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Activation of neurokinin-1 receptors up-regulates substance P and neurokinin-1 receptor expression in murine pancreatic acinar cells

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Abstract

Acute pancreatitis (AP) has been associated with an up-regulation of substance P (SP) and neurokinin-1 receptor (NK1R) in the pancreas. Increased SP-NK1R interaction was suggested to be pro-inflammatory during AP. Previously, we showed that caerulein treatment increased SP/NK1R expression in mouse pancreatic acinar cells, but the effect of SP treatment was not evaluated. Pancreatic acinar cells were obtained from pancreas of male swiss mice (25–30 g). We measured mRNA expression of *preprotachykinin-A* (PPTA) and *NK1R* following treatment of SP (10^{-6}M). SP treatment increased *PPTA* and NK1R expression in isolated pancreatic acinar cells, which was abolished by pretreatment of a selective NK1R antagonist, CP96,345. SP also time dependently increased protein expression of NK1R. Treatment of cells with a specific NK1R agonist, GR73,632, up-regulated SP protein levels in the cells. Using previously established concentrations, pre-treatment of pancreatic acinar cells with Gö6976 (10 nM), rottlerin (5 μM), PD98059 (30 μM), SP600125 (30 μM) or Bay11-7082 (30 μM) significantly inhibited up-regulation of SP and NK1R. These observations suggested that the PKC-ERK/JNK-NF-κB pathway is necessary for the modulation of expression levels. In comparison, pre-treatment of CP96,345 reversed gene expression in SP-induced cells, but not in caerulein-treated cells. Overall, the findings in this study suggested a possible auto-regulatory mechanism of SP/NK1R expression in mouse pancreatic acinar cells, *via* activation of NK1R. Elevated SP levels during AP might increase the occurrence of a positive feedback loop that contributes to abnormally high expression of SP and NK1R.

Keywords: substance p • neurokinin-1-receptor • acute pancreatitis • gene expression • auto-regulation

Introduction

Acute pancreatitis (AP) is the sudden inflammation of the pancreas. The outcome of AP ranges from a mild, self-limiting disease to a potentially fatal illness characterized by local and systemic complications. Currently the incidence of AP is on the rise [1]. Improved understanding of the underlying mechanisms that lead to AP is essential in the management and treatment of this disease. Dysregulated activation of digestive enzymes, especially trypsinogen, followed by an excessive inflammatory response was generally accepted as a key event of AP [1]. However, the exact mechanisms that distinguish the severity and outcome of AP are still unclear.

Substance P (SP) is an 11-amino acid peptide and is now a well-recognized pro-inflammatory mediator. SP has been implicated in inflammatory disorders such as polymicrobial sepsis, rheumatoid arthritis, asthma and colitis [2–5]. Encoded by the preprotachykinin-A (PPTA) gene, SP mainly binds to neurokinin-1 receptor (NK1R), which leads to downstream pro-inflammatory responses. SP is highly expressed in the peripheral neurons but can also be produced by cells of non-neural origin, such as leukocytes, Leydig cells and, as we have

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shown, in isolated pancreatic acinar cells [6–9]. SP-NK1R interaction plays a key role in the pathogenesis of experimental AP. Antagonism of NK1R with CP96,345 or deletion of the PPTA gene showed markedly improved outcome of pancreatic damage and associated lung injury [10,11]. Moreover, inhibition of SP release from the primary sensory neurons also protected rodents against experimental AP [12,13]. SP promotes inflammatory conditions by inducing the expression of cytokines and chemokines, such as macrophage inflammatory protein-1α (MIP-1α) and monocyte chemotactic protein-1 (MCP-1) *via* a NF-κB dependant pathway [14,15]. SP was also suggested to increase neutrophil recruitment and adhesion by up-regulating expression of chemokine receptors in mouse neutrophils [14]. These all point to an important role of SP-NK1R interaction in AP.

Although the importance of SP-NK1R interaction in AP and other inflammatory diseases has been brought into focus, mechanisms that regulate their expression were less explored. The expression of both SP and NK1R were shown to be up-regulated in the pancreas and lung in caerulein-induced AP [11]. Similar up-regulation was observed when isolated mouse pancreatic acinar cells were incubated with caerulein (10^{-7}M). In another study, antagonism of NK1R with CP96,345 significantly reversed AP-induced PPTA/NK1R mRNA up-regulation in the pancreas [16]. This observation hinted the possibility of an auto-regulatory mechanism in the pancreas, where activation of NK1R contributed to the overall elevated expression of SP and NK1R.

We have recently demonstrated that treatment of mouse pancreatic acinar cells with caerulein induced SP/NK1R up-regulation *via* activation of CCK_A receptors [9]. The response is rapid, with a significant up-regulation occurring within 1 hr of stimulation. Activation of CCK_A receptors causes subsequent protein kinase C (PKC) – mitogen activated protein kinase (MAPK; specifically ERK and JNK pathway) – nuclear factor-κB (NF-κB) activation, and leads to up-regulation of SP and NK1R expression [9,17]. On the other hand, activation of NK1R is also known to activate similar pathways *via* its G-protein coupled downstream signalling [18]. In mouse pancreatic acinar cells, it was shown that treatment of cells with SP up-regulated chemokine expression by a Src-Ca^{2+}-PKC-MAPK-NF-κB dependent pathway [19]. Hence, there are similarities between NK1R and CCK_A receptors with regard to their downstream signalling pathways.

In the present investigation, we aim to examine the relationship between exposure to SP and the expression of SP and NK1R in isolated mouse pancreatic acinar cells. We assessed both protein and mRNA expression levels of SP and NK1R after pancreatic acinar cells were treated with SP or a selective NK1R agonist, GR73,632. In some experiments, we used CP96,345, a selective NK1R antagonist, or specific inhibitors of signalling molecules to elucidate the signalling pathway involved. Finally, we also compared the differences between SP-mediated responses with caerulein-mediated responses.

Materials and methods

Animals and chemicals

All experimental procedures were approved by the Animal Ethics Committee of the National University of Singapore and carried out in accordance with established International Guiding Principles for Animal Research. Swiss mice (male, 25–30 grams) were acclimatized in a controlled environment with an ambient temperature of 23°C and a 12:12-hour light-dark cycle. SP and caerulein was purchased from Bachem (Torrance, CA, USA). Glucose, HEPES and soybean trypsin inhibitor were obtained from Sigma-Aldrich (St. Louis, MO, USA). Type IV collagenase was purchased from Worthington (Freehold, NJ, USA). PD98059 (mitogen-activated protein kinase kinase (MEK) inhibitor), SP600125 (JNK inhibitor), Bay11-7082 (NF-κB inhibitor), Gö6976 (PKCα/β inhibitor) and rottlerin (PKCδ inhibitor) were purchased from Calbiochem (Darmstadt, Germany). CP96,345 (selective NK1R antagonist) was obtained from Sigma-Aldrich. All chemicals were purchased with the highest purity available.

Preparation of pancreatic acini

Pancreatic acini were obtained from mouse pancreas by collagenase treatment as described previously [10]. Briefly, Swiss mice were killed by a lethal dose of sodium pentobarbitone (150 mg/kg). Fresh pancreases were infused with buffer A (mM: 140 NaCl, 4.7 KCl, 1.13$MgCl_2$, 1$CaCl_2$, 10 glucose, and 10 HEPES, and 0.5 mg/ml soybean trypsin inhibitor, pH 7.3) containing 200 IU/ml Type IV collagenase. The pancreas was then minced and placed in 12 ml of *buffer A* containing 200 IU/ml Type IV collagenase, and incubated in a shaking water bath for 10 min. at 37°C with shaking. To obtain dispersed acini, the digested tissue was passed through small pipette tips. The cells were then passed through a solution of 50 mg/ml bovine serum albumin (Dissolved in *buffer A*) and then washed twice with buffer A before further experiments. The viability of pancreatic acinar cells was determined by trypan blue exclusion assay. Cell preparations with at least 95% viability were used for further experiments.

Treatment of pancreatic acinar cells

Freshly isolated pancreatic acinar cells were stimulated with caerulein (10^{-7}M), SP (10^{-6}M) or GR73,632 (10^{-6}M-10^{-10}M). The concentration of caerulein and SP used in this study was shown to activate NF-κB dependent gene expression in pancreatic acinar cells [9,20]. For time-dependent studies, cells were treated with caerulein or SP for 30, 60, 90, 120 min. in a 37°C water bath. For other experiments, cells were pre-treated with either Gö6976 (10 nM), rottlerin (5 μM), PD98059 (30 μM), SP600125 (30 μM), Bay11-7082 (30 μM) or CP96,345 for 30 min. before addition of caerulein, SP or GR73,632 (10^{-7}M). The concentration of these inhibitors was previously established and showed to be effective in caerulein-treated pancreatic acinar cells [9,17]. Gö6976, rottlerin, PD98059, SP600125, Bay11-7082 and CP96,345 were dissolved in dimethyl sulfoxide (DMSO) and diluted with *buffer A*. The final concentration of DMSO in the cell culture is less than 1%. Other chemicals were dissolved in normal saline and diluted with *buffer A*. Cell viability for all treatment groups averaged at 80–90% after the experimental protocol, as determined by trypan blue exclusion assay.

Substance P extraction and detection

Treated pancreatic acinar cells were homogenized in ice-cold SP assay buffer and SP was concentrated by adsorbing on C_{18} cartridge columns (Bachem) as described previously [11]. The adsorbed peptides were eluted with 1.5 ml of 75% acetonitrile. The eluates were freeze-dried overnight and reconstituted with SP assay buffer. SP content was then determined with an ELISA kit (Bachem) according to manufacturer's instructions. The results were quantified by spectrophotometry at 450 nm. The results were then normalized with DNA content of the acinar cell samples. DNA assay was performed fluorometrically by using Hoechst dye 33256 and calf thymus DNA as a standard [21]. SP expression was corrected as ng/μg NA.

Quantitative real time PCR analysis

Total RNA from the pancreatic acinar cells was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. All the steps were performed on ice-cold conditions. The integrity of RNA was verified by ethidium bromide staining for the presence of distinct 28S and 18S bands on a 1.2% agarose gel following electrophoresis. One microgram (1 μg) of total RNA was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) in a total volume of 20 μl. The reaction was commenced at 25°C for 5 min. and 42°C for 30 min., followed by 85°C for 5 min. 2 μl of cDNA was used as a template for PCR amplification by using SYBR-green PCR master mix from Roche Diagnostics (Singapore). No template controls and intron spanning primers were used. All reactions were done in duplicates. PCR reaction mix was first subjected to 95°C for 5 min., followed by a 45 cycles of amplification. Each cycle consisted of 95°C for 20 sec., annealing temperature of 57°C for 20 sec. and elongation temperature of 72°C for 20 sec. The murine primer sequences are as follows:

PPTA (282bp; GenBank accession no: NM_009311): Forward: 5'-CGCGATGCAGAACTACGAAA-3', Reverse: 5'-GCTTGGACAGCTCCTTCATC-3'. NK1R (223bp; GenBank accession no: NM_009313): Forward: 5'- GCTGCCCTTCCACATCTTCT-3', Reverse: 5'-TTCCAGCCCCTCATAATCAC-3'

β-actin (165bp; GenBank accession no: NM_007393), Forward: 5'-TGTTACCAACTGGGACGACA-3', Reverse: 5'-GGGGTGTTGAAGGTCTCAAA-3' *β-actin* was used as a housekeeping gene to normalize the mRNA expression of PPTA and NK1R. Expression of PPTA, NK1R and β-actin was determined using the 'crossing point (Cp)' of the sample, where Cp is the point (cycle number) at which the fluorescence of a sample rises above the background fluorescence.

Whole cell lysate preparation and Western blot analysis

After treatment of pancreatic acinar cells, they were homogenized on ice in radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich) and phosphatase inhibitor cocktail (Sigma-Aldrich). Protein concentrations were determined by the Bradford protein assay. Protein samples (80 μg) were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. Non-specific binding was blocked by 1-hr incubation of the membranes in 5% nonfat dry milk in PBST (0.05% Tween 20 in PBS). The blots were then incubated overnight with primary antibody against NK1R (Abcam, Cambridge, UK) or hypoxanthine-guanine phosphoribosyltransferase (HPRT) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 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 2 hrs 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. Membranes were washed and then incubated in SuperSignal™ West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) before exposure to X-ray films (CL-Xposure™, Pierce). HPRT was applied as an internal control to normalize protein loading. The intensity of bands was quantified using LabWorks™ Image Analysis software (UVP, Upland, CA, USA).

Statistical analysis

The data were expressed as the mean ± standard error of mean (S.E.M.). The significance of changes among groups was evaluated by using ANOVA with Tukey's *post-hoc* test. A P value ≤ 0.05 was considered as statistically significant.

Results

Substance P induces PPTA and NK1R mRNA expression in murine pancreatic acinar cells

In order to understand the role of SP-NK1R interaction in SP/NK1R expression, we first examine the effect of exogenous SP on PPTA/NK1R mRNA expression in pancreatic acinar cells. As we recently showed that PPTA and NK1R mRNA expression could be induced by caerulein [9], gene expression levels of caerulein-induced cells were compared with SP-induced cells. To do this, freshly isolated pancreatic acinar cells were treated with either SP (10^{-6}M) or caerulein (10^{-7}M) for up to 120 min. After incubation of cells with SP, both PPTA and NK1R mRNA expression showed a time-dependant increase, as determined by real-time PCR assay. Expression levels start to rise significantly after 30 min., and peaked in 1–2 hrs after treatment with SP (Fig. 1A and B). In comparison, both SP-induced cells and caerulein-induced cells showed a similar 2-fold increase of PPTA mRNA expression (Fig. 1A). On the other hand, NK1R expression in SP-treated cells (1.6-fold increase at 60 min.) was considerably lower when compared to caerulein-treated cells (2.5-fold increase at 60 min.) ($P < 0.05$; Fig. 1B). In our studies, mRNA expression of β-actin, which was used as a housekeeping gene, showed no significant changes in expression levels in all experiments groups (data not shown).

CP96,345 down-regulates exogenous SP-induced PPTA and NK1R mRNA expression

We have previously shown that SP up-regulated the expression of PPTA and NK1R in pancreatic acinar cells. To test whether this effect is due to SP-NK1R interaction, we pre-treated cells with CP96,345,

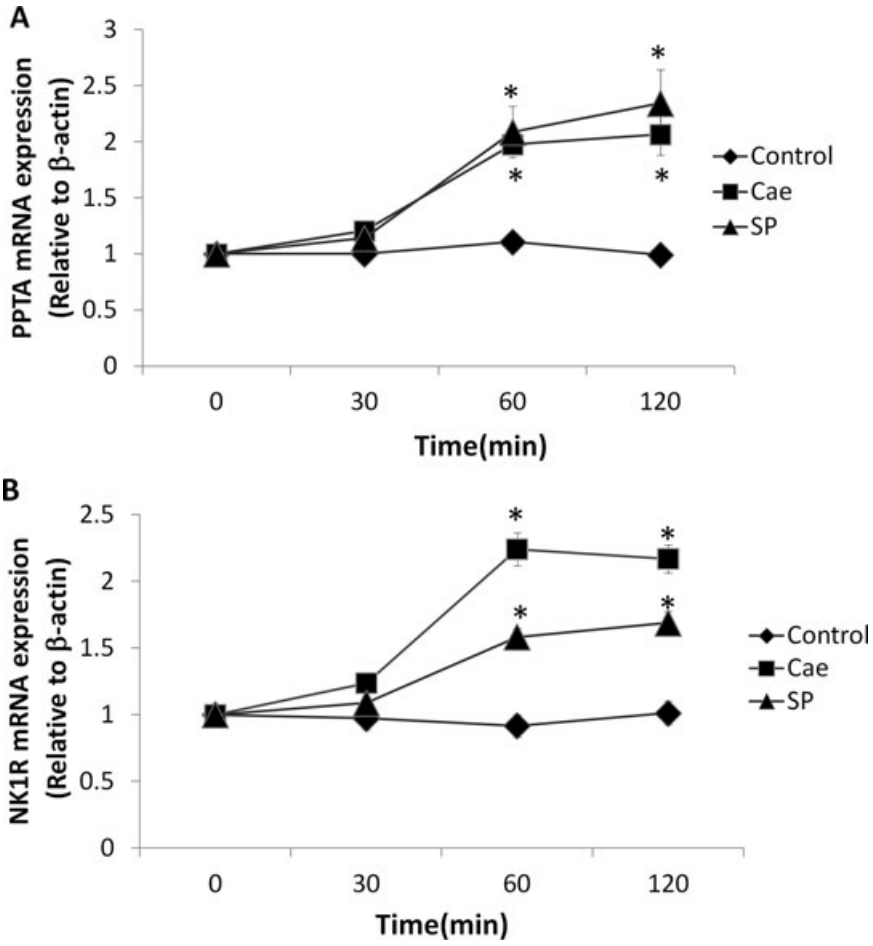

Fig. 1 SP-induced gene expression of PPTA and NK1R in murine pancreatic acinar cells. Freshly prepared pancreatic acinar cells were treated with either SP (10^{-6} M) or Cae (10^{-7} M) for 0–120 min. PPTA/NK1R mRNA expression was determined with real time PCR following TRIzol reagent extraction and normalized with β-actin expression in the samples. (**A**) Time course effect of SP and Cae treatments on PPTA mRNA expression. (**B**) Time course effect of SP and Cae treatments on NK1R mRNA expression. Results are expressed as means ± S.E.M. from 4–6 independent experiments. *$P < 0.05$ *versus* control. SP: substance P; Cae: caerulein.

a selective NK1R antagonist, before induction with SP [22]. Pretreatment of cells with CP96,345 inhibited PPTA and NK1R mRNA expression in a concentration-dependent manner, when compared to their vehicle-treated SP-induced cells. At a low concentration of 0.2 μM, CP96,345 showed a trend of inhibition ($0.05 < P < 0.15$) but at higher concentrations (5 μM), it completely abolished SP-induced up-regulation of PPTA/NK1R mRNA expression (Fig. 2A and B). Treatment of CP96,345 in the absence of SP did not alter expression levels when compared to the baseline control (data not shown), suggesting that NK1R activation is not involved in maintaining the basal expression level of PPTA and NK1R.

Caerulein-induced PPTA and NK1R gene expression in murine pancreatic acinar cells does not involve the activation of NK1R

Previous results have demonstrated an increase in PPTA and NK1R mRNA expression in the pancreas when mice were induced with AP. On the other hand, antagonism of NK1R with CP96,345 significantly reduced their expression [16]. This suggested that activation of NK1R partly attributed to the overall PPTA/NK1R up-regulation observed in caerulein-treated animals. We aim to reproduce this observation in our cellular model of isolated pancreatic acinar cells. To do this, cells were treated with CP96,345 (1 μM) for 30 min. before treatment with stimulatory concentration of caerulein (10^{-7} M). Caerulein treatment significantly increased the expression of PPTA and NK1R. However, contrary to the animal model of caerulein-induced AP, antagonism of NK1R did not reduce mRNA expression of PPTA/NK1R in isolated pancreatic acinar cells (Fig. 2A and B).

Activation of NK1R induces expression levels of SP peptides

Since PPTA and NK1R mRNA expression can be regulated through activation of NK1R, we further investigated SP peptide expression in pancreatic acinar cells. Unlabelled exogenous SP was discovered to confound with endogenously produced SP using conventional ELISA

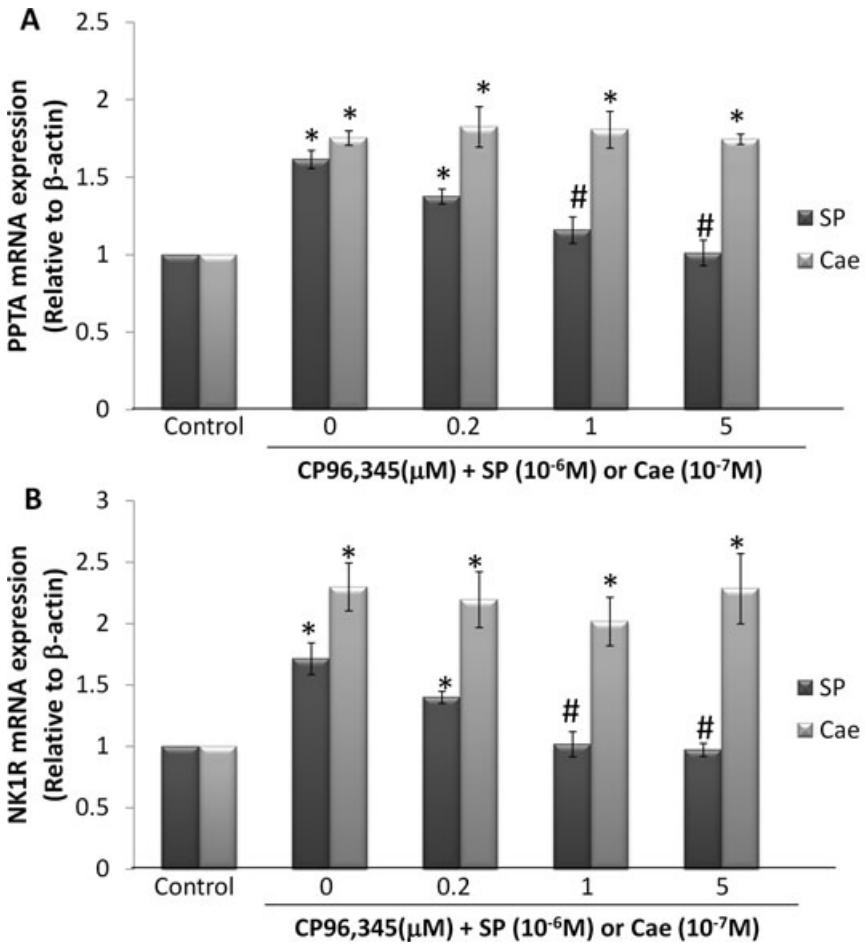

Fig. 2 SP-induced, but not Cae-induced PPTA/NK1R up-regulation is abolished by antagonism of NK1R. Pancreatic acinar cells were pre-treated with CP96,345 (0.2–5 μM) for 30 min. before treatment with Cae (10^{-7} M) or SP (10^{-6} M) for 60 min. Subsequently, PPTA and NK1R mRNA expression was determined with real time PCR following TRIzol reagent extraction and normalized with β-actin expression in the samples. (**A**) Pre-treatment of CP96,345 reversed SP-induced, but not Cae-induced PPTA up-regulation. (**B**) Pre-treatment of CP96,345 reversed SP-induced, but not Cae-induced NK1R up-regulation. Results are expressed as the means ± S.E.M. from 6 independent experiments. $^{*}P < 0.05$ *versus* control, $^{\#}P < 0.05$ *versus* Cae. SP: substance P; Cae: caerulein.

assays (data not shown), and very likely with other protein assays. Therefore, we used a specific NK1R agonist, GR73,632, to investigate its effects on SP peptide expression. GR73,632 had no detectable reactivity in the ELISA assay at concentrations up to 10 μg/ml. Isolated pancreatic acinar cells were cultured for 60 min. in the presence of GR73,632 (10^{-10} M to 10^{-6} M). Our results showed that treatment of isolated murine pancreatic acinar cells with GR73,632 significantly increased SP peptide levels at concentrations larger than 10^{-8} M (Fig. 3A). To further confirm that the observations were mediated through NK1R, we treated cells with GR73,632 (100 nM) and CP96,345 (1 μM). Pre-treatment of CP96,345 completely abolished the effects of GR73,632, indicating that the effect is specifically mediated by activation of NK1R (Fig. 3B).

Effect of substance P treatment on protein expression of NK1R

We also evaluated the effect of exogenous SP on NK1R protein expression. To do this, we performed a time course study of NK1R *via* Western blot. Using a specific antibody against NK1R, we confirmed that NK1R protein is expressed in the pancreatic acinar cells. Cells cultured in control medium did not show a change in background expression of NK1R. On the other hand, a time-dependant up-regulation of NK1R protein expression was observed in whole cell lysates after treatment with SP (Fig. 4). After 2 hrs of SP stimulation, NK1R band intensity reached a peak of 2.5-fold increase when compared to the control.

SP up-regulates SP and NK1R expression *via* PKC, MAPK and NF-κB dependant pathways

It was previously demonstrated that SP induces the expression of chemokines *via* the Src family kinases (Sfk) – ERK/JNK – NF-κB pathway in pancreatic acinar cells [19]. The PKC family was also found to be activated after cells were stimulated with SP [23]. These activated signalling molecules share similarities with the

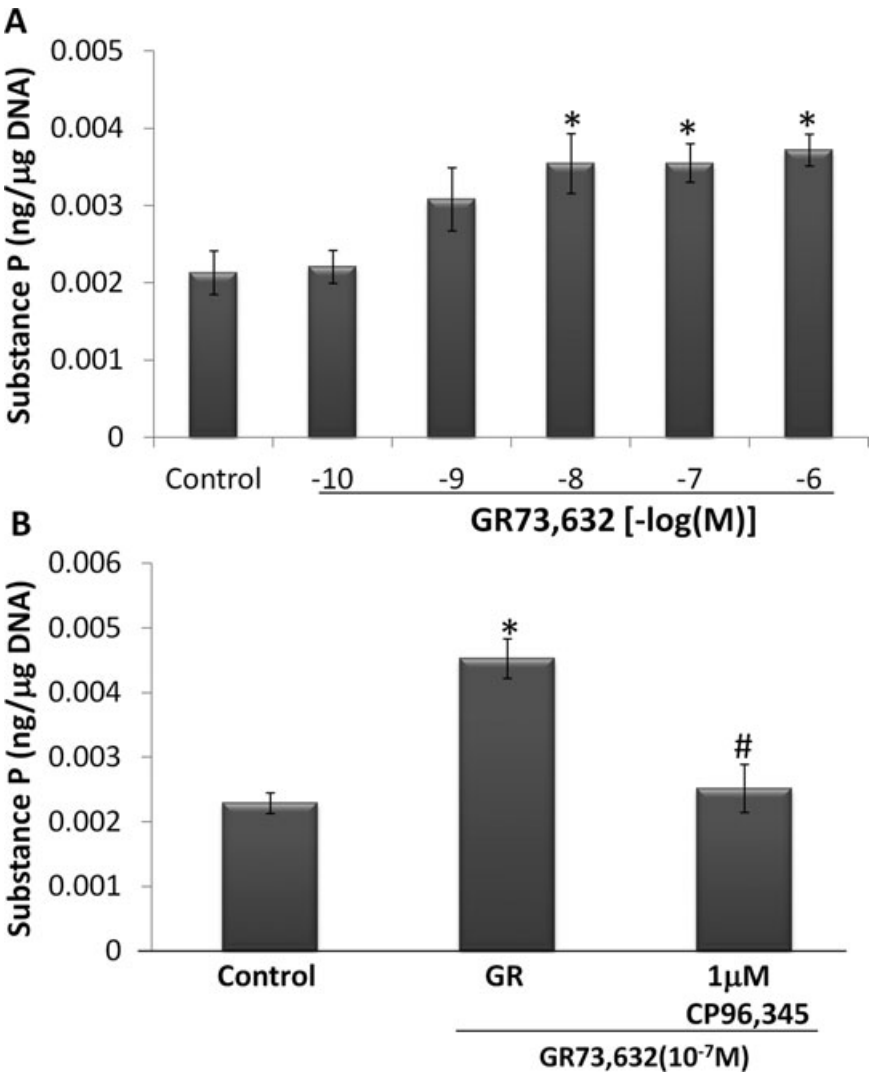

Fig. 3 Activation of NK1R up-regulated SP peptide expression in isolated murine pancreatic acinar cells. Freshly prepared pancreatic acinar cells were treated with GR73,632 (10^{-6}–10^{-10} M) for 60 min. In some experiments, pancreatic acinar cells were pre-treated with CP96,345 (1 μM) before treatment with GR73,632 (10^{-7} M). SP peptide levels were determined using a commercially available ELISA kit and normalized with the DNA content of the samples. (**A**) Concentration dependant effect of GR73,632 on SP expression. (**B**) Pre-treatment of cells with CP96,345 reversed GR73,632-induced SP up-regulation. Results are expressed as means ± S.E.M. from 4–6 independent experiments. $*P < 0.05$ *versus* control. SP: substance P.

PKC-MAPK-NF-κB pathway involved in caerulein-mediated responses [17]. To determine the signalling pathway in SP-induced expression of SP/NK1R, pancreatic acinar cells were pre-treated with specific inhibitors of PKCα (Gö6976, 10nM), PKCδ (Rottlerin, 5μM), MEK/ERK pathway (PD98059, 30μM), JNK (SP600125,30μM) or NF-κB (Bay11-7082, 30μM) before induction with SP (10^{-6}M, 60 min.). The addition of these inhibitors almost completely abolished SP-induced PPTA/NK1R mRNA up-regulation in the pancreatic acinar cells (Figs 5A and 6A). Protein expression of SP and NK1R was also abrogated by the addition of PKC, MAPK or NF-κB inhibitors (Figs 5B and 6B). These results confirm the similarity in downstream signalling pathways between caerulein-induced and SP-induced gene expression in the pancreatic acinar cells.

Discussion

SP-NK1R interaction was defined as a pro-inflammatory process that contributes to the progression of AP [11,24]. Both pancreatic SP and NK1R expression are elevated in caerulein-induced AP models. We have previously reported caerulein-induced expression of SP and NK1R in isolated mouse pancreatic acinar cells and the mechanisms that led to their up-regulation [9,17]. In the present study, we sought to investigate the effect of SP on the expression of SP and NK1R in isolated pancreatic acinar cells.

Herein we demonstrated that SP treatment increased PPTA and NK1R mRNA expression in isolated murine pancreatic acinar cells. We observed that the response was rapid, with significant up-regulations occurred within 1 hr after administration of SP. This is

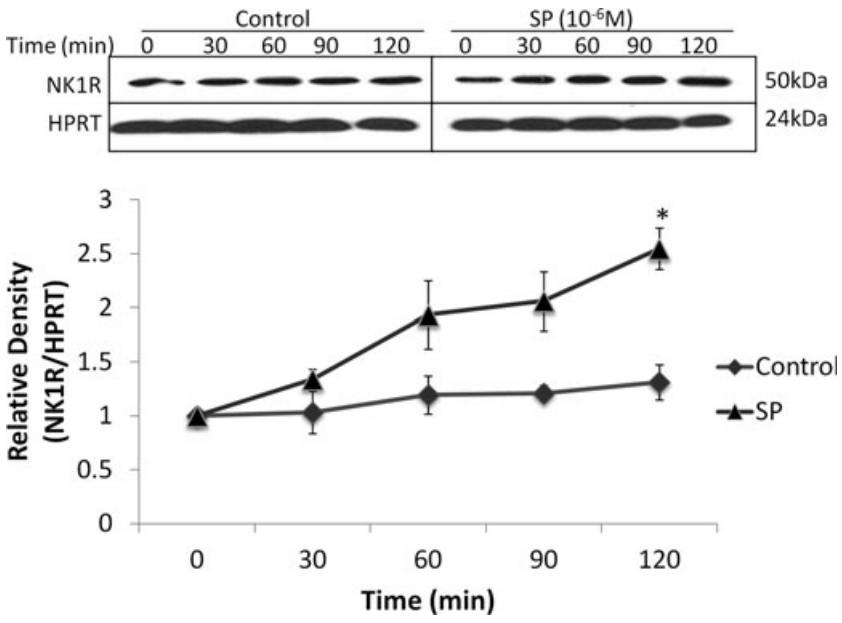

Fig. 4 SP up-regulates NK1R protein expression in a time-dependent manner. Freshly prepared pancreatic acinar cells were treated with SP for 0–120 min. After treatment, whole cell lysates were prepared and 50 μg of protein were fractionated on 10% sodium dodecyl sulfate-polyacrylamide gels for Western blot analysis of NK1R and HPRT. NK1R protein expression was normalized with HPRT expression. Results are expressed as means ± S.E.M. from 4–6 independent experiments. $^{*}P < 0.05$ *versus* control. SP: substance P.

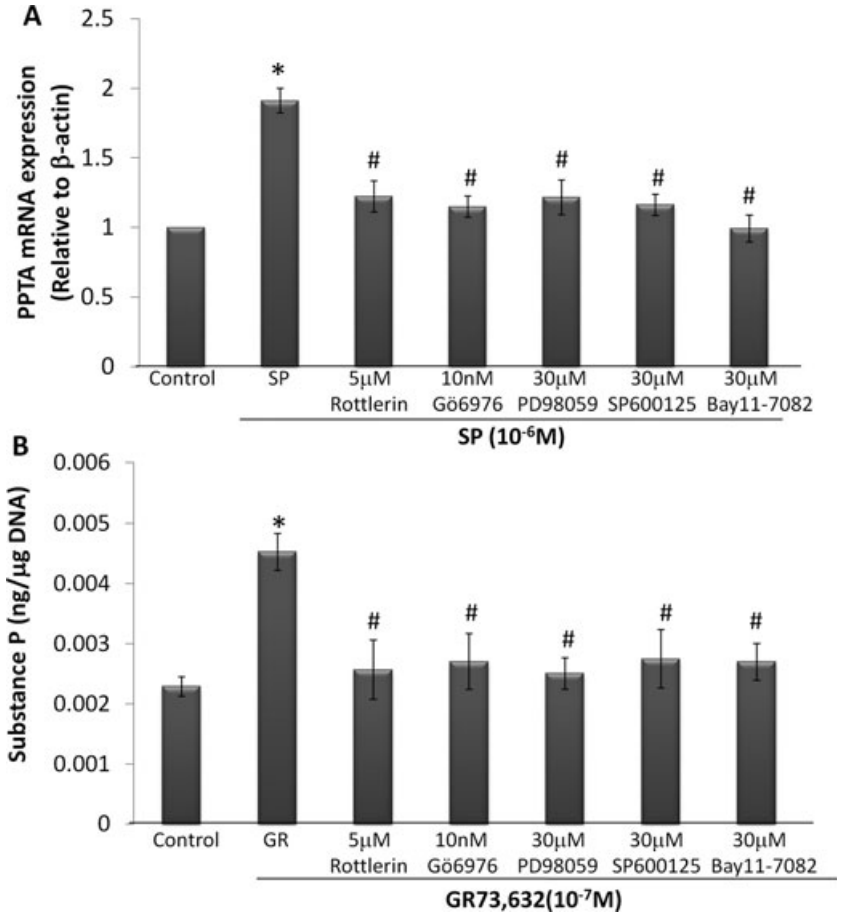

Fig. 5 PKC, MAPK and NF-κB are involved in SP-induced SP up-regulation in murine pancreatic acinar cells. Freshly prepared pancreatic acinar cells were pretreated with either Gö6976 (10 nM), rottlerin (5 μM), PD98059 (30 μM), SP600125 (30 μM) or Bay11-7082(30μM) for 30 min. before stimulation with SP (10^{-6} M) or GR73,632 (10^{-7} M) for 60 min. PPTA expression was determined with real time PCR and SP levels were determined by a commercially available ELISA kit. PPTA mRNA expression was normalized with β-actin expression and SP levels was normalized with DNA content in the samples. (**A**) PPTA mRNA expression. (**B**) SP expression. Results are expressed as means ± S.E.M. from 4–6 independent experiments. $^{*}P < 0.05$ *versus* control, $^{\#}P < 0.05$ *versus* SP/GR73,632 treated groups. Gö6976: a conventional PKC inhibitor; Rottlerin: a PKCδ inhibitor; PD98059: an ERK pathway inhibitor; SP600125: a JNK inhibitor; Bay11-7082: a NF-κB inhibitor; SP: substance P.

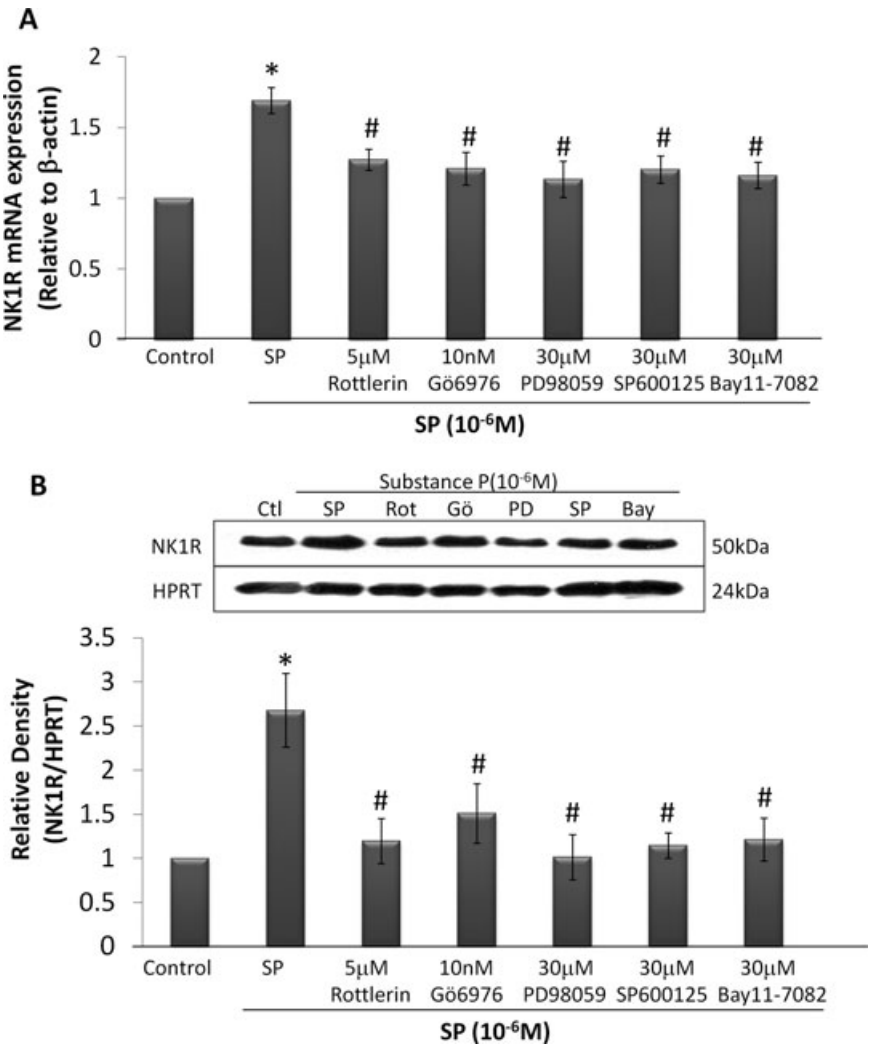

Fig. 6 PKC, MAPK and NF-κB are involved in SP-induced SP up-regulation in murine pancreatic acinar cells. Freshly prepared pancreatic acinar cells were pretreated with either Gö6976 (10 nM), rottlerin (5 μM), PD98059 (30 μM), SP600125 (30 μM) or Bay11-7082 (30μM) for 30 min. before stimulation with SP (10^{-6} M) for 60 min. NK1R mRNA expression was determined with real time PCR. Whole cell lysates were prepared and 50 μg of protein were fractionated on 10% sodium dodecyl sulfate-polyacrylamide gels for Western blot analysis of NK1R and HPRT. NK1R mRNA expression was normalized with β-actin expression and NK1R protein expression was normalized with HPRT expression. (**A**) NK1R mRNA expression. (**B**) NK1R protein expression. Results are expressed as means ± S.E.M. from 4–6 independent experiments. $^{*}P < 0.05$ *versus* control, $^{\#}P < 0.05$ *versus* SP treated groups. Gö6976: a conventional PKC inhibitor; Rottlerin: a PKCδ inhibitor; PD98059: an ERK pathway inhibitor; SP600125: a JNK inhibitor; Bay11-7082: a NF-κB inhibitor; SP: substance P.

in agreement with previous studies, which also showed rapid increases in chemokine expression when pancreatic acinar cells were stimulated with SP (10^{-6}M) [15]. SP treatment also induced a gradual increase in protein levels of NK1R. These rapid changes might enhance SP-NK1R-dependent pro-inflammatory responses in early phase of AP. SP was widely reported to act through stimulation of NK1R, including isolated pancreatic acinar cells [15]. Pre-treatment of cells with the selective NK1R antagonist CP96,345 completely reversed SP-induced up-regulation of PPTA and NK1R mRNA expression. Therefore, we suggest the observations were dependent on the activation of NK1R. These results proposed the presence of an 'auto-regulatory' mechanism, in which activation of NK1R increases the expression of SP and NK1R, thus potentially creating a positive feedback loop. However, the occurrence and extent of this feedback loop is currently unclear in physiological conditions.

GR73,632, a specific agonist of NK1R, induced a concentration-dependant increase of endogenous SP levels. Concurrent treatment of cells with an agonist and antagonist of NK1R completely abrogated the effects of NK1R activation. These observations confirmed that GR73,632 acts specifically through NK1R, and further demonstrated that NK1R activation is one possible mechanism that contributes to increased SP levels. The EC_{50} of GR73,632 was published as 2nM in guinea pig vas deferens, but information on pancreatic acinar cells was not available [25]. In agreement with previously published EC_{50} concentrations, our results showed that SP levels were induced with low nanomolar concentrations of GR73,632. On the other hand, previous work showed that the addition of exogenous SP (10^{-8}M) increased PPTA expression in cultured human normal fibroblasts [26]. In light of these observations, the proposed self-perpetuating feedback loop might also occur at lower concentrations of SP depending on the cell type used.

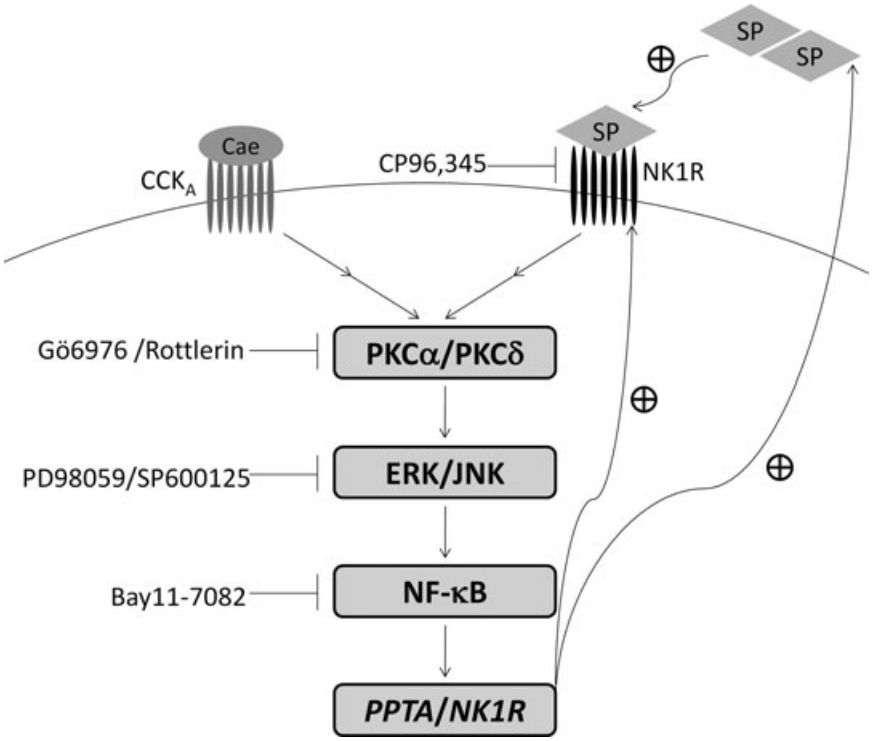

Fig. 7 A schematic model summarizing the results of the study. SP/NK1R expression auto-regulation could potentially up-regulate their expression to a much higher level.

Our results have suggested that caerulein stimulated CCK_A receptors and SP stimulated NK1R receptors in mouse pancreatic acinar cells. Current findings also suggested that CCK_A and NK1R downstream signalling may involve a similar pathway [17,19]. Upon stimulation with caerulein, both PPTA and NK1R gene were up-regulated by a PKC-MAPK-NF-κB dependent pathway in the pancreatic acinar cells. Ramnath *et al.* also showed SP-induced chemokine expression *via* the activation of PKC, MAPK and NF-κB pathway. These similarities are not surprising as both CCK_A receptors and SP receptors are both G-protein coupled receptors, acting *via* Gq/11 pathways [27,28]. In reference to previously established effective concentrations, pre-treatment of cells with specific inhibitors of PKC, ERK/JNK and NF-κB abolished SP-induced up-regulation of SP/NK1R in both protein and mRNA levels. In this case, activation of NK1R may lead to the activation of PKC and subsequent MAPK activation, followed by NF-κB transcription factor activation, which ultimately up-regulated expression of SP and NK1R (Fig. 7). The role of MAPKs on the activation of NF-κB is poorly understood, but current evidence suggests that ERK/JNK could act upstream of NF-κB by modulating IκB or IκB kinase [29–31]. Despite these observations, the similarities in CCK_A signalling pathway and NK1R signalling pathway should not be extrapolated to other models, as they exhibit very different responses in physiological conditions.

Previous studies have hinted the presence of an auto-regulatory mechanism of SP/NK1R expression in AP. In the pancreas of caerulein-treated mice, up-regulation of PPTA and NK1R mRNA expression was partially abolished when they were pre-treated with a selective NK1R antagonist, CP96,345 [16]. This is interesting because caerulein is not known to mediate through NK1R. Hence, we investigated whether similar observations could be reproduced in isolated pancreatic acinar cells. Unfortunately, CP96,345 neither reduced nor raised caerulein-induced up-regulation of PPTA/NK1R expression in these cells. Therefore, caerulein itself has no direct effects on the NK1R receptors. The difference between animal model and cellular model setting might be attributed to additional SP sources *in vivo*. SP can be released by nerve endings and also by infiltrating leukocytes, increasing its local levels so high that enabled the activation of NK1R to induce SP/NK1R up-regulation in pancreatic acinar cells. On the other hand, SP produced in pancreatic acinar cells alone did not reach sufficient concentrations to trigger stimulatory effects on the NK1R in our design.

Autocrine regulation of SP and NK1R may have its significance in regulating the severity of AP and other inflammatory diseases. In immune cells, autocrine regulation of SP was suggested to be involved in T-lymphocyte proliferation [32]. During intestinal inflammation, it was suggested that autocrine/paracrine regulation of cytokine secretion by SP led to a worsening of inflammation conditions [33]. Besides involvement in inflammatory conditions, SP auto-regulation was also shown in other physiological functions. In one previous report, exogenously administered SP in sutured rat Achilles tendon rupture had a booster effect on endogenous SP for fibroblast proliferation *via* autocrine/paracrine

stimulation [34]. With SP currently known to promote chemokine production in macrophages and pancreatic acinar cells, this auto-regulatory mechanism might be of significance. Especially when pancreatic acinar cells consisted of the bulk of pancreatic mass, SP contributed by these cells cannot be discounted. Further work could be done to elucidate the occurrence and extent of this auto-regulatory effect during the course of AP.

The observation that activation of NK1R up-regulates the expression of SP and/or NK1R is very likely to depend on the cell type or model used. SP was found to cause a 13-fold increase in PPTA expression in human mononuclear phagocytes [35]. Continuous administration of thiorphan, a specific neutral endopeptidase inhibitor, reduced SP degradation and enhanced rat thymocyte PPTA and NK1R mRNA expression as well as SP and NK1R protein levels in an NK1R-dependent manner [36]. It was also previously shown that stem cell factor treatment on bone marrow stroma induced PPTA and NK1R expression leading to autocrine and/or paracrine cell activation [37]. Previous studies have also shown direct evidence of an auto-regulatory role of SP/NK1R expression in human keratinocytes and human mononuclear phagocytes [27,38]. The auto-regulatory mechanism could also be activated by a N-terminal metabolite of SP. In that study, only NK1R expression, but not SP expression, was up-regulated by treatment of a SP metabolite on rat spinal cord [39]. Although there was evidence to suggest an auto-regulatory role of SP and NK1R expression, there were also several reports that showed otherwise. Hegde *et al*. showed that treatment of mice with SR140333, a selective NK1R antagonist, did not abolish sepsis-induced SP up-regulation in the lung [40]. Even in caerulein-induced AP model itself, it was shown that lung NK1R expression was further up-regulated when mice were treated with CP96,345, suggesting antagonism of NK1R further enhanced NK1R expression [16]. Palmer *et al*. also reported that disruption of the NK1R gene does not prevent up-regulation of PPTA mRNA in the spinal cord of mice following peripheral inflammation, when SP levels were greatly up-regulated [41]. Therefore, much work needs to be done to elucidate the different mechanisms that regulate SP/NK1R expression in different cell types. Investigators should be cautious when extrapolating the results to all models.

Collectively, the results in this study describe a novel mechanism of the contribution of SP-NK1R interaction during AP. Auto-regulation of SP/NK1R expression was occasionally reported but it has not been brought to focus. Current knowledge suggests that the SP increase in the pancreas could be contributed to increased release from sensory nerve fibres, infiltrating leukocytes, and possibly from the pancreatic acinar cells. Together, these sources contribute to elevated SP levels and up-regulate the expression of cytokines and chemokines *via* activating the NF-κB pathway. Further work could be done to investigate the significance of each of these sources of SP and defining other mechanisms that lead to SP/NK1R up-regulation. It is also useful to study the expression of SP/NK1R in peripheral neurons following NK1R activation, as they were considered to be a major source of endogenously produced SP.

In conclusion, we report evidence for the auto-regulatory mechanisms of SP and NK1R expression. By activation of the NK1R receptors, it is possible to up-regulate the expression of SP and NK1R in a PKC-MAPK-NF-κB dependant pathway (Fig. 7). With further understanding on the mechanisms that modulate physiological SP levels, new approaches may be achieved for the management of AP and the accompanying systemic inflammation.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

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