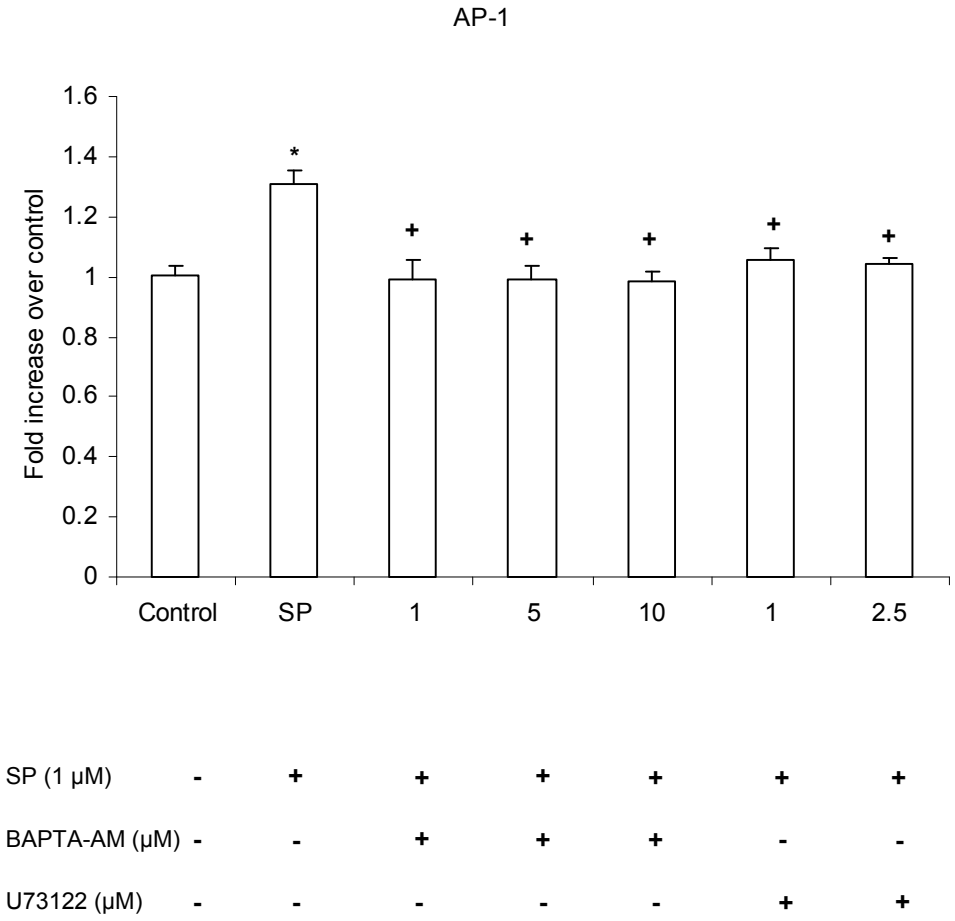
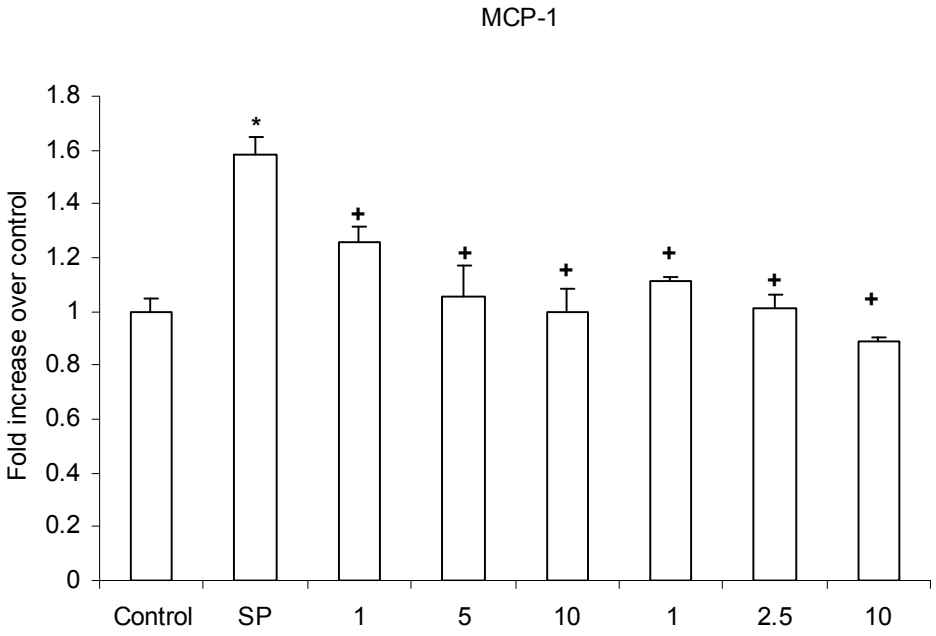


Figure 5.5 PLC and Ca^{2+} are involved in substance P (SP)-induced NFκB p65 and AP-1 c-Jun activation in pancreatic acinar cells. Freshly isolated pancreatic acini were pre-incubated with BAPTA-AM, ranging from 1 to 10 μM, or U73122 at 1 μM, 2.5 μM or vehicle (DMSO) followed by stimulation with 1 μM of SP for 45 min. The cells were separated from incubation medium by centrifugation. The pellet (cells) was used for (a) NFκB and (b) AP-1 nuclear extraction. NFκB p65 and AP-1 c-Jun DNA-binding assays were then carried out. The results are representative of three independent (n=3) experiments. Results shown are the means + SE. *$P < 0.05$ when compared to control, +$P < 0.05$ when compared to SP.

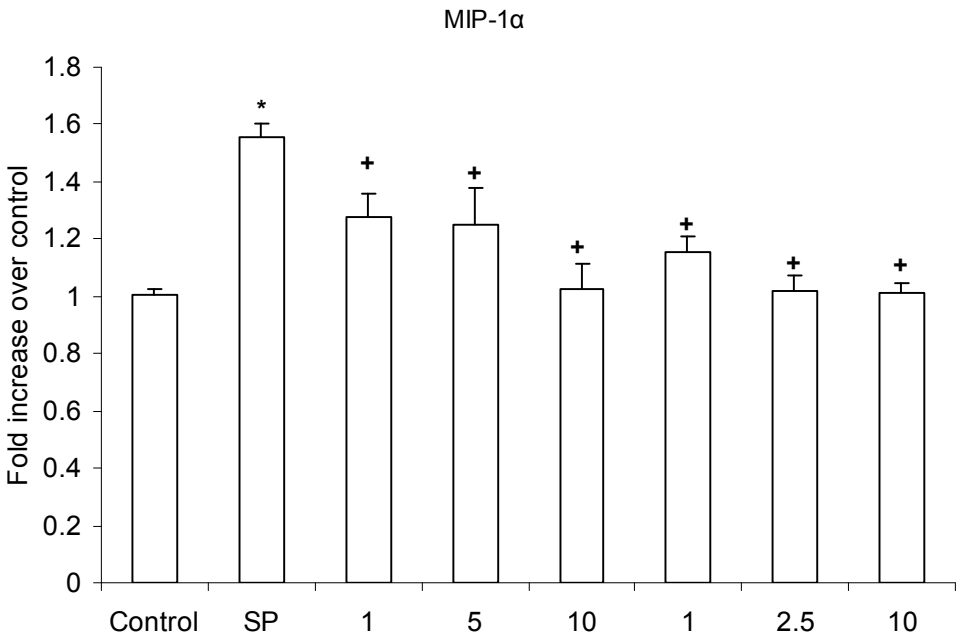
Figure 5.6

(a)



SP (1 μM)	-	+	+	+	+	+	+	+
BAPTA-AM (μM)	-	-	+	+	+	-	-	-
U73122 (μM)	-	-	-	-	-	+	+	-
Chelerythrine chloride (μM)	-	-	-	-	-	-	-	+

(b)



SP (1 μM)	-	+	+	+	+	+	+	+
BAPTA-AM (μM)	-	-	+	+	+	-	-	-
U73122 (μM)	-	-	-	-	-	+	+	-
Chelerythrine chloride (μM)	-	-	-	-	-	-	-	+

(c)

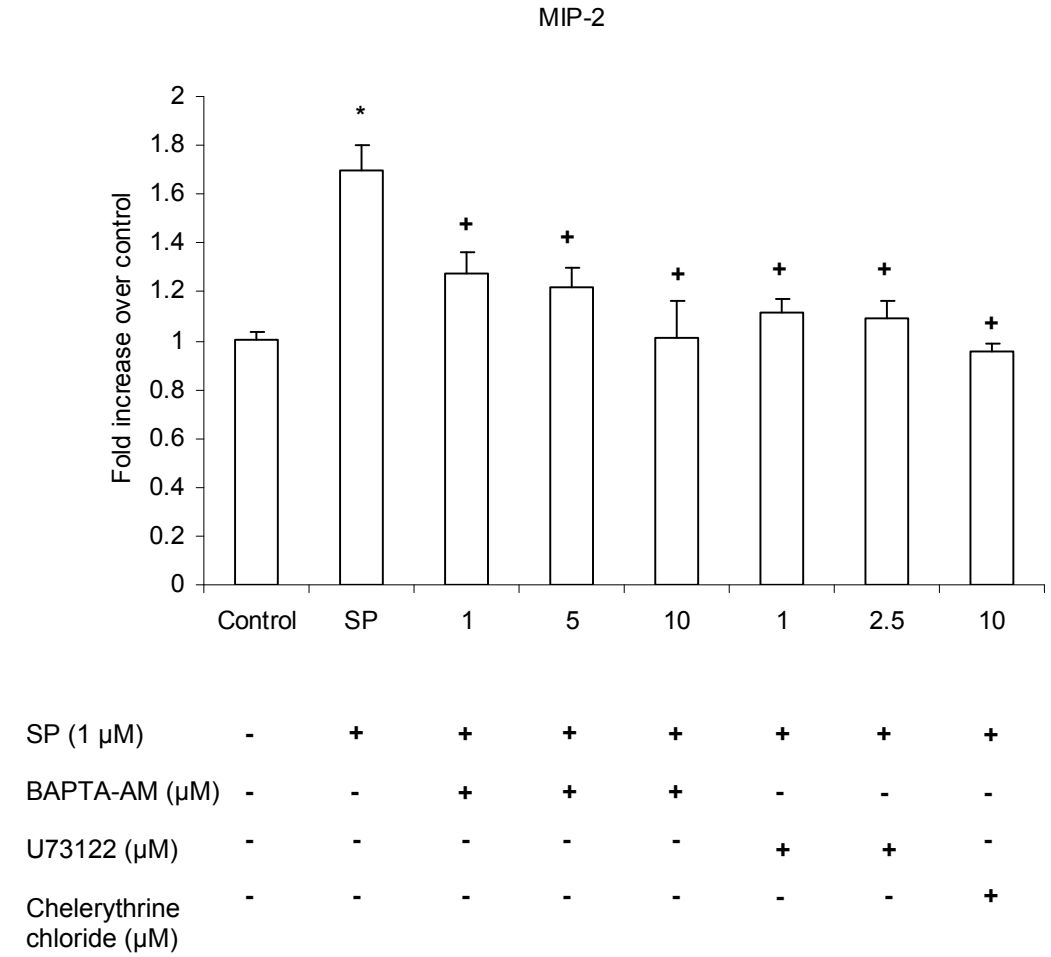


Figure 5.6 PLC, Ca^{2+} and PKC are involved in the secretion of several pro-inflammatory chemokines in pancreatic acinar cells. Freshly isolated pancreatic acini were pre-incubated with BAPTA-AM, ranging from 1 to 10 μM, or U73122 at 1 μM, 2.5 μM or chelerythrine chloride at 10 μM or vehicle (DMSO) for 30 min followed by stimulation with 1 μM of substance P (SP) for 45 min. The supernatant was used to measure (a) MCP-1, (b) MIP-1α and (c) MIP-2 levels by ELISA. The results are representative of three independent (n=3) experiments. Results shown are the means + SE. *$P < 0.05$ when compared to control, +$P < 0.05$ when compared to SP.

CHAPTER 6

INVOLVEMENT OF SRC FAMILY KINASES IN SUBSTANCE P-INDUCED CHEMOKINE PRODUCTION IN MOUSE PANCREATIC ACINAR CELLS, AND ITS SIGNIFICANCE IN ACUTE PANCREATITIS

6.1 INTRODUCTION

Acute pancreatitis is increasing in incidence and is often a fatal human disease, in which the pancreas digests itself and its surroundings (Bhatia, Brady *et al.* 2000; Bhatia and Moochhala 2004; Bhatia, Neoptolemos *et al.* 2001). Most cases are secondary to gallstones or excess alcohol consumption. Regardless of the cause, activation of digestive enzymes within pancreatic acinar cells is thought to be a critical initiating event. Pancreatic damage results in a local inflammatory response, which if pronounced leads to a systemic inflammatory response. Thus inflammatory mediators play a key role in the pathogenesis of acute pancreatitis (Bhatia 2002; Bhatia 2004; Bhatia, Brady *et al.* 2000; Bhatia and Moochhala 2004; Bhatia, Neoptolemos *et al.* 2001).

SFKs specifically Src has been widely studied in tumorigenesis. However, latest studies have revealed that SKFs are among the most important families for the intracellular signal transduction related to acute inflammatory responses (Armstrong 2004; Lowell 2004; Yuan 2002). Several animal studies have shown that inhibition of SFKs with small chemical inhibitors prevented ischemia-reperfusion-induced injury in the brain and heart.

Moreover, blockade of SFKs attenuated sepsis, acute lung injury, and other organ damage (Akiyama, Yuguchi *et al.* 2004; Khadaroo, He *et al.* 2004; Kusaka, Ishikawa *et al.* 2004; Lennmyr, Ericsson *et al.* 2004; Paul, Zhang *et al.* 2001; Severgnini, Takahashi *et al.* 2005; Weis, Shintani *et al.* 2004).

SFKs are activated in response to the stimulation of a variety of cell surface receptors (Thomas and Brugge 1997). One class of receptor is GPCR. The neuropeptide bombesin has been shown to induce a rapid and transient increase in the activation of SFK (Rodríguez-Fernández and Rozengurt 1996). Once activated Src is then capable of interacting with and activating several substrates, such as Shc, Rho GTPase-activating protein p190 and the transcription factor STAT3 (Brown and Cooper 1996). Previous studies have demonstrated that both v-Src and c-Src are capable of activating STAT3 in fibroblasts (Bromberg, Horvath *et al.* 1998; Turkson, Bowman *et al.* 1999). The STAT family of proteins has been implicated in the function of a wide range of cells (Akira 2000; Bromberg and Darnell 2000) and it is known to activate various key inflammatory mediators, for example the cytokine signaling pathway (Severgnini, Takahashi *et al.* 2004).

Accumulating experimental evidence has suggested that substance P/NK1R as well as chemokines play critical roles in the pathogenesis of acute pancreatitis. I have shown, in chapter 2, that substance P causes an increased synthesis in CC and CXC chemokines in pancreatic acinar cells. Moreover, blockade of NK1R attenuated chemokine production in pancreatic acinar cells, as demonstrated in chapter 3, and protected mice from acute pancreatitis (Lau, Wong *et al.* 2005; Sun and Bhatia 2007).

Limited knowledge is available on the role of SFKs in chemokine production in acute pancreatitis. Therefore, the aim is to investigate the role of SFKs in mediating substance P-induced chemokine production in pancreatic acinar cells and also the underlying signal transduction mechanisms involved. Moreover, I sought to test the significance of my *in vitro* findings in an *in vivo* model of acute pancreatitis.

6.2 MATERIALS AND METHODS

6.2.1 Animal Ethics

Please refer to section 2.2.1.

6.2.2 Test system used

Mouse pancreatic acinar cells were prepared, as previously described in section 2.2.2.

6.2.3 Viability of mouse pancreatic acinar cells

The viability of mouse pancreatic acinar cells was assessed, as previously described in section 2.2.3.

6.2.4 *In vitro* experimental design

Pancreatic acinar cells (500 µl of cell suspension) were treated with substance P (Sigma-Aldrich) at a concentration of 1 µM for 0, 3, 5, 10, 15, 30 and 45min at 37˚C. Following treatment with substance P, the cells were lysed to detect for SFK activation by Western blot analysis. In some experiments, cells were either pre-treated with a potent and selective inhibitor of the Src family of protein tyrosine kinases, 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine, PP2, (Calbiochem) at 1 and 10 µM or a negative control for the Src family protein tyrosine kinase inhibitor, 4-Amino-7-phenylpyrazol [3,4-d] pyrimidine, PP3, (Calbiochem) at 1 µM for 30 min followed by stimulation with 1 µM substance P or vehicle for 10 or 45 min at 37°C. Subsequently, the supernatant was used for chemokine detection and the pellet was used for either nuclear extract, to detect STAT3, NFκB (p65) and AP-1(c-Jun) activation, or cell lysis for Western blot analysis to detect SFKs, ERK and JNK. In another experiment, isolated pancreatic acinar cells were pre-incubated with the selective NK1R antagonist, CP96345,

at 1 μM (Pfizer Diagnostics) for 30 min followed by treatment with 1 μM substance P or vehicle for 10 min at 37°C. Subsequently, the cells were lysed and used for Western blot analysis to detect SFKs.

6.2.5 Preparation of total cell lysates for Western blot analysis

Total cell lysates for Western blot analysis were prepared, as previously described in section 3.2.5.

6.2.6 Western blot analysis

Cell lysates (50 μg of protein) were separated on 12% SDS-polyacrylamide gel and electrophoretically transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Non-specific binding was blocked by 1 h incubation of the membranes, at room temperature, in 5% nonfat dry milk in phosphate buffered saline Tween 20 (PBST) (0.05% Tween 20 in phosphate buffered saline). The blots were then incubated overnight at 4°C with the primary antibodies (at a 1:1000 dilutions in the buffer containing 2.5% nonfat dry milk in PBST) phospho-Src family, phospho-ERK1/2, phospho-SAPK/JNK, (Cell Signaling Technology) and HPRT, purchased from Santa Cruz Biotechnology. HPRT was used as the housekeeping protein. Phospho-Src Family (Tyr416) antibody detects endogenous levels of Src only when phosphorylated at tyrosine 416. The antibody may cross-react with other Src family members (Lyn, Fyn, Lck, Yes and Hck) when phosphorylated at equivalent sites. It does not cross-react with Src phosphorylated at tyrosine 527. After which, the membranes were washed four times with PBST, and finally incubated for 1 h at room temperature with goat anti-rabbit HRP-conjugated secondary antibody (Santa Cruz Biotechnology) at 1:2000 dilutions in the buffer containing 2.5% nonfat dry milk in PBST. The blots were developed for visualization

using enhanced chemiluminescence (ECL) detection kit (Pierce, Rockford, IL). The densities of the bands were quantified using a UVP GelDoc-It Imaging Systems.

6.2.7 Nuclear extract preparation

Nuclear cell extract was prepared, as previously described in section 2.2.6.

6.2.8 STAT3 DNA-binding activity

The binding of STAT3 to DNA was measured in nuclear extracts with ELISA-based TransAM STAT3 assay kit (Active Motif, SciMed, Asia). This assay uses multi-well plates coated with an unlabeled oligonucleotide containing the consensus binding site for STAT (5'-TTCCCGGAA-3'). Nuclear proteins (5 μg) were added to each well and incubated for 1 h at room temperature to allow STAT3 DNA binding. Subsequently, by using an antibody that is directed against STAT3 subunit, the STAT3 complex bound to the oligonucleotide is detected. Addition of the secondary antibody conjugated to horseradish peroxidase (HRP) provides sensitive colorimetric readout that is quantified by spectrophotometry. Absorbance was read at 450 nm within 5 min by using a 96-well microplate reader (Tecan Systems, San Jose, CA). The wild-type consensus oligonucleotide is provided as a competitor for STAT3 binding to monitor the specificity of the assay. Results were expressed as fold increase over the control group.

6.2.9 NFκB DNA-binding activity

The binding of NFκB to DNA was measured in nuclear extracts, as previously described in section 2.2.7.

6.2.10 AP-1 DNA-binding activity

The binding of AP-1 to DNA was measured in nuclear extracts, as previously described in section 3.2.9.

6.2.11 Chemokine detection

Pancreatic acinar cell supernatants and pancreatic homogenate were assayed for MCP-1, MIP-1α and MIP-2 using a sandwich ELISA, as previously described in section 2.2.5.

6.2.12 Induction of Acute Pancreatitis

Swiss mice (20-25 g) were randomly assigned to control or experimental groups using 10 or more animals for each group. Animals were given hourly intra-peritoneal (i.p.) injections of normal saline or saline containing caerulein (50 μg/kg) for 10 h. PP2 at doses of 0.5, 1.0 and 1.5 mg/kg or PP3 at a dose of 1.0 mg/kg was administered to mice i.p. either 1h before or 1 h after the first caerulein injection. 1 h after the last caerulein injection animals were sacrificed by an i.p. injection of a lethal dose of pentabarbitone. Harvested heparinized blood was centrifuged, the plasma removed and stored at -80°C. Random cross-sections of the head, body, and tail of the pancreas were fixed in 4% neutral phosphate-buffered formalin then embedded in paraffin wax. Freshly harvested sample of pancreas was weighed and then dried and reweighed to determine pancreatic water content (Bhatia, Saluja *et al.* 1998). Samples of pancreas were stored at -80°C for subsequent measurement of tissue myeloperoxidase (MPO) activity, to perform ELISA and Western blot analysis.

6.2.13 Amylase estimation

Amylase activity was measured using a kinetic spectrophotometric assay. Plasma samples were incubated with the substrate, 4, 6-ethylidene (G_7)-p-nitrophenyl (G_1)-1-D-

maltoheptoside (Sigma, St. Louis, MO, USA) for 2 minutes at 37°C and absorbance measured every minute for the subsequent 2 minutes at 405 nm (Bhatia, Brady *et al.* 2000; Bhatia, Saluja *et al.* 1998; Pierre, Tung *et al.* 1976). The change in absorbance was used to calculate the amylase activity.

6.2.14 Myeloperoxidase estimation

Neutrophil sequestration in pancreas was quantified by measuring tissue MPO activity (Bhatia, Brady *et al.* 2000; Bhatia, Saluja *et al.* 1998). Tissue samples were thawed, homogenised in 20 mM phosphate buffer (pH 7.4), centrifuged (10,000 × g, 10 min, 4°C) and the resulting pellet resuspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma, St. Louis, MO, USA). The suspension was subjected to four cycles of freezing and thawing and further disrupted by sonication (40 sec). The sample was then centrifuged (10,000 × g, 5 min, 4°C) and the supernatant used for the MPO assay. The reaction mixture consisted of the supernatant, 1.6 mM tetramethylbenzidine (Sigma, St. Louis, MO, USA), 80 mM sodium phosphate buffer (pH 5.4), and 0.3 mM hydrogen peroxide. This mixture was incubated for 120 sec, the reaction terminated with 2M H_2SO_4 and the absorbance measured at 450 nm. This absorbance was then corrected for the protein content of the tissue sample.

6.2.15 Morphological Examination

Paraffin-embedded pancreas samples were sectioned (5 μm), stained with hematoxylin/eosin, and examined under light microscopy at ×400 magnifications. For these studies, 8 randomly chosen microscopic fields (x125) were examined for each tissue sample. The criteria used while choosing the pictures were the presence of acinar-cell ghosts, vacuolization, swelling of acinar cells and the destruction of the

histoarchitecture of whole or parts of the acini, all of which had been associated with inflammatory reaction.

6.2.16 Statistical Analysis

Data are expressed as the mean + standard error of the mean (SEM). In all figures, vertical bars denote the SEM and the absence of such bars indicates that the SEM is too small to illustrate. The significance of changes was evaluated by using Student's *t* test when the data consisted of only two groups or by analysis of variance (ANOVA) when comparing three or more groups. If ANOVA indicated a significant difference, the data were analyzed by using Tukey's method as a post hoc test for the difference between groups. A P value of < 0.05 was considered to indicate a significant difference.

6.3 RESULTS

6.3.1 Substance P/NK1R induces a rapid and transient increase in phosphorylation of Src family (Tyr 416) in mouse pancreatic acinar cells

Pancreatic acinar cells were stimulated with 1 μM substance P or vehicle (saline) for 0, 3, 5, 10, 15, 30 and 45 min at 37˚C. Cells were then lysed, and cell proteins were subjected to Western blot analysis. As shown in Figure 6.1 (a, b), substance P induced phosphorylation of Src family (Tyr 416) in a time dependent manner up to 10 min, followed by a time-dependent decrease. Densitometric analysis of Western blot experiments revealed a significant increase in phosphorylation Src family (Tyr 416) at 10 and 15 min when compared to 0 min control. No significant change was observed when pancreatic acinar cells were treated with the vehicle at different time points. Pancreatic acini were also pre-treated with 1 μM of CP96345, a selective NK1R antagonist, for 30 min followed by stimulation with 1 μM of substance P for 10 min. Cells were then lysed, and cell proteins were subjected to Western blot analysis. As demonstrated in Figure 6.1 (c, d), CP96345 significantly reduced substance P-induced phosphorylation of Src family (Tyr 416) in pancreatic acinar cells. No significant change was detected in the housekeeping protein HPRT. This shows that substance P-induced phosphorylation of Src family is mediated through substance P/NK1R in pancreatic acinar cells.

6.3.2 Involvement of SFKs in substance P-induced MAP Kinases in pancreatic acinar cells.

Stimulation of pancreatic acinar cells with 1 μM substance P significantly upregulated phosphorylation of Src family (Tyr 416), ERK and JNK. Pancreatic acinar cells were pre-treated for 30 min with the selective inhibitor of the Src family of protein tyrosine

kinases, PP2, at 1 and 10 μM, followed by stimulation with 1 μM of substance P for 10 min. Cells were then lysed, and cell proteins were subjected to Western blot analysis. As shown in Figure 6.2, PP2 decreased substance P-induced phosphorylation of Src family (Tyr 416), hence confirming its inhibitory effect. Moreover, PP2 attenuated substance P-induced phosphorylation of ERK and JNK in pancreatic acinar cells. No significant change was detected in the housekeeping proteins HPRT.

6.3.3 Substance P-induced SFKs is involved in activation of STAT3, NFκB and AP-1 in pancreatic acinar cells.

Pancreatic acinar cells were pre-incubated for 30 min with PP2, at 1 and 10 μM followed by stimulation with 1 μM of substance P for 45 min. STAT3, NFκB and AP-1 DNA-binding assay revealed that treatment with substance P led to a notable increase in the activity of STAT3, NFκB and AP-1 in pancreatic acinar cell. As shown in Figure 6.3 (a, b, c), pre-treatment with PP2 attenuated substance P-induced DNA-binding activity of STAT3, NFκB and AP-1.

6.3.4 SFKs mediate substance P-induced production of CC and CXC chemokines in pancreatic acinar cells.

Treatment of pancreatic acini with 1 μM substance P resulted in increased production of CC chemokine MCP-1, MIP-1α and CXC chemokine MIP-2. Pancreatic acinar cells were pre-treated with either PP2 at 1 and 10 μM or its negative control PP3 at 1 μM for 30 min followed by stimulation with 1 μM of substance P for 45 min. The protein levels of these chemokines were determined by ELISA. Figure 6.4 (a, b, c) shows that pre-treatment with PP2 markedly decreased MCP-1, MIP-1α, and MIP-2 production. However,

treatment with the negative control PP3 had no significant effect on chemokine production when compared to substance P only treated cells.

6.3.5 Effect of prophylactic and therapeutic treatment with PP2 on the severity of caerulein-induced acute pancreatitis.

Acute pancreatitis was induced by intraperitoneal administration of caerulein at a concentration of 50 μg/kg hourly for 10 h. Evidence of pancreatic injury in acute pancreatitis mice was confirmed by a rise in plasma amylase, Figure 6.5 (a, b); and an increase in pancreatic edema as evidenced by elevated pancreatic water content, Figure 6.5 (c, d). Animals were administered either PP2 or PP3 1h before or after starting caerulein injection. Both prophylactic and therapeutic treatment with PP2 significantly attenuated plasma amylase levels and pancreatic edema when compared with animals treated with caerulein alone, Figure 6.5 (a, c). Histological examination of pancreas sections confirmed a protection by PP2 treatment on acute pancreatitis in terms of acinar cell injury/necrosis, neutrophil infiltration as well as edema, Figure 6.6. However, prophylactic and therapeutic treatment with PP3 had no protective effect on pancreatic injury in acute pancreatitis when compared to mice treated with caerulein alone.

6.3.6 Involvement of SFKs in the mobilization of neutrophils and chemokines in acute pancreatitis.

Induction of acute pancreatitis by caerulein hyperstimulation resulted in heightened pancreatic MPO, a measure of neutrophil infiltration. Acute pancreatitis also resulted in increased level of pancreatitc CC chemokine MCP-1, MIP-1α and CXC chemokine MIP-2 when compared to vehicle-treated animals. Prophylactic treatment with PP2 resulted in a dose-dependent decrease in MPO activity, MCP-1, MIP-1α and MIP-2 levels in the

pancreas when compared to caerulein-treated animals. Moreover, mice administered with 1mg/kg PP2 therapeutically had a significant reduction in pancreatic MPO activity, chemokines MCP-1, MIP-1α and MIP-2 levels when compared to caerulein treated mice, Figure 6.7.

6.3.7 Inhibition of SFKs attenuated the activation of pancreatic STAT3, NFκB, AP-1 and MAP Kinases in acute pancreatitis

Caerulein-induced acute pancreatitis resulted in significant activation of transcription factors STAT3, NFκB and AP-1 in pancreas when compared to vehicle-treated animals. Treatment with PP2 both prophylactically and therapeutically significantly attenuated the activation of pancreatic STAT3, NFκB and AP-1 when compared to caerulein-treated animals, Figure 6.8 (a, b, c). To further explore the signaling pathways mediated by SRC tyrosine kinases in acute pancreatitis, I have investigated the MAP kinases' pathway. Acute pancreatitis increased phosphorylation of MAP kinases ERK and JNK in pancreas when compared to vehicle-treated animals. Both prophylactic and therapeutic treatment with PP2 significantly attenuated activation of ERK and JNK when compared to caerulein-treated mice, Figure 6.8 (d, e, f). No significant change was observed in the housekeeping protein HPRT in the pancreas.

6.4 DISCUSSION

SFKs are signaling proteins that participate in several cell signaling pathways and mediate a wide spectrum of cellular activities such as proliferation, differentiation, survival, adhesion, and migration. Moreover, SFKs have been shown to play a key role in cytokine signaling and inflammatory response (Chaturvedi, Reddy *et al.* 1998; Song, Turkson *et al.* 2003). However, the involvement of SFKs in substance P-induced chemokine production and also its role in acute pancreatitis have not been investigated yet.

In this study, I have demonstrated that substance P induced a rapid and transient activation of SFKs as evidence by the phosphorylation of Src family (Tyr 416) in pancreatic acinar cells. I further confirmed that activation of SFKs was indeed mediated by substance P. I observed a significant reduction in phosphorylation of Src family (Tyr 416) when pancreatic acinar cells were pre-treated with its selective NK1R antagonist. SFKs also mediated substance P-induced activation of MAP Kinases ERK and JNK. My results are in agreement with several other studies which have found that Src activation was induced by GPCR agonists, and it has also been implicated in promoting Ras dependent ERK activation (Della Rocca, van Biesen *et al.* 1997). Src also acted as a mediator of substance P-stimulated ERK1/2 phosphorylation in human U373 MG glioblastoma cells (Yamaguchi, Kugimiya *et al.* 2005). Src was found to be a major component of the substance P receptor signaling pathway as blockade of the Src inhibited the activation of ERK1/2 and PKC δ in U373 MG human glioblastoma cells (Yamaguchi, Richardson *et al.* 2005). Another group has shown that substance P activation of NK1R stimulated the formation of a scaffolding complex comprising of the internalized

receptor, beta-arrestin, Src and ERK1/2 to mediate its downstream effects (DeFea, Vaughn 2000).

It is known that SFKs plays an important role in cytokine signaling, however only a limited number of studies have invesstigated the role of SFKs in chemokine production. In the current study, I have illustrated that substance P-induced phosphorylation of Src tyrosine kinases is involved in activation of of transcription factors STAT3, NFκB, AP-1 and production of chemokines MCP-1, MIP-1α and MIP-2 in pancreatic acinar cells. Studies have shown that MAP Kinase 1, 2 phosphorylates Ser727 on STAT3 and phosphorylation of this residue is thought to modulate the transcriptional activity of STAT3 (Chung, Uchida *et al.* 1997). Additionally, activation of JNK/SAPK is thought to be required for v-Src activation of STAT3 (Turkson, Bowman *et al.* 1999). In parallel with NFκB, STATs also regulate the expression of genes that are critically involved in inflammatory and immune responses (Akira 2000).

As shown in chapters 2 and 3, NFκB and AP-1 are involved in substance P induced chemokine synthesis. Substance P-induced activation of SFKs most likely is mediating chemokine production through the MAP Kinases ERK and JNK induced transcription factors NFκB and AP-1. However, whether substance P-induced STAT3 activation is directly involved in the production of chemokines remained to be investigated. On one hand, studies have suggested that STAT3 plays a pivotal role in orchestrating inflammatory responses by increasing the expression of inflammatory mediators, cytokines and chemokines (Schumann, Kirschning *et al.* 1996; Takeda, Clausen *et al.* 1999). STAT3 might be playing a part in the initiation of lung injury as it was found to be activated very early in the lungs in different models of acute lung injury (Gao, Guo *et al.*

2004; Severgnini, Takahashi *et al.* 2004). However, on the other hand, studies have revealed that STAT3 modulates anti-inflammatory responses (Matsukawa, Takeda *et al.* 2003). STAT3 was found to mediate IL 10 production (Opal and DePalo 2000) which is a potent anti-inflammatory cytokine. STAT3 expressed in macrophages and neutrophils might negatively regulate inflammation (Riley, Taked *et al.* 1999). Studies have also shown that STAT3-/- mice were susceptible to endotoxemia due to high levels of inflammatory cytokines TNFα, IL1β and IFNγ (Takeda, Clausen *et al.* 1999). Hence, more work need to be carried out to elucidate the complexity of STAT3 signaling mechanisms in acute pancreatitis.

I have used primary culture of isolated pancreatic acinar cells as my model to show that substance P-induced activation of Src tyrosine kinases is involved in MAP Kinases ERK and JNK mediated STAT3, NFκB and AP-1 driven chemokine synthesis. I further sought to test the significance of SFKs signaling pathway in acute pancreatitis. My results show that treatment of animals with the potent and selective inhibitor of the Src family of protein tyrosine kinases PP2 but not its negative inhibitor PP3 (either prophylactic or therapeutic) reduces the severity of pancreatitis as evidenced by a significant attenuation of hyperamylasemia, pancreatic MPO activity, chemokines and water content which is a measure of edema. Moreover, histological evidence of diminished pancreatic injury such as acinar cell injury/necrosis, neutrophil infiltration as well as edema also confirmed the protective effect of the inhibition of SFKs on acute pancreatitis. In line with my study, Src tyrosine kinase inhibitors were found to suppress inflammatory responses *in vivo* by blocking the function of inflammatory cells including neutrophils, monocytes and macrophages (Okutani, Lodyga *et al.* 2006). Previous studies have demonstrated that Src

tyrosine kinases are rapidly activated in the lungs in an LPS model of acute lung injury. Systemic inhibition of Src tyrosine kinases using specific small molecule inhibitor PP2 significantly attenuated LPS-induced lung injury by blocking LPS-dependent cytokine and chemokine production in the lungs (Severgnini, Takahashi *et al.* 2005)

Using the *in vivo* model of acute pancreatitis, I further explored the molecular mechanisms through which SFKs protected against acute pancreatitis, I found that SFKs mediate their protective effects through the same signaling pathway that I have shown in my *in vitro* model of isolated acinar cells. Acute pancreatitis resulted in elevated activation of pancreatic MAP kinases ERK and JNK, transcription factors STAT3, NFκB, and AP-1. Both prophylactic and therapeutic treatment with PP2 significantly attenuated activation of ERK, JNK, STAT3, NFκB and AP-1 and hence resulted in protection against acute pancreatitis.

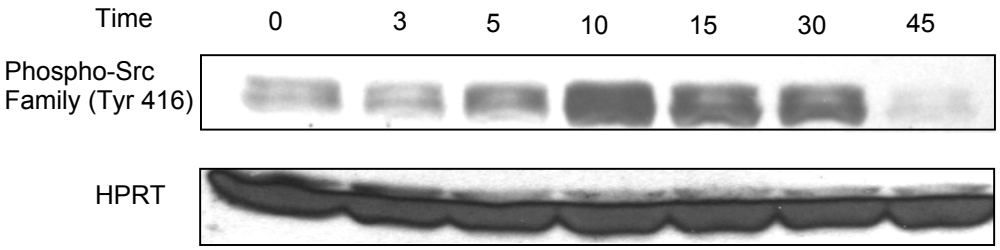
Based on my results I proposed that elevated levels of substance P, which is produced as a result of acute pancreatitis, bind to NK1R to activate several signaling molecules to mediate chemokine production. One such signaling complex is SFKs and its blockade attenuated MAP Kinases, STAT3, NFκB, AP-1 and chemokines, thus resulting in protection against acute pancreatitis. It is difficult to determine solely on the basis of pharmacological studies which group of Src family members is involved in acute pancreatitis as the selectivity of these inhibitors to different Src protein tyrosine kinases has not been fully tested. The proposed signaling pathway through which substance P mediates acute pancreatitis is through substance P/NK1R - SFKs - (ERK, JNK) - (STAT3, NFκB, AP-1) - (MCP-1, MIP-1α, MIP-2). Moreover, these results further

confirmed the credibility of using isolated pancreatic acinar cells as valid model to test pathogenesis of acute pancreatitis

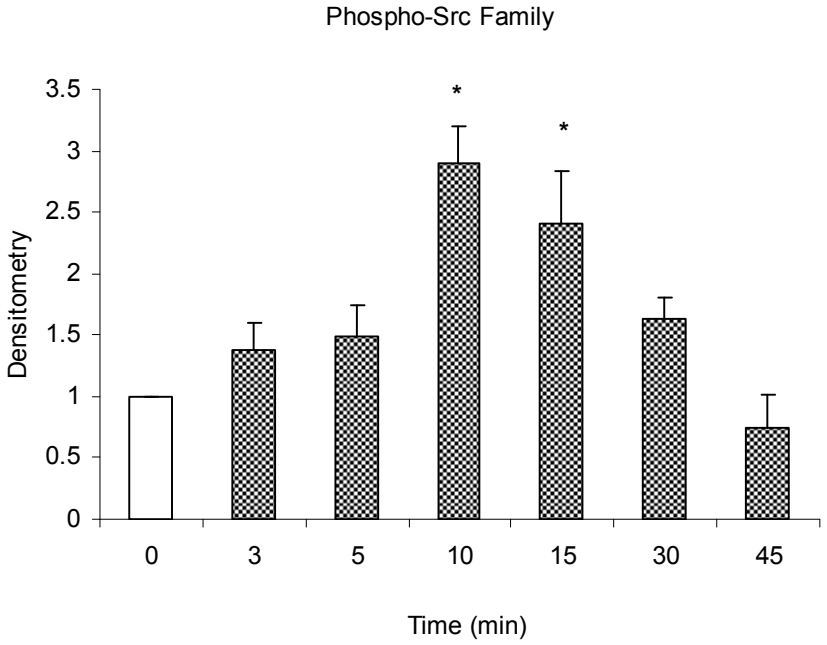
The mechanism of activation of SFKs in acute pancreatitis is most likely to be multifactorial, however one of the factors involved is substance P. Using a clean system of isolated pancreatic acinar cells I were able to show that substance P/NK1R indeed induces activation of SRC tyrosine kinases. Blockade of SFKs attenuated chemokine production both *in vitro* and *in vivo* as well as protected the mice against acute pancreatitis. The study presented in this chapter gives us a deeper insight into the mechanisms by which substance P contributes to acute pancreatitis. Increased understanding of the signal transduction mechanisms involved in acute pancreatitis would facilitate the discovery of novel therapeutic compounds useful in treating inflammatory disorders such as acute pancreatitis.

Figure 6.1

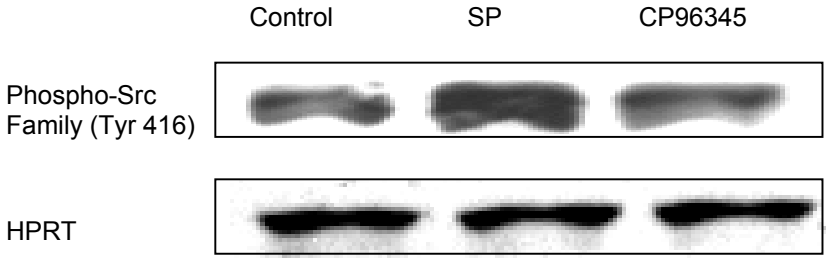
(a)


Time
0
3
5
10
15
30
45
Phospho-Src Family (Tyr 416)
HPRT

(b)


Phospho-Src Family
Densitometry
3.5
3
2.5
2
1.5
1
0.5
0
*
*
0
3
5
10
15
30
45
Time (min)

(c)



(d)

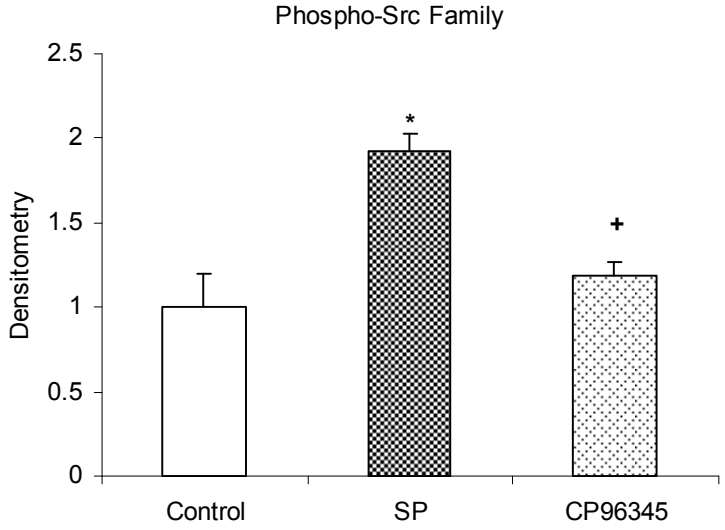
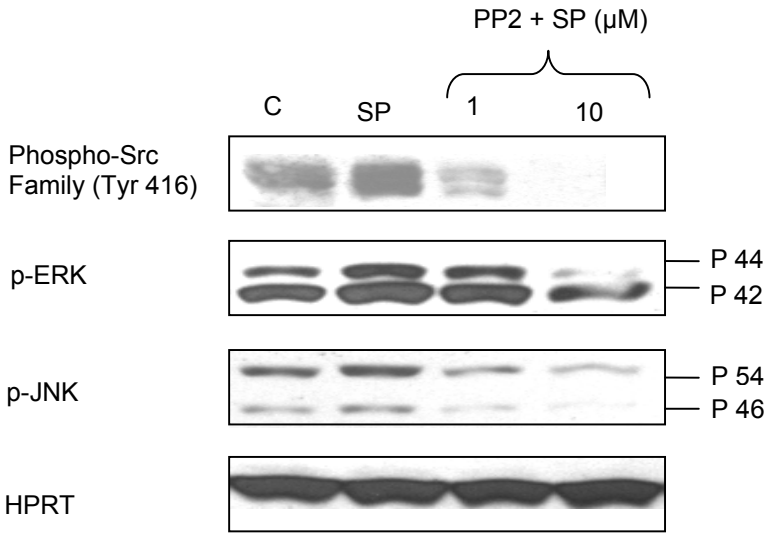


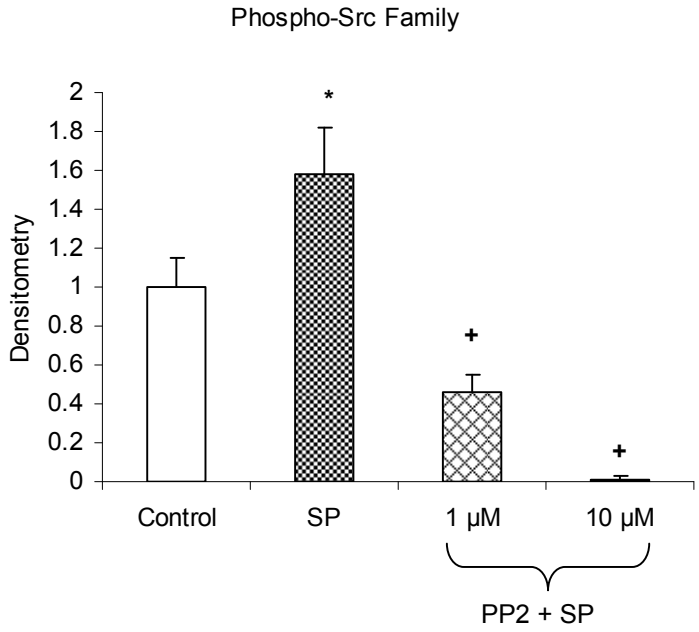
Figure 6.1 Substance P (SP)/NK1R induces a time dependent increase and decrease in phosphorylation of Src family (Tyr 416) in mouse pancreatic acinar cells. Freshly isolated pancreatic acini were incubated with either 1 μM SP/vehicle (saline) for 0, 3, 5, 10, 15, 30 and 45 min at 37˚C or pre-incubated with 1 μM CP96345 for 30 min followed by stimulation with 1 μM SP for 10 min. The cells were lysed, and cell proteins were subjected to Western blot analysis using antibodies against (a, c) phospho-Src family (Tyr 416) and HPRT. MW in (kDa) for Phospho-Src Family is 60 and HPRT is 24. Densitometric analysis of Western blot experiments were performed and the group data from 3 independent preparations (n=3) are presented in (b, d). The results are representative of three independent (n=3) experiments. Results shown are the means + SE. *$P < 0.05$ when compared to 0 min control, +$P < 0.05$ when compared to SP.

Figure 6.2

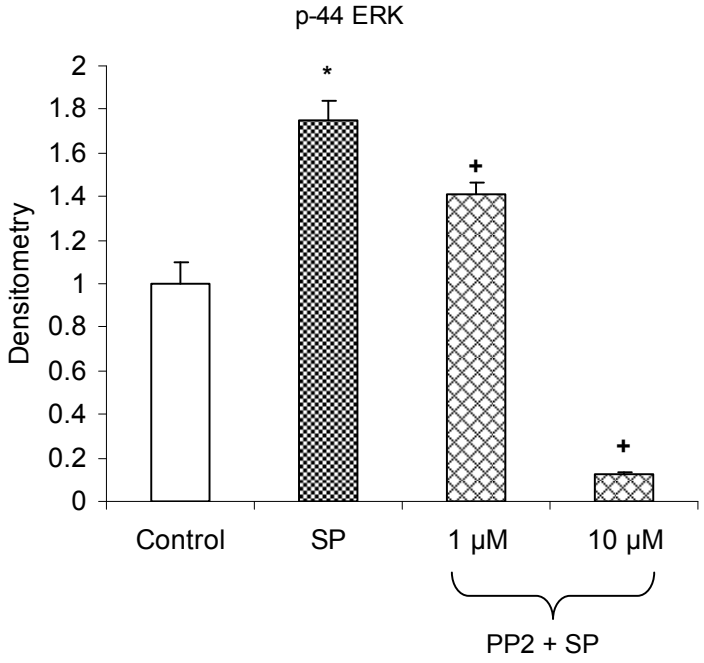
(a)

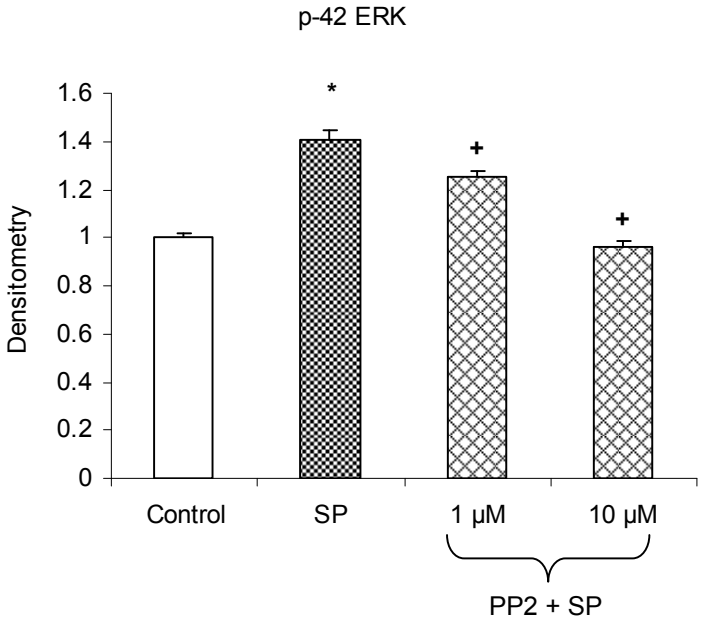

PP2 + SP (μM)
C
SP
1
10
Phospho-Src Family (Tyr 416)
p-ERK
P 44
P 42
p-JNK
P 54
P 46
HPRT

(b)

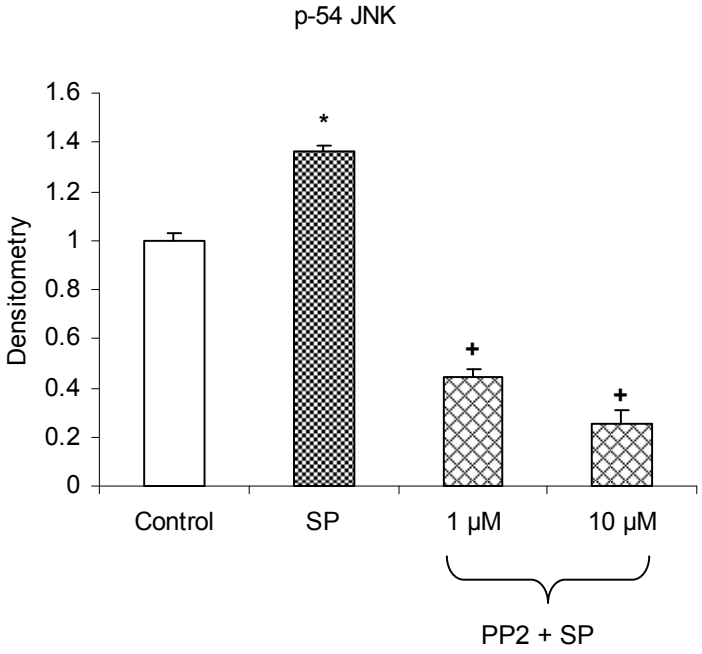

Phospho-Src Family
Densitometry
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
+
+
Control
SP
1 μM
10 μM
PP2 + SP

(c)


p-44 ERK
Densitometry
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
+
+
Control
SP
1 μM
10 μM
PP2 + SP


p-42 ERK
Densitometry
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
+
+
Control
SP
1 μM
10 μM
PP2 + SP

(d)


p-54 JNK
Densitometry
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
+
+
Control
SP
1 μM
10 μM
PP2 + SP

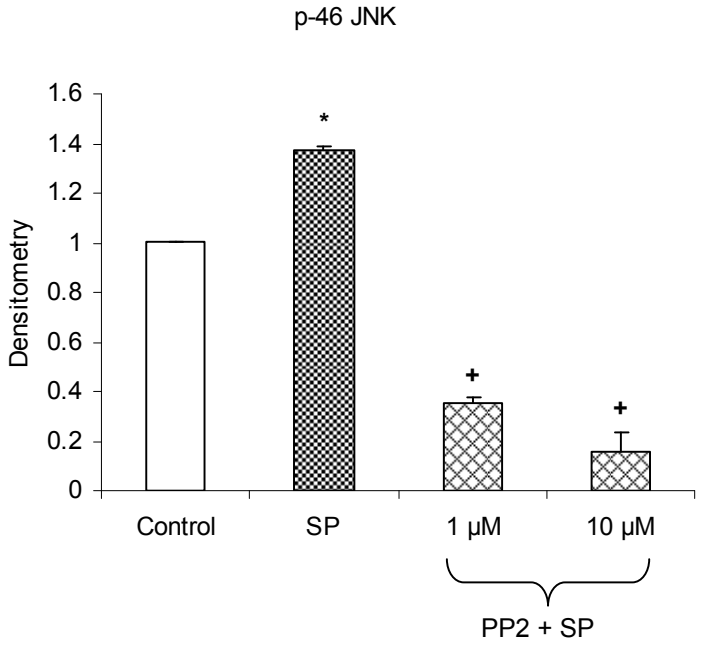
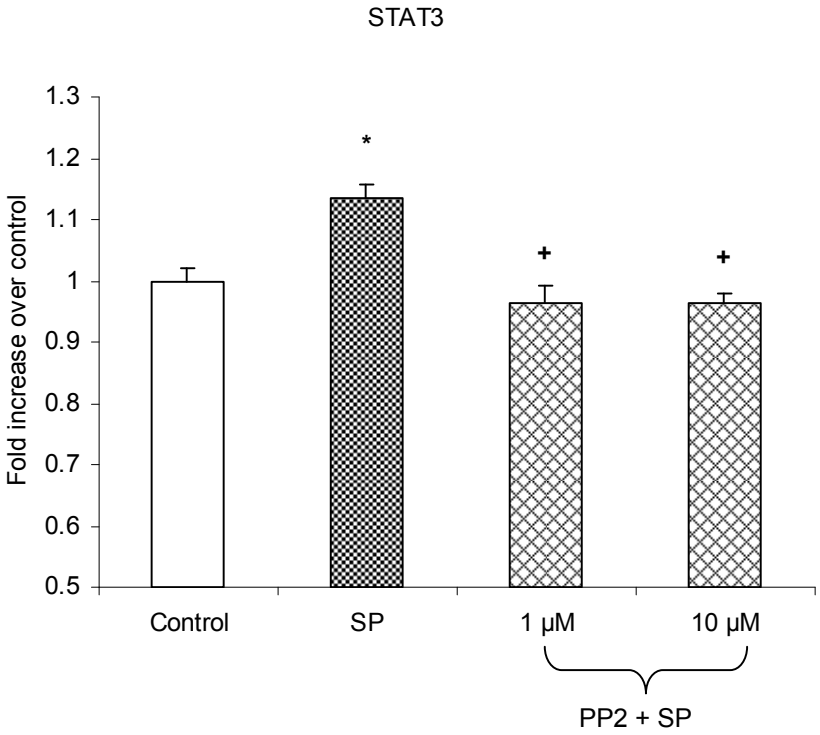

p-46 JNK
Densitometry
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
+
+
Control
SP
1 μM
10 μM
PP2 + SP

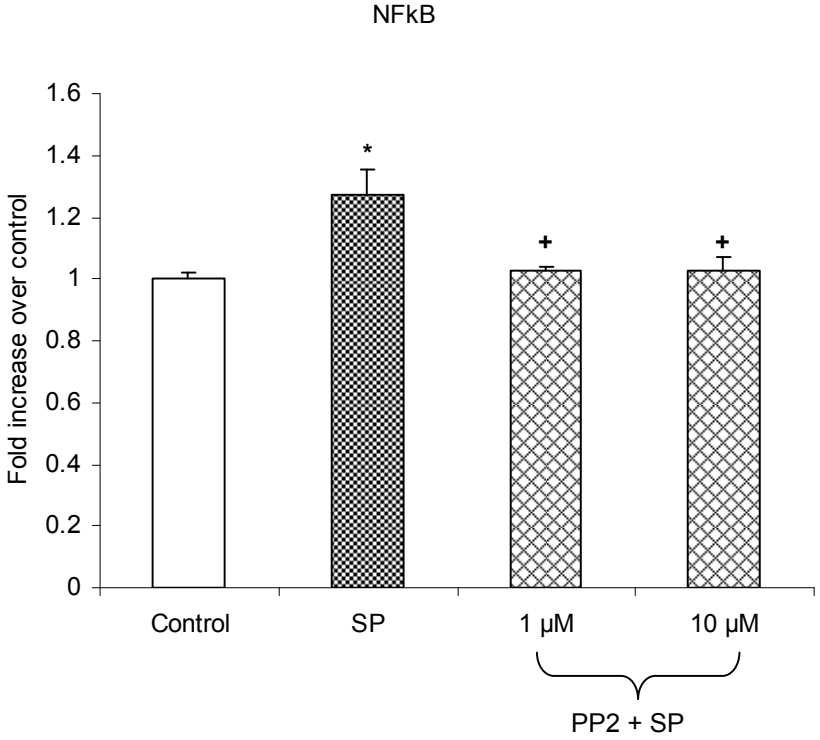
Figure 6.2 Substance P (SP)-induced activation of ERK and JNK is mediated by SFKs in pancreatic acinar cells. Freshly isolated pancreatic acini were pre-incubated with either PP2, at 1 μM, 10 μM or vehicle (DMSO) for 30 min at 37˚C followed by stimulation with 1 μM SP for 10 min at 37˚C. Cells were subsequently lysed, and cell proteins were subjected to Western blot analysis using antibodies against (a) phospho-Src family (Tyr 416), phospho-ERK, phospho-JNK and HPRT. MW in (kDa) for phospho-Src family is 60, JNK 2/1 is 54/46, ERK1/2 is 44/42 and HPRT is 24.The phosphorylated sub-units such as p-Src family, p-44 ERK, p-42 ERK, p-54 JNK and p-46 JNK have been quantified. Densitometric analysis of Western blot experiments were performed and the group data from 3 independent preparations (n=3) are presented in (b, c, d). Results shown are the means + SE. *P < 0.05 when compared to control, +P < 0.05 when compared to SP.

Figure 6.3

(a)


STAT3
Fold increase over control
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
*
+
+
Control
SP
1 μM
10 μM
PP2 + SP

(b)


NFkB
Fold increase over control
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
+
+
Control
SP
1 μM
10 μM
PP2 + SP

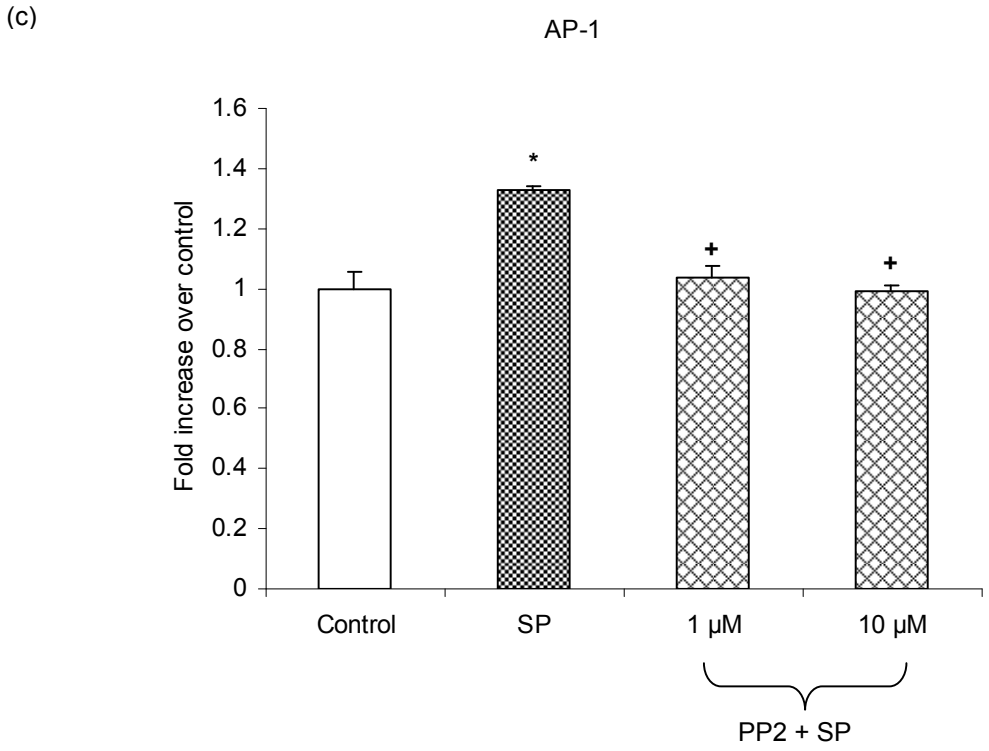
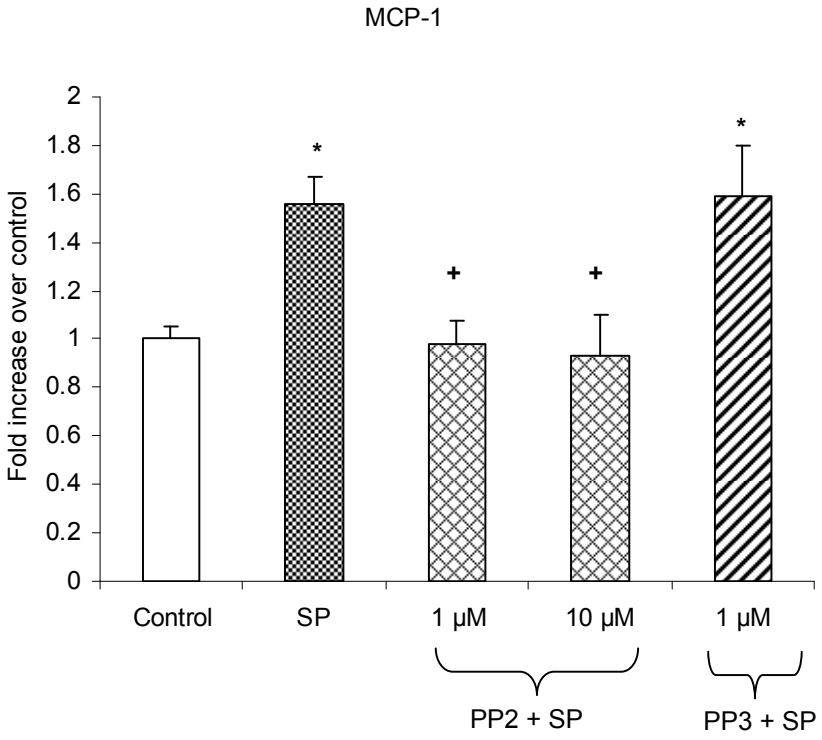


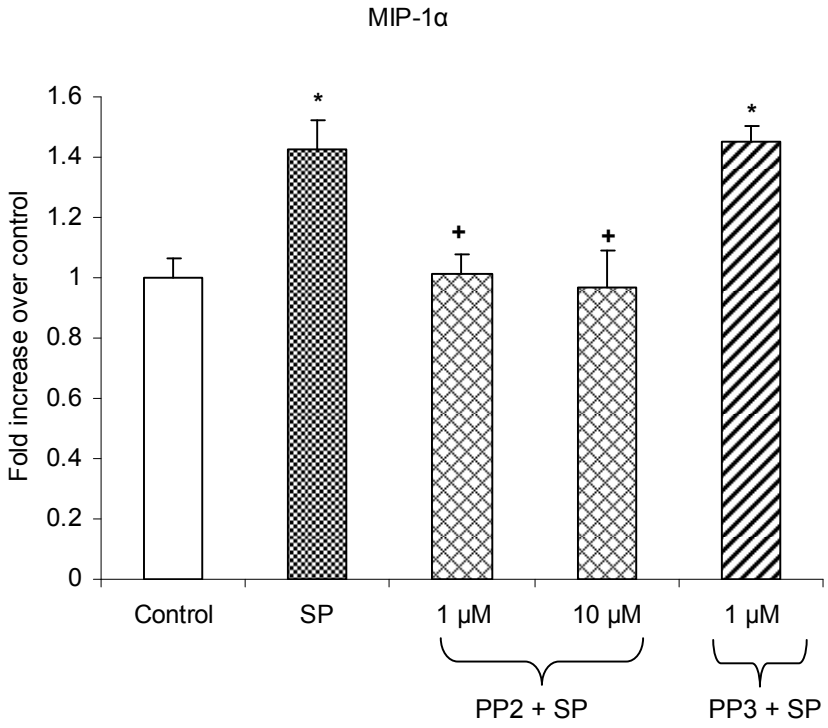
Figure 6.3 SFKs are involved in substance P (SP)-induced STAT3, NFκB and AP-1 activation in pancreatic acinar cells. Freshly isolated pancreatic acini were pre-incubated with PP2 at 1 and 10 μM of PP2 or vehicle (DMSO) followed by stimulation with 1 μM of SP for 45 min. The cells were separated from incubation medium by centrifugation. The pellet (cells) was used for (a) STAT3, (b) NFκB and (c) AP-1 nuclear extraction. STAT3, NFκB and AP-1 DNA-binding assays were then carried out as described in MATERIALS AND METHODS. The results are representative of three independent (n=3) experiments. Results shown are the means + SE. $*P < 0.05$ when compared to control, $+P < 0.05$ when compared to SP.

Figure 6.4

(a)


MCP-1
Fold increase over control
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
+
+
Control
SP
1 μM
10 μM
1 μM
PP2 + SP
PP3 + SP

(b)


MIP-1α
Fold increase over control
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
+
+
Control
SP
1 μM
10 μM
1 μM
PP2 + SP
PP3 + SP

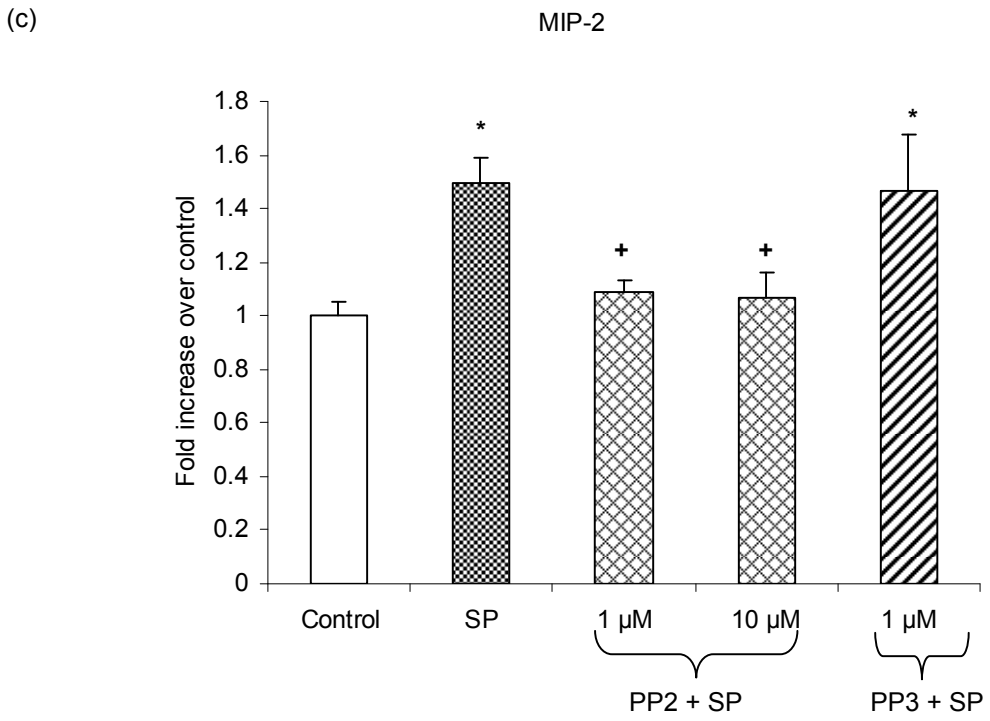
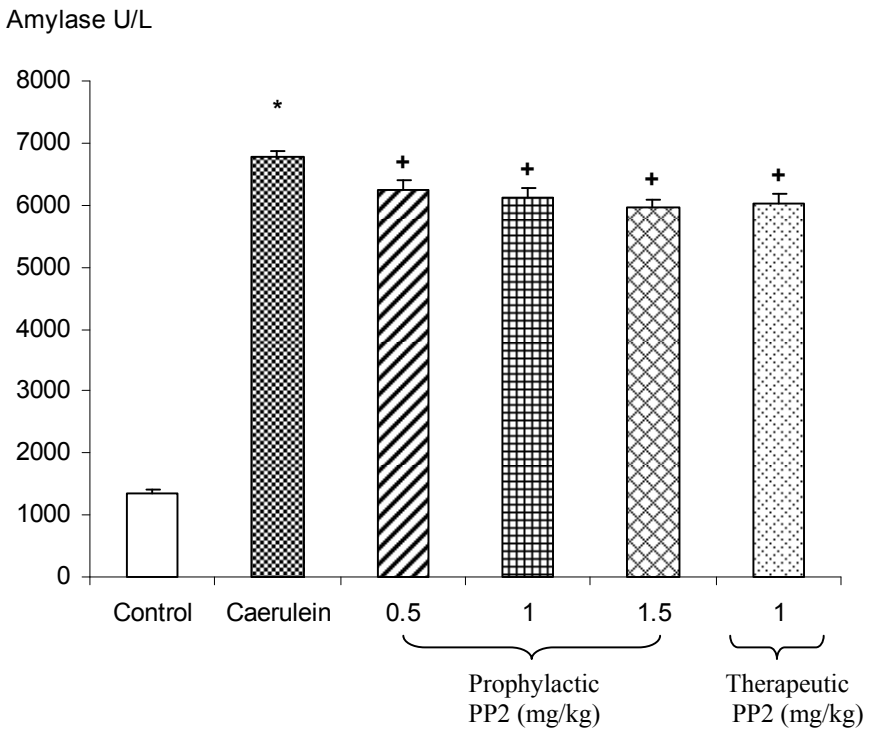


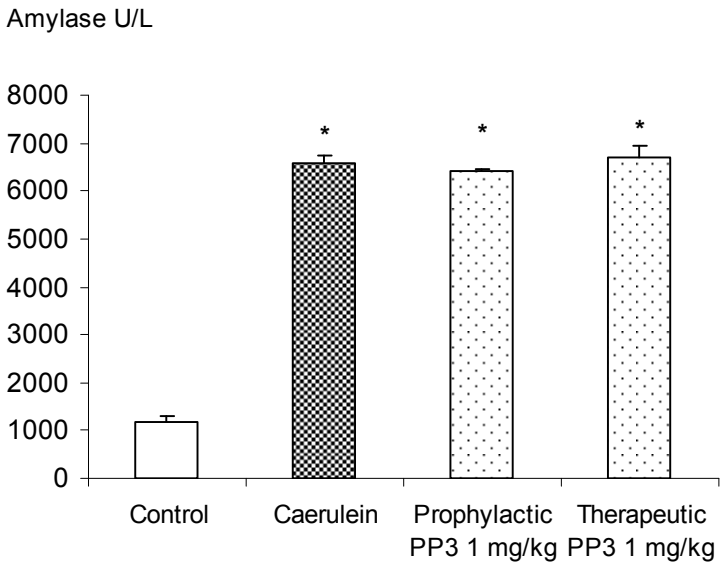
Figure 6.4 SFKs are involved in the secretion of CC and CXC chemokines in pancreatic acinar cells. Freshly isolated pancreatic acini were pre-incubated with either PP2 at 1 and 10 µM, or PP3 at 1 µM for 30 min followed by stimulation with 1 µM of substance P (SP) for 45 min. The supernatant was used to measure (a) MCP-1, (b) MIP-1α and (c) MIP-2 levels by ELISA as described in MATERIALS AND METHODS. The results are representative of three independent (n=3) experiments. Results shown are the means + SE. *****$P < 0.05$ when compared to control, +$P < 0.05$ when compared to SP.

Figure 6.5

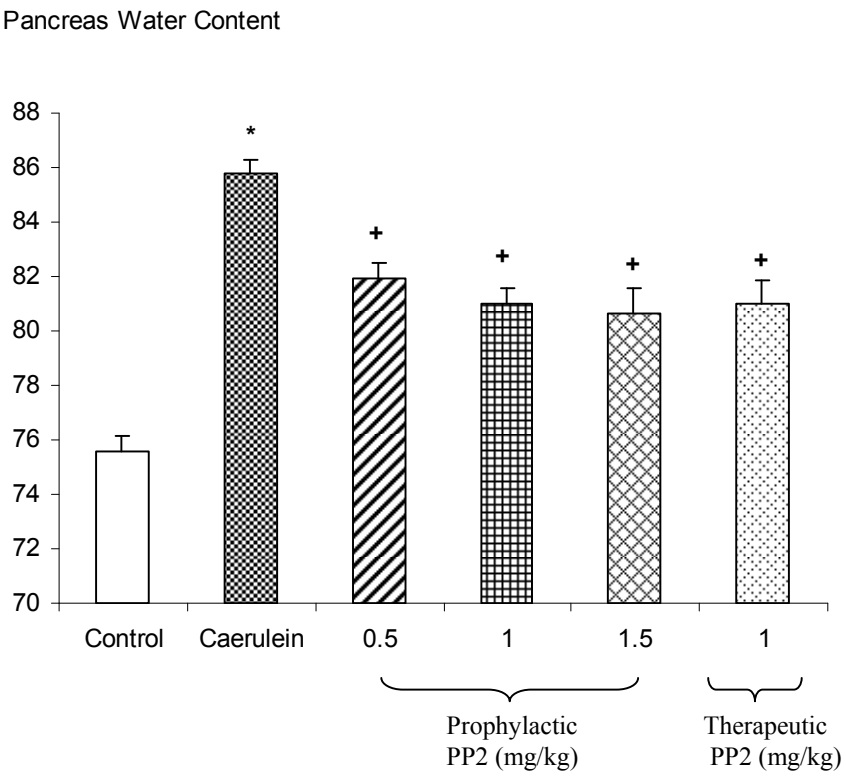
(a)


Amylase U/L
8000
7000
6000
5000
4000
3000
2000
1000
0
*
+
+
+
+
Control
Caerulein
0.5
1
1.5
1
Prophylactic
PP2 (mg/kg)
Therapeutic
PP2 (mg/kg)

(b)


Amylase U/L
8000
7000
6000
5000
4000
3000
2000
1000
0
*
*
*
Control
Caerulein
Prophylactic
PP3 1 mg/kg
Therapeutic
PP3 1 mg/kg

(c)


Pancreas Water Content
88
86
84
82
80
78
76
74
72
70
*
+
+
+
+
Control
Caerulein
0.5
1
1.5
1
Prophylactic
PP2 (mg/kg)
Therapeutic
PP2 (mg/kg)

(d)

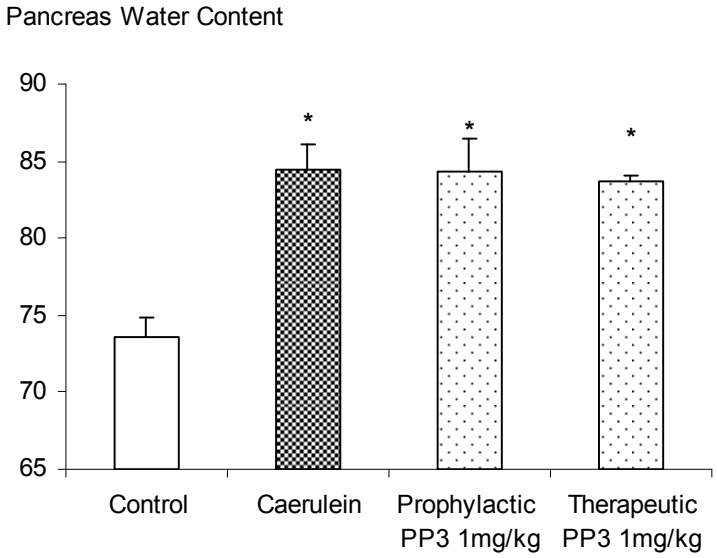

Pancreas Water Content
90
85
80
75
70
65
*
*
*
Control
Caerulein
Prophylactic
PP3 1mg/kg
Therapeutic
PP3 1mg/kg

Figure 6.5 Effects of prophylactic and therapeutic PP2 administration on the severity of acute pancreatitis. Mice (n =10 in each group) were given 10 hourly injections of caerulein (50 μg/kg i.p.). PP2 or PP3 was administered to mice i.p. either prophylatically (1h before) or therapeutically (1h after) the first caerulein injection. 1h after the last caerulein injection, mice were sacrificed by an i.p. injection of a lethal dose of pentabarbitone. Plasma amylase activity (a, b) and pancreatic edema (water content c, d) were determined as described in MATERIALS AND METHODS. Results shown are the means + SE. *$P < 0.05$ when caerulein or PP3-treated animals were compared with vehicle-treated animals. +$P < 0.05$ when PP2-treated animals were compared to caerulein-treated animals.

Figure 6.6

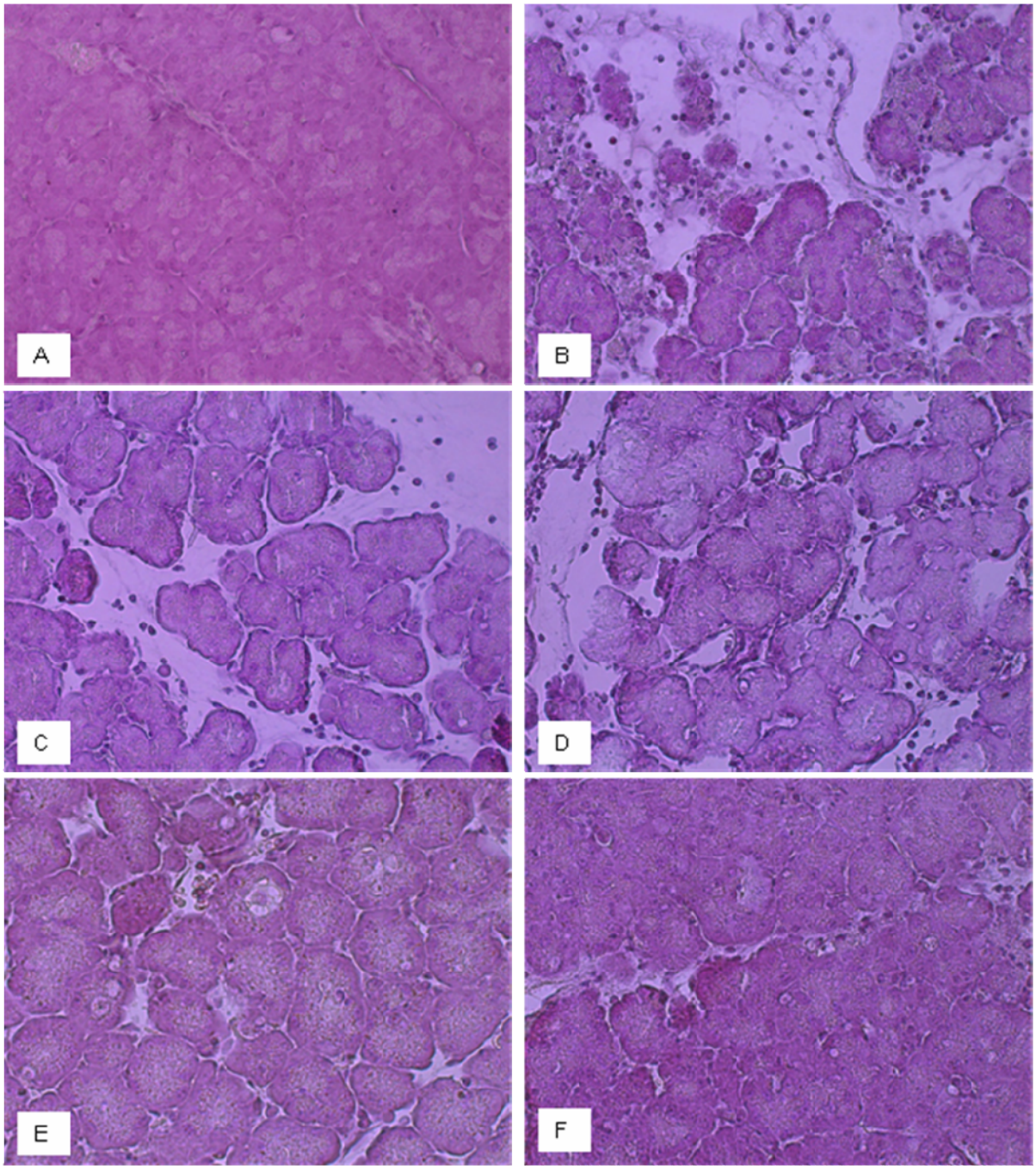
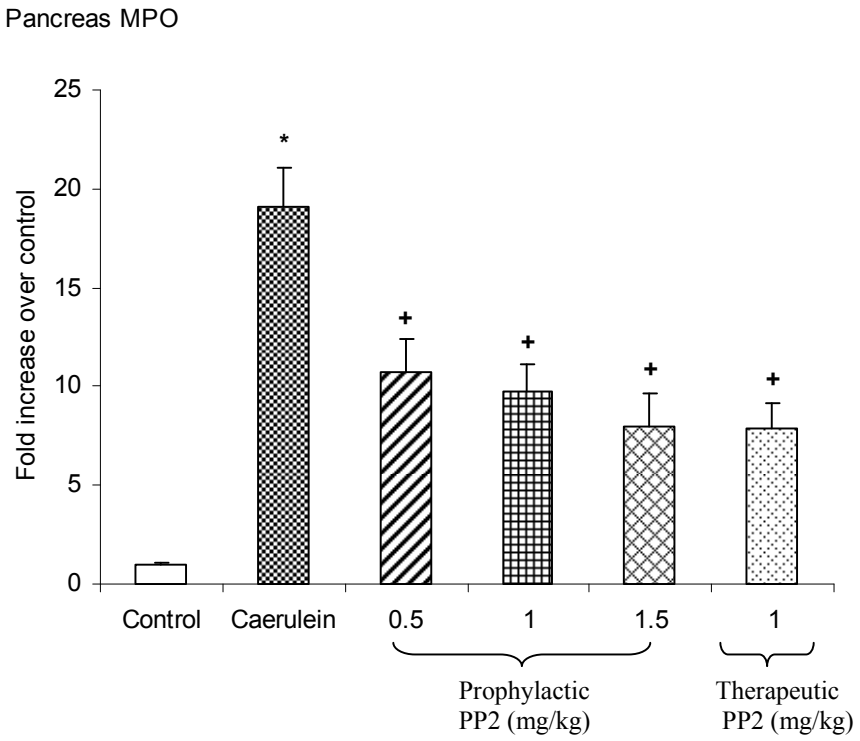


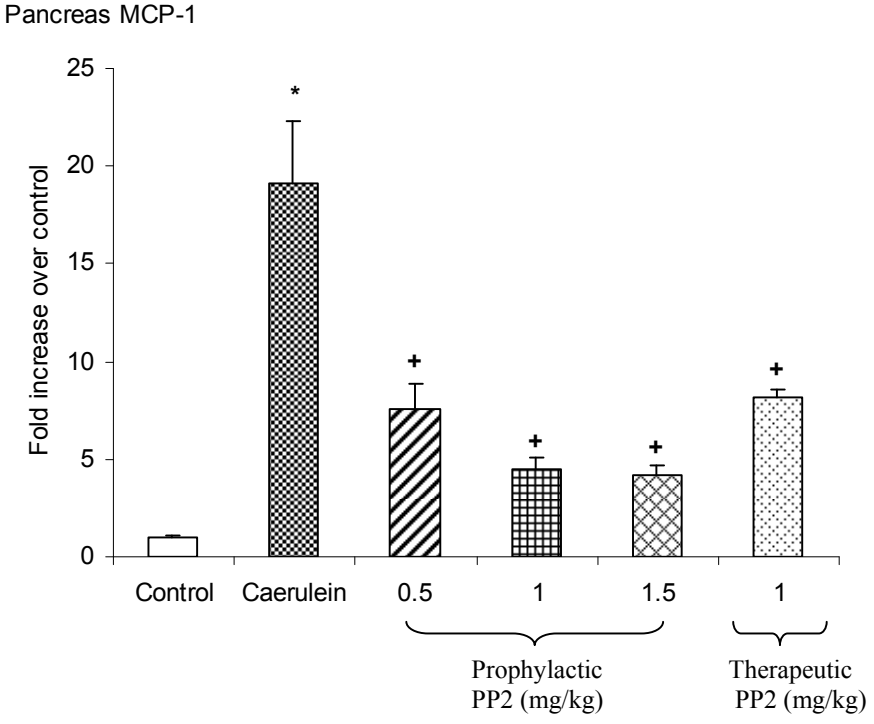
Figure 6.6 Morphological changes in mouse pancreas on induction of acute pancreatitis with/without prophylactic and therapeutic treatment with PP2 or PP3. A: control; no pancreatitis. B: caerulein-induced acute pancreatitis. C: caerulein-induced acute pancreatitis in mice administered PP3 1mg/kg (prophylactic). D: caerulein-induced acute pancreatitis in mice administered PP3 1mg/kg (therapeutic). E: caerulein-induced acute pancreatitis in mice administered PP2 1mg/kg (prophylactic). F: caerulein-induced acute pancreatitis in mice administered PP2 1mg/kg (therapeutic). The criteria used were the presence of neutrophil infiltration, edema, necrosis, acinar-cell ghosts, vacuolization and the destruction of the histoarchitecture of whole or parts of the acini, all of which had been associated with inflammatory reactions in acute pancreatitis.

Figure 6.7

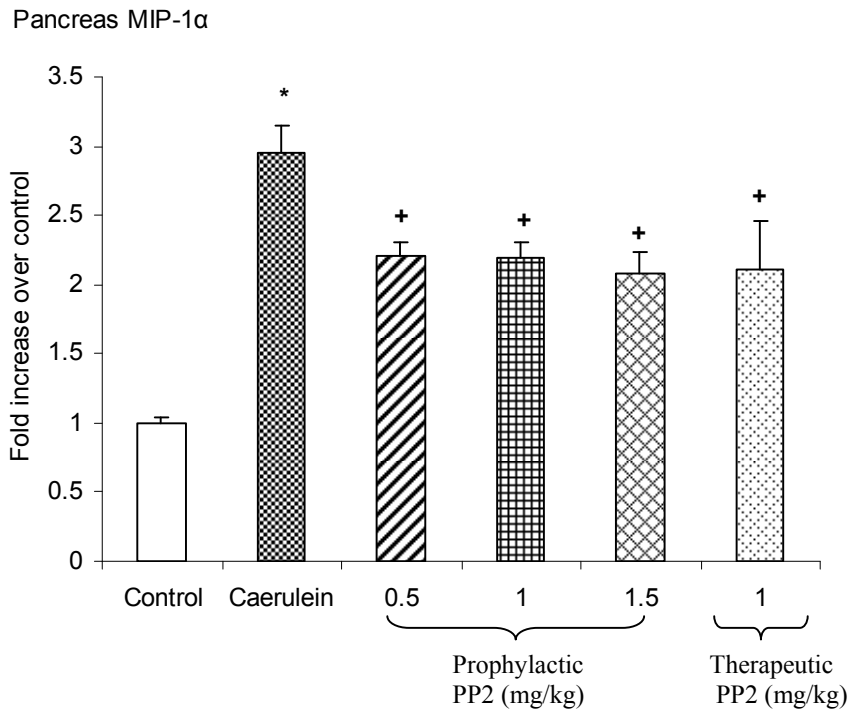
(a)


Pancreas MPO
Fold increase over control
25
20
15
10
5
0
*
+
+
+
+
Control
Caerulein
0.5
1
1.5
1
Prophylactic
PP2 (mg/kg)
Therapeutic
PP2 (mg/kg)

(b)


Pancreas MCP-1
Fold increase over control
25
20
15
10
5
0
*
+
+
+
+
Control
Caerulein
0.5
1
1.5
1
Prophylactic
PP2 (mg/kg)
Therapeutic
PP2 (mg/kg)

(c)


Pancreas MIP-1α
Fold increase over control
3.5
3
2.5
2
1.5
1
0.5
0
*
+
+
+
+
Control
Caerulein
0.5
1
1.5
1
Prophylactic
PP2 (mg/kg)
Therapeutic
PP2 (mg/kg)

(d)

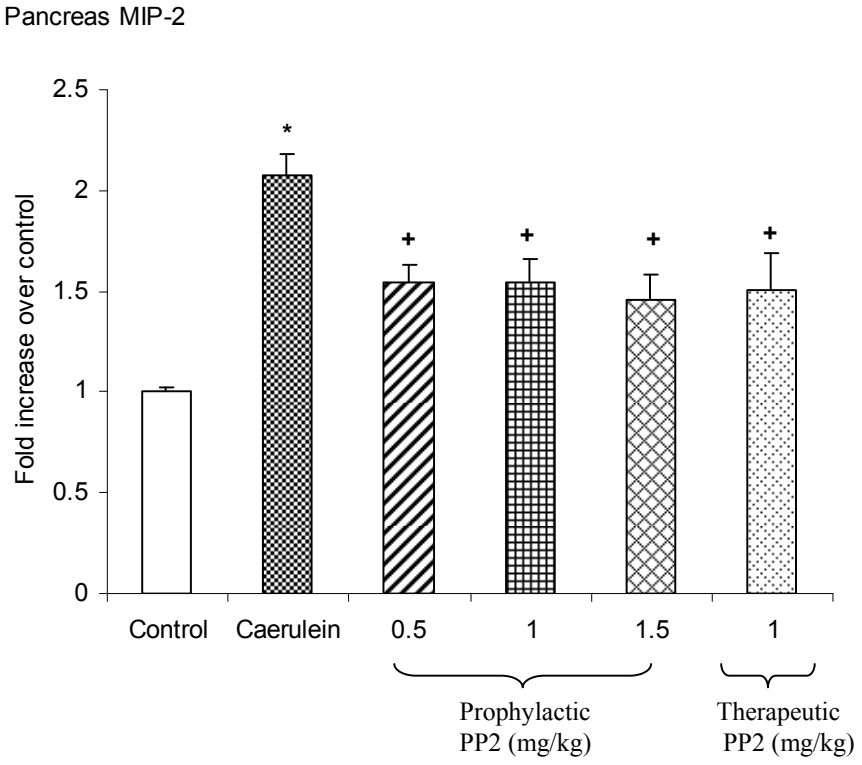
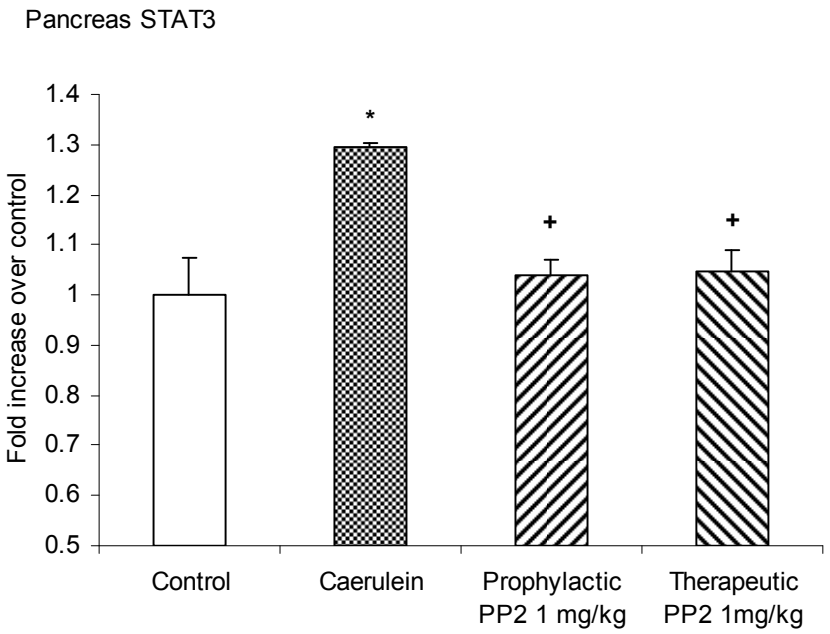

Pancreas MIP-2
Fold increase over control
2.5
2
1.5
1
0.5
0
*
+
+
+
+
Control
Caerulein
0.5
1
1.5
1
Prophylactic
PP2 (mg/kg)
Therapeutic
PP2 (mg/kg)

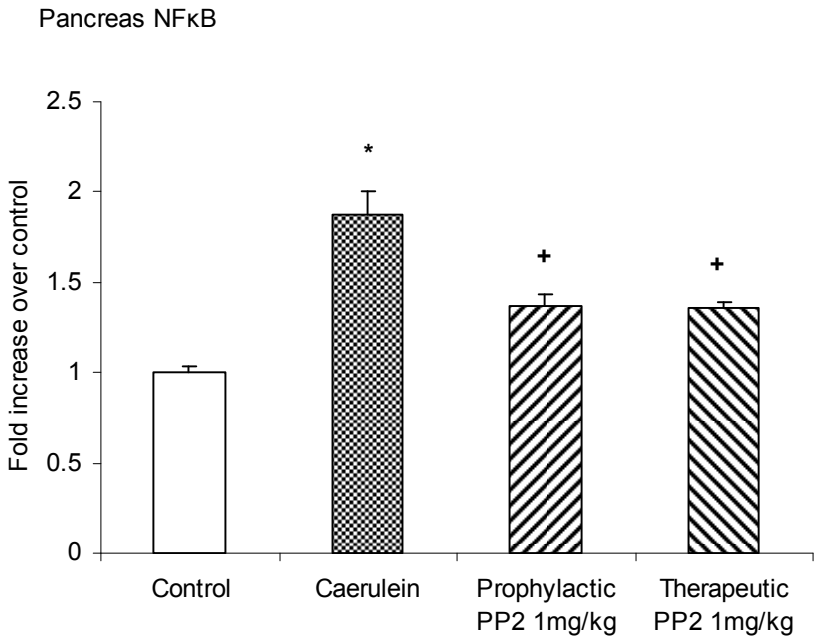
Figure 6.7 Involvement of SFKs in the mobilization of pancreatic neutrophils and chemokines in acute pancreatitis. Mice (n=10 in each group) were given 10 hourly injections of caerulein (50 μg/kg i.p). PP2 was administered in mice at doses of 0.5, 1, 1.5 mg/kg i.p. 1h before or at a dose of 1 mg/kg 1 h after the first caerulein injection. One hour after the last caerulein injection, mice were sacrificed by an intraperitoneal injection of a lethal dose of pentabarbitone, and pancreatic MPO, MCP-1, MIP-1α and MIP-2 levels were measured, as described in MATERIALS AND METHODS. Results shown are the means + SE. $*P < 0.05$ when caerulein-treated animals were compared with vehicle-treated animals. $+P < 0.05$ when PP2-treated animals were compared to caerulein-treated animals.

Figure 6.8

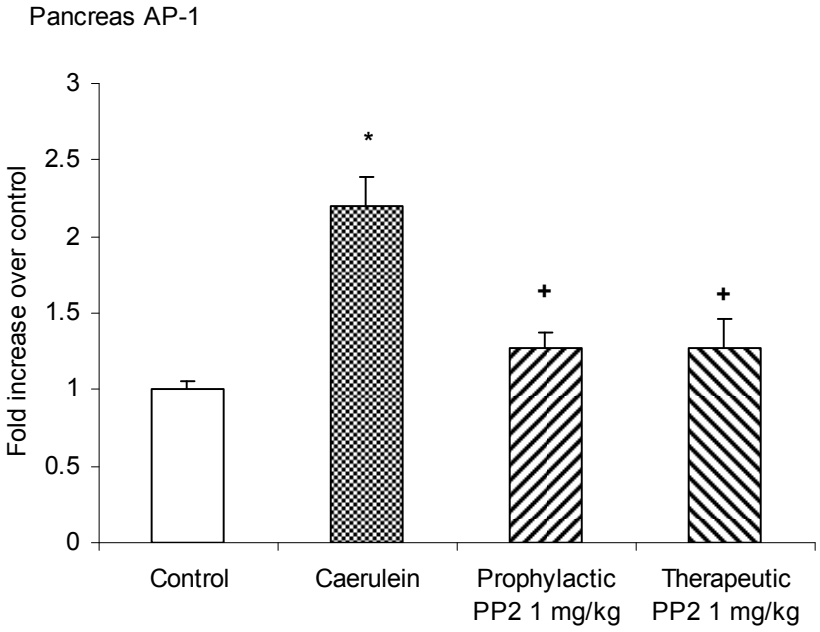
(a)


Pancreas STAT3
Fold increase over control
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
*
+
+
Control
Caerulein
Prophylactic PP2 1 mg/kg
Therapeutic PP2 1mg/kg

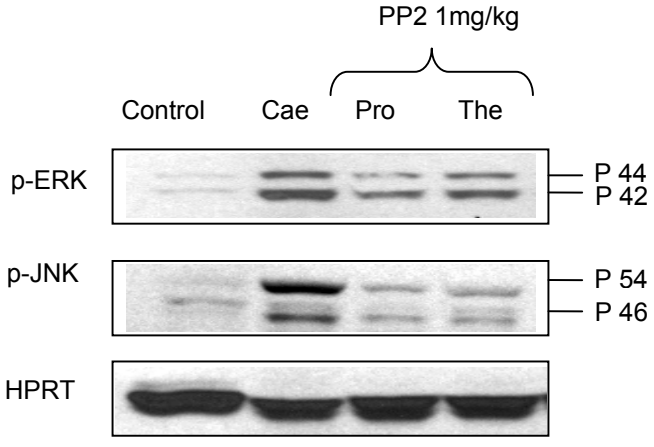
(b)


Pancreas NFκB
Fold increase over control
2.5
2
1.5
1
0.5
0
*
+
+
Control
Caerulein
Prophylactic PP2 1mg/kg
Therapeutic PP2 1mg/kg

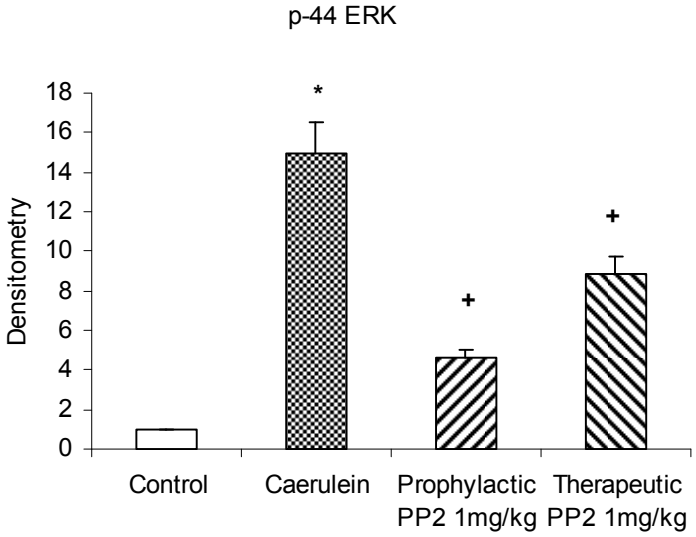
(c)

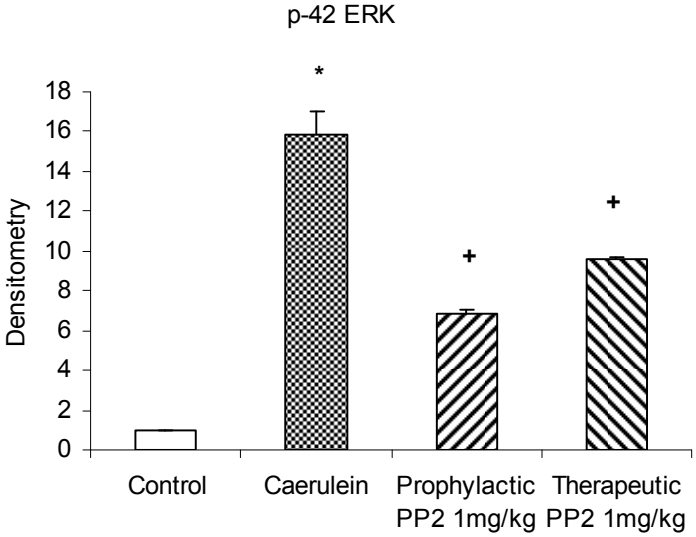

Pancreas AP-1
Fold increase over control
3
2.5
2
1.5
1
0.5
0
*
+
+
Control
Caerulein
Prophylactic PP2 1 mg/kg
Therapeutic PP2 1 mg/kg

(d)


PP2 1mg/kg
Control
Cae
Pro
The
p-ERK
P 44
P 42
p-JNK
P 54
P 46
HPRT

(e)


p-44 ERK
Densitometry
18
16
14
12
10
8
6
4
2
0
*
+
+
Control
Caerulein
Prophylactic PP2 1mg/kg
Therapeutic PP2 1mg/kg


p-42 ERK
Densitometry
18
16
14
12
10
8
6
4
2
0
*
+
+
Control
Caerulein
Prophylactic PP2 1mg/kg
Therapeutic PP2 1mg/kg

(f)

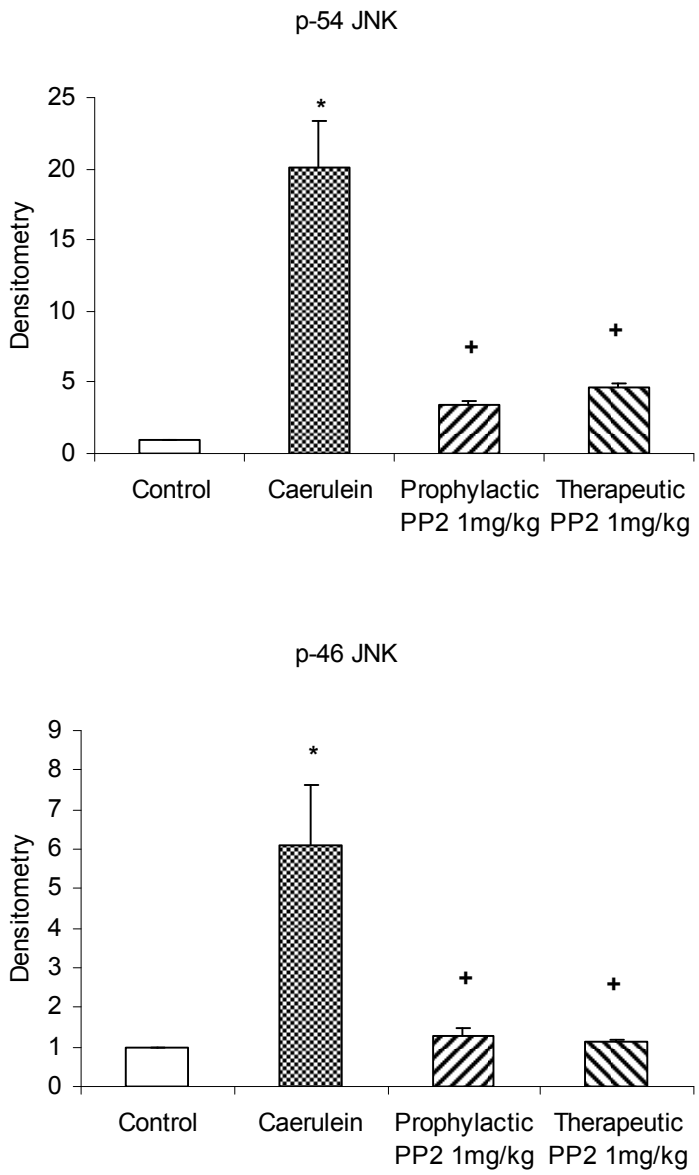


Figure 6.8 Inhibition of SFKs attenuated the activation of pancreatic STAT3, NFκB, AP-1 and MAP Kinases in acute pancreatitis. Mice (n=10 in each group) were given 10 hourly injections of caerulein (50 μg/kg i.p). PP2 was administered to mice at a dose of 1 mg/kg i.p. 1h before or 1 h after the first caerulein injection. One hour after the last caerulein injection, mice were sacrificed by an intraperitoneal injection of a lethal dose of pentabarbitone. The activation of pancreatic STAT3, NFκB, AP-1 and MAP Kinases was quantified as described in MATERIALS AND METHODS. MW in (kDa) for JNK 2/1 is 54/46, ERK1/2 is 44/42 and HPRT is 24. Results shown are the means + SE. *$P < 0.05$ when caerulein-treated animals were compared with vehicle-treated animals. +$P < 0.05$ when PP2-treated animals were compared to caerulein-treated animals.

CHAPTER 7

CONCLUSIONS AND IMPLICATIONS

Even though much advance has been made in elucidating the pathogenesis of acute pancreatitis, yet no specific treatment is currently available.

Previously, it has been shown that during acute pancreatitis, pancreatic levels of substance P and pancreatic acinar cell expression of NK1R are both increased during secretagogue-induced experimental pancreatitis (Bhatia, Saluja *et al.* 1998). Genetic deletion of NK1R reduces the severity of pancreatitis and pancreatitis-associated lung injury (Bhatia, Saluja *et al.* 1998). Similarly, blockade of substance P receptor with its potent and selective antagonist, CP96345, protected mice from acute pancreatitis by attenuating the increase in CC chemokines MCP-1, MIP-1α, and CXC chemokine MIP-2 production in pancreas (Lau and Bhatia 2007; Sun and Bhatia 2007). Substance P, acting through NK1R, plays an important pro-inflammatory role in regulating the severity of acute pancreatitis. However, the exact mechanism by which substance P contributes to the pro-inflammatory signaling in acute pancreatitis is not completely understood.

In chapter 2, I have demonstrated that substance P, by itself, induced synthesis of CC chemokines MCP-1, MIP-1α and CXC chemokine MIP-2 via NFκB dependent pathway in mouse pancreatic acinar cells. This is the first direct evidence of the role of substance P, acting via NK1R present on mouse pancreatic acini, in inflammation and points to the mechanism by which substance P contributes to inflammation in acute pancreatitis.

Looking at the signaling pathways, I showed, in chapter 3, that substance P induced the activation of MAP Kinases ERK and JNK as well as transcription factor AP-1 in mouse pancreatic acinar cells. To my knowledge, this is the first study that shows that substance P-induced activation of both ERK and JNK cascades are essential for NFκB and AP-1 activation, resulting in increased production of chemokines MCP-1, MIP-1α and MIP-2 in mouse pancreatic acini. This study gives us a further insight into the mechanism by which substance P contributes to the inflammatory responses in acute pancreatitis.

Going deeper into the signaling mechanisms I demonstrated, in chapter 4, that substance P/NK1R stimulated the activation of PKCδ as well as MEKK1. My findings indicated that PKCδ acts as an important pro-inflammatory signal transducer in substance P/NK1R-induced production of pro-inflammatory mediators MCP-1, MIP-1α and MIP-2 in pancreatic acinar cells. The secretion of these pro-inflammatory mediators was mediated through the signaling cascade of PKCδ-MEKK1-(ERK, JNK)-(NFκB, AP-1), thereby contributing to local inflammation and consequently leading to systemic inflammation in acute pancreatitis.

Moreover, in chapter 5, substance P stimulated pancreatic acinar cells to release chemokines MCP-1, MIP-1α, and MIP-2 through a PLC dependent mechanism. Substance P induced an increase in $[Ca^{2+}]i$ which resulted in the phosphorylation of PKCα/βII, ERK and JNK; consequently leading to the activation of NFκB, AP-1 and ultimately to chemokine production. This study reveals the critical role of calcium in substance P-induced chemokine production in pancreatic acinar cells and also points to another mechanism through which calcium could be mediating acute pancreatitis.

To my knowledge, this is the first study, as shown in chapter 6, which illustrates the involvement of non receptor tyrosine kinases SFKs in substance P-induced chemokine production in pancreatic acinar cells. Substance P-induced activation of SFKs was also involved in the phosphorylation of ERK, JNK as well as activation of STAT3, NFκB and AP-1. I also tested the significance of my *in vitro* results in a more complex *in vivo* system of caerulein-induced acute pancreatitis. I demonstrated that SFKs mediated protection against acute pancreatitis through the same signaling pathway that I had shown in my *in vitro* model of isolated acinar cells, which is through substance P/NK1R-SFKs-(ERK, JNK)-(STAT3, NFκB, AP-1)-(MCP-1, MIP-1α, MIP-2).

Based on my results, I propose that elevated levels of substance P, which is produced as a result of acute pancreatitis, bind to pancreatic NK1R to activate several intracellular signaling molecules which lead to chemokine production. One such signaling complex is SFKs. Another signaling cascade that mediates chemokine production is through PLC-induced elevated intracellular calcium and conventional PKCα/βII activation. Novel PKCδ and MEKK1 are equally responsible for substance P-induced chemokine production in pancreatic acinar cells. The activation of all these upstream signaling molecules converges towards the phosphorylation of MAP Kinases ERK and JNK, leading to the activation of transcription factors NFκB, AP-1 and STAT3 which then up regulate both CC chemokines MCP-1, MIP-1α and CXC chemokine MIP-2. In addition, I have shown that inhibition of SFKs which blocked the activation of pancreatic ERK, JNK, NFκB, AP-1 and STAT3 also protected mice against acute pancreatitis. My proposed signaling pathways through which substance P mediates chemokine production in acute pancreatitis is illustrated in Figure 7.1

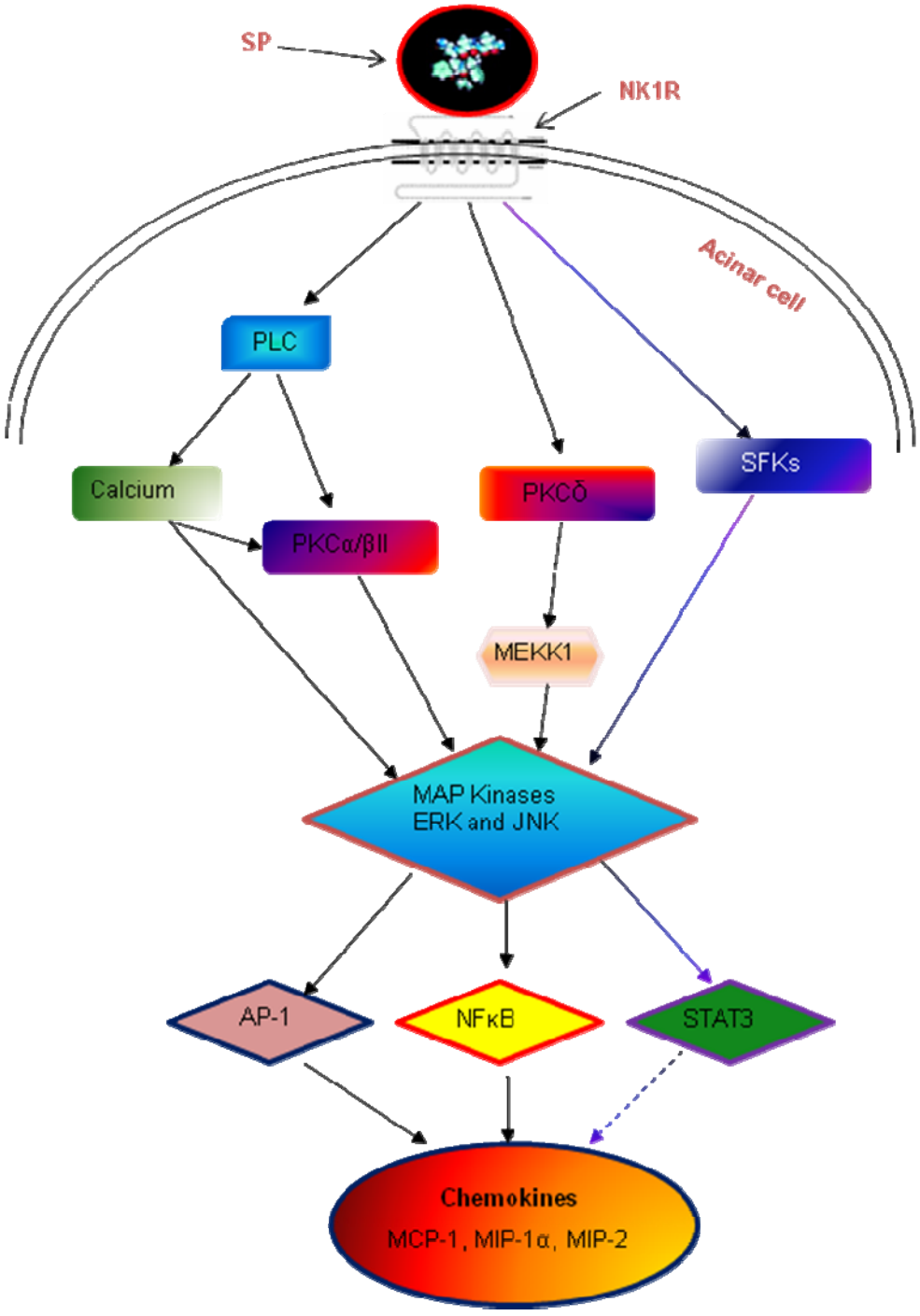


Figure 7.1 Schematic representation of the proposed cascades through which substance P stimulates the production of chemokines in acute pancreatitis.Substance P interacts

with its preferred receptor NK1 to induce activation of PLC, which causes the release of $[Ca^{2+}]i$ and activation of PKCα/βII. $[Ca^{2+}]i$ also causes activation of PKCα/βII, leading to phosphorylation of MAP Kinases ERK and JNK, thereby activating transcription factors NFκB, AP-1, resulting in chemokine production. Substance P, through NK1R induces phosphorylation of PKCδ, activation of MEKK1 which results in phosphorylation of ERK and JNK, activation of NFκB, AP-1, consequently leading to chemokine production. Substance P via NK1R induces activation of SFKs, resulting in phosphorylation of ERK and JNK, leading to activation of transcription factors STAT3, NFκB, AP-1, resulting in the production of CC chemokines MCP-1, MIP-1α and CXC chemokine MIP-2.

It is clear that substance P and NK1R play important roles in acute pancreatitis and that NK1R antagonist represents a promising therapeutic tool for the treatment of acute pancreatitis. However, care should be taken as blocking the GPCR NK1R altogether can have some adverse effects due to the promiscuity of GPCR. In light of my study, I proposed that drugs targeting the substance P/NK1R mediated signaling pathways could prove beneficial in improving the treatment of acute pancreatitis.

Understanding the mechanisms that mediate acute pancreatitis is crucial for the discovery and development of more effective treatment strategies. The cascade of cellular and molecular pathways mediating acute pancreatitis is intricate and not fully understood. Using the information obtained from single signaling pathways and building up complex signaling networks can enable us to better understand the mechanisms and physiological outcome of substance P/NK1R mediated responses. An understanding of the mechanisms

by which substance P/NK1R modulate its downstream effects would facilitate the discovery and design of strategies by which specific points in the signaling network can be modulated, leading to specific changes in the physiological functions. This in turn would help to prevent disease progression and/or improve treatment efficacy by developing clinically effective anti-inflammatory therapies.

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