NK-1 receptor desensitization and neutral endopeptidase terminate SP-induced pancreatic plasma extravasation

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Maa, John, Eileen F. Grady, Ed H. Kim, Shandra K. Yoshimi, Matthew M. Hutter, Nigel W. Bunnett, and Kimberly S. Kirkwood. NK-1 receptor desensitization and neutral endopeptidase terminate SP-induced pancreatic plasma extravasation. Am J Physiol Gastrointest Liver Physiol 279: G726–G732, 2000.—Substance P (SP) induces plasma extravasation and neutrophil infiltration by activating the neurokinin-1 receptor (NK1-R). We characterized the mechanisms regulating this response in the rat pancreas. Anesthetized rats were continuously infused with SP, and plasma extravasation was quantified using Evans blue (EB) dye. Continuous infusion of SP (8 nmol·kg⁻¹·h⁻¹) resulted in a threshold increase in EB at 15 min, a peak effect at 30 min (150% increase), and a return to baseline by 60 min. The NK1-R antagonist CP-96,345 blocked SP-induced plasma extravasation. After 60 min, the NK1-R was desensitized to agonist challenge. Resensitization was first detected at 20 min and increased until full recovery was seen at 30 min. Inhibition of the cell-surface protease neutral endopeptidase (NEP) by phosphoramidon potentiated the effect of exogenous SP; therefore endogenous NEP attenuates SP-induced plasma extravasation. Thus the continuous infusion of SP stimulates plasma extravasation in the rat pancreas via activation of the NK1-R, and these effects are terminated by both desensitization of the NK1-R and the cell-surface protease NEP.

neurogenic inflammation; Evans blue; phosphoramidon; resensitization; tachykinins

SUBSTANCE P (SP) BELONGS TO the tachykinin family of neuropeptides and is found in the C fiber subpopulation of primary afferent nerves in many tissues (25). After its release from nerve endings, SP binds to the neurokinin-1 receptor (NK1-R), a seven transmembrane domain G protein-coupled receptor (11). Activation of the NK1-R on endothelial cells of postcapillary venules induces the formation of gaps between endothelial cells through which plasma proteins, fluid, and neutrophils extravasate (22). This process, which is a critical component of neurogenic inflammation, is important in animal models of asthma (2, 21), inflammatory bowel disease (7), arthritis (16), and bronchitis (5). The biological effects of SP are terminated by enzymatic degradation and receptor uncoupling from G proteins (4). Neurogenic inflammation is regulated by 1) the action of the cell surface enzyme neutral endopeptidase (NEP), which degrades SP in the extracellular space and thereby terminates its proinflammatory effects (18), and 2) desensitization of the NK1-R on endothelial cells (6). Genetic deletion of NEP results in increased levels of plasma protein leakage due to the impaired regulation of the proinflammatory effects of SP (17). Although NEP is found in the pancreas (28), its role in modulating SP-induced pancreatic inflammation has not been studied.

In the pancreas, SP immunoreactivity has been demonstrated in primary afferent nerves surrounding blood vessels and ductules (27). The NK1-receptor has been identified on pancreatic acinar and endothelial cells (29). We and others (14) have shown that SP modulates pancreatic exocrine secretion. The importance of SP and the NK1-R in pancreatic inflammation is suggested by several recent findings (3, 8, 12, 23). First, the bolus administration of SP induces pancreatic plasma extravasation in mice (8) and rats (23). Second, the plasma extravasation accompanying caerulein-induced pancreatitis is blocked by antagonism of the NK1-R in both rats and mice (12). Similarly, genetic deletion of the NK1-R reduces the severity of acute pancreatitis (12) and pancreatitis-associated lung injury (3) in caerulein-induced pancreatitis. Although SP plays an important proinflammatory role in regulating the severity of acute pancreatitis via activation of the NK1-R, it is not clear how this occurs.

To better understand the mechanism(s) by which SP promotes pancreatic inflammation, we sought to more closely mimic a pathophysiological inflammatory state in which low doses of endogenous SP could be released over longer periods of time. The aims of our study were to 1) characterize the contribution of SP to neurogenic pancreatic inflammation, 2) determine the receptor specificity of SP-induced plasma extravasation in rat pancreas, and 3) examine the mechanisms that terminate the proinflammatory effects of SP in the pancreas. We studied the effects of continuous, low-dose admin-
istration of SP on plasma extravasation of the albumin-bound dye Evans blue in the rat. We also determined if excitation of sensory nerves induces SP-mediated pancreatic plasma extravasation. Finally, we examined the contributions of receptor desensitization and enzymatic degradation of SP to the termination of neurogenic pancreatic edema.

**MATERIALS AND METHODS**

**Animals.** Male Sprague-Dawley rats weighing 250–400 g were obtained from Charles River Laboratories (Wilmington, MA). Rats were kept in a temperature-controlled environment with standard laboratory food and water freely available. They were housed in air-conditioned rooms with a 12:12-h light-dark cycle. Experiments were approved by and performed according to the rules of the University of California at San Francisco Committee on Animal Research.

**Materials.** Xylazine was from Phoenix Pharmaceuticals (St. Joseph, MO), and ketamine was from Abbott Laboratories (North Chicago, IL). SP, bradykinin (BK), Evans blue, formamide phosphoramidon, capsaicin (8-methyl-N-vanillyl- nonenamide), Tween 80, hexadecyltrimethylammonium bromide (HTAB), and 3,3',5,5'-tetramethylbenzidine substrate were from Sigma Chemical (St. Louis, MO). The NK1-R receptor antagonist CP-96,345 and its inactive enantiomer CP-96,344 were a gift of Pfizer Pharmaceuticals, courtesy of Dr. Saul Kadin (Groton, CT). SP ELISA kits were from Peninsula Laboratories (Belmont, CA).

**Extravasation of Evans blue.** We used Evans blue to quantify plasma extravasation. Evans blue binds to plasma proteins (principally albumin) and remains within the vasculature until gaps form between endothelial cells where the Evans blue-protein complex leaks into peripheral tissues (26). We (12) have previously confirmed that SP-induced increases in pancreatic Evans blue reflect plasma extravasation using Monastral blue pigment deposition in pancreatic endothelium. Rats were anesthetized with xylazine (5 mg/100 g im) and ketamine (10 mg/100 g im). A cervical incision was made, and a polyethylene catheter (PE-10) was inserted into the right external jugular vein. SP (4–20 nmol·kg⁻¹·h⁻¹ at a rate of 1 ml/h) or carrier (1 ml/h saline) was infused continuously (0–60 min). Evans blue (30 mg/kg of a 3% solution in 0.9% NaCl, 0.3 ml) was then injected through the jugular catheter. After 7 min, a cannula was placed into the left ventricle with its tip in the aorta, the right atria was cut open, and the rat was perfused with 50 ml PBS (10 mM phosphate buffer, 120 mM NaCl, and 2.7 mM KCl, pH 7.4) containing 0.2 U/ml heparin sodium, followed by 200 ml of 1% paraformaldehyde in 50 mM citrate buffer, pH 3.5. The pancreas was removed, rinsed in saline, gently blotted, and weighed. One-half of each tissue was dried by incubation at 120°C for 48 h and weighed (dry weight). The wet-to-dry ratio of the pancreas is a measure of pancreatic edema.

**Myeloperoxidase determination.** Neutrophil sequestration in pancreas and lung was quantified measuring tissue myeloperoxidase (MPO) activity. For these measurements, fresh tissue samples were homogenized in 2 ml of 20 mM phosphate buffer (pH 6.0) containing 0.5% HTAB and centrifuged (10,000 g for 20 min at 4°C), and the supernatant was used for the MPO assay. The reaction mixture consisted of a 50-µl aliquot of this extracted enzyme, 1.6 mM 3,3',5,5'-tetramethylbenzidine, 80 mM sodium phosphate buffer (pH 5.4), and 0.3 mM hydrogen peroxide. The absorbance at 655 nm was then corrected for the dry weight of the tissue sample used, and results are expressed as activity per unit of dry weight.

**Measurement of SP levels.** Rat pancreatic fragments were homogenized in 2 ml of ice-cold 0.1 M HCl for 20 s. The homogenates were centrifuged (15,000 g for 5 min at 4°C), and the supernatants were adsorbed on Sep-Pak cartridge columns. The peptides were freeze-dried and reconstituted in sample buffer: SP content was then determined with an ELISA kit according to the manufacturer's instructions and expressed as picograms per micromgram of DNA.

**Blood pressure measurements.** Under certain circumstances, SP administration lowers blood pressure (25). To exclude the potentially confounding effects of hypotension on Evans blue extravasation, mean arterial pressure was measured continuously during SP infusion. A polyethylene catheter (PE-10) containing heparin (100 U/ml) was inserted into the left femoral artery of anesthetized rats and connected to a pressure transducer (Custom Transpac; Abbott Systems) and a Squibb-Vitatek digital blood pressure analyzer (Hillsboro, OR).
Statistical analysis. Results are expressed as means ± SE. Differences between multiple groups were examined using ANOVA and the Student-Newman-Keuls-test. *P < 0.05 was considered significant.

RESULTS

**SP-stimulated extravasation of Evans blue.** Basal extravasation of Evans blue in carrier-treated animals was low in the pancreas (9 ± 2 ng/mg). The background signal, measured in rats that did not receive Evans blue, was 2 ng/mg. SP stimulated extravasation of Evans blue in the pancreas in a dose-dependent fashion. The threshold response occurred after a 30-min infusion of 6 nmol·kg⁻¹·h⁻¹ SP (17 ± 4 vs. 9 ± 2 ng/mg for 6 nmol·kg⁻¹·h⁻¹ SP vs. saline, respectively). The maximal effect occurred with 8 nmol·kg⁻¹·h⁻¹ SP for 30 min, which resulted in a 200% increase in Evans blue extravasation (Fig. 1). A diminished effect was seen at doses higher than 8 nmol·kg⁻¹·h⁻¹, with a return to baseline Evans blue accumulation after 20 nmol·kg⁻¹·h⁻¹ SP. Blood pressure was constant throughout the entire period of infusion at all doses.

Because 8 nmol·kg⁻¹·h⁻¹ SP produced the peak effect, we selected this dose for time-course experiments. Peak extravasation of Evans blue occurred after 30 min of continuous SP infusion (8 nmol·kg⁻¹·h⁻¹) (Fig. 2). With a return to baseline Evans blue accumulation after 20 nmol·kg⁻¹·h⁻¹ SP. Pretreatment with the NK1-R antagonist CP-96,345 abolished Evans blue extravasation after 20 nmol·kg⁻¹·h⁻¹ SP. The continuous infusion of SP resulted in a reduced Evans blue response after 30 min, rather than the expected plateau effect (Fig. 2). We therefore determined if desensitization of SP-induced plasma extravasation accounts for this observation. Excitation of sensory nerves with capsaicin induced a near fourfold increase in pancreatic plasma extravasation (42 ± 10 vs. 8 ± 8 ng/mg for capsaicin vs. saline, respectively; *P < 0.05, n = 3/group). This effect was due to activation of the NK1-R because it was completely blocked by CP-96,345 (CP-96,345 + capsaicin, 3 ± 1 ng/mg; *P < 0.05, n = 3).

**Desensitization.** The continuous infusion of SP resulted in a reduced Evans blue response after 30 min, rather than the expected plateau effect (Fig. 2). We therefore determined if desensitization of SP-induced plasma extravasation accounts for this observation. Alternatively, one could hypothesize that endothelial cell gaps became mechanically blocked by extravasated protein, precluding further extravasation.

A bolus of SP (10 nmol/kg) administered immediately after 60 min of continuous infusion of SP (8 nmol·kg⁻¹·h⁻¹) had no effect on Evans blue (Fig. 4), whereas a bolus of SP after 60 min of saline increased Evans blue extravasation by 2.5-fold (10 ± 1 vs. 25 ± 3 ng/mg, respectively; *P < 0.05). Thus physiological des-

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Fig. 1. Effects of continuous infusion of graded doses of substance P (SP) or saline for 30 min on Evans blue (EB) extravasation in rat pancreas. EB was injected, and tissue was collected 7 min later (n = 4–5 animals/group). Values are means ± SE. *P < 0.05 vs. saline.

Fig. 2. Time course of SP-induced EB dye accumulation in pancreas. SP (8 nmol·kg⁻¹·h⁻¹; hatched bars) or saline (open bars) was infused for 15–60 min. EB was injected, and tissue was collected 7 min later (n = 4–6 animals/group). Values are means ± SE. *P < 0.05 vs. saline.

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Summary of results:

- **Basal Extravasation:**
  - Evans blue background = 2 ng/mg.
  - SP (8 nmol·kg⁻¹·h⁻¹) increased extravasation to 200%.

- **Continuous Infusion:**
  - Peak effect after 30 min.
  - Desensitization observed.

- **Desensitization:**
  - Capsaicin induced near fourfold increase.
  - NK1-R block by CP-96,345.

- **Bolus Infusion:**
  - Immediate bolus after continuous infusion had no effect.
  - Bolus after 60 min on saline increased extravasation.

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Statistical Analysis:

- Data expressed as means ± SE.
- ANOVA and Student-Newman-Keuls-test.
- *P < 0.05 considered significant.
sensitization of SP-induced Evans blue accumulation had occurred. To determine if plasma extravasation could still be induced using a different stimulus, a 10 nmol/kg bolus of BK was injected after 60 min of SP infusion. BK stimulated a threefold increase in Evans blue extravasation (Fig. 4), which was not affected by pretreatment with the NK1-R antagonist CP-96,345 (data not shown). Thus BK-induced plasma extravasation occurred via activation of the BK B2 receptor and did not involve activation of the NK1-R. Continuous exposure of the NK1-R to SP results in desensitization of SP-induced plasma extravasation; however, the endothelium remains sensitive to other agonists.

Resensitization and recovery. To determine the time course for recovery of the pancreatic endothelium after SP-induced desensitization, SP (8 nmol·kg⁻¹·h⁻¹) was infused for 1 h. Animals were allowed to recover under anesthesia for increasing periods of time (0–30 min) after which a test bolus of SP (10 nmol/kg) was administered. Recovery was first detectable after 20 min, and full resensitization occurred by 30 min (Fig. 5). Thus desensitization of NK1-R-mediated plasma extravasation is rapidly reversed by removal of the ligand.

NEP antagonism. Because NEP degrades SP, we determined whether inhibiting NEP increases pancreatic plasma extravasation. First, we confirmed that the NEP inhibitor phosphoramidon increases basal levels of SP in the pancreas. Injection of phosphoramidon 15 min before a 30-min infusion of saline increased pancreatic SP levels by 100%. Phosphoramidon also increased basal extravasation from 9 ± 2 to 20 ± 3 ng/mg (Fig. 6), indicating tonic regulation by NEP. We then determined if NEP regulates SP-induced plasma extravasation. Because we expected a significant enhancement of SP-induced Evans blue with phosphoramidon, we selected a submaximal dose of 6 nmol·kg⁻¹·h⁻¹ SP for these experiments. Phosphoramidon pretreatment (15 min before SP) increased Evans blue extravasation by 142% (17 ± 4 vs. 41 ± 4 ng/mg, SP vs. phosphoramidon + SP, respectively; P < 0.05) (Fig. 6). The effect of phosphoramidon on pancreatic plasma extravasation was abolished by pretreatment with the NK1-R antagonist CP-96,345. Thus NEP modulates SP-induced plasma extravasation, presumably by regulating the local concentration of SP available to interact with the NK1-R.

Blood pressure. In contrast to the observed effects of the bolus administration of SP, which produces significant hypotension, mean arterial pressure did not change significantly with continuous infusion of tachykinins (8 nmol·kg⁻¹·h⁻¹ SP) or treatment with recep-
tor antagonists (Fig. 7). Modulation of blood pressure using Nipride (160 µg·kg⁻¹·min⁻¹) and Neo-Synephrine (16 µg·kg⁻¹·min⁻¹) produced expected alterations in mean arterial pressure. Thus the observed effects of SP on Evans blue accumulation are not attributable to systemic hemodynamic effects of tachykins.

Amylase and pancreatic wet-to-dry weight ratios. The continuous infusion of SP did not result in significant increases of either serum amylase, pancreatic water content, or neutrophil accumulation over the 1-h period of study.

DISCUSSION

In this study, we have characterized the initiation, termination, and receptor specificity of plasma extravasation induced by SP in the rat pancreas. Pancreatic plasma extravasation occurred with low-dose infusion of the tachykinin SP (8 nmol·kg⁻¹·h⁻¹ or 0.1 nmol·kg⁻¹·min⁻¹ for 30 min) or after excitation of sensory nerves using capsaicin. Pancreatic plasma extravasation induced by both SP and capsaicin was mediated by activation of the NK1-R because it was abolished by NK1-R antagonism. Maximal plasma extravasation occurred after 30 min of continuous SP infusion, whereas by 60 min NK1-R-mediated desensitization of this response was observed. Thus SP-induced plasma extravasation is both rapid in onset and rapidly attenuated despite the continued presence of agonist. Desensitization was rapidly reversed by removal of SP, and resensitization of SP-induced plasma extravasation was complete after a 30-min recovery period. The cell-surface peptidase NEP terminates the proinflammatory effects of SP via enzymatic degradation of the ligand. Inhibition of NEP increased pancreas SP levels and potentiated the effects of both endogenous and exogenous SP, presumably due to the increased local concentration of agonist available to interact with the NK1-R. SP infusion over 1 h did not result in increased pancreas weight or a significant increase in neutrophil infiltration. It is possible that a longer infusion period would be necessary to observe these effects.

We chose the continuous route of tachykinin administration rather than bolus injection. Bolus administration of SP has been shown (23) to induce Evans blue accumulation in the rat pancreas, which supports our findings. However, interpretation of results from these studies is rendered more difficult by the pharmacological levels of SP that may be achieved. Administration of neuropeptides via the continuous route presents two primary advantages over the administration via the bolus route. First, continuous infusion of low doses of peptides reduces the effect of these agents on heart rate and mean arterial pressure. Indeed, SP had no hemodynamic effects at the doses used in the present study. Second, the continuous method of delivery may more closely mimic the pathophysiological state of acute pancreatitis, wherein sensory nerves are presumably continuously stimulated to release tachykins.

After 60 min of continuous SP infusion, plasma extravasation could no longer be induced by SP, whereas the response to an alternative stimulus, BK, was preserved. In isolated neurons, cellular responses to SP desensitize after repeated challenge with SP and resensitize after 30 min of recovery (19). Desensitization prevents the uncontrolled stimulation of cells, whereas resensitization allows cells to maintain their ability to respond over time. After SP activates the NK1-R, G protein receptor kinases (GRK)²⁵ phosphorylate the receptor (15). Subsequently, β-arrestin-1 and -2 redistribute from the cytosol to the plasma membrane, where they interact with GRK-phosphorylated receptors and disrupt their association with heterotrimeric G proteins and terminate signal transduction. β-arrestins remain associated with the SP-NK1-R complex, which is then internalized into endosomes (19, 20). Although internalization is not required for desensitization, resensitization of cellular responses to SP requires endocytosis, recycling of the NK1-R, and phosphatase activity (9). Moreover, desensitization and resensitization of plasma extravasation to repeated challenge with SP in the rat trachea is temporally correlated with internalization and recycling of the NK1-R in endothelial cells of postcapillary venules (6). Thus responses to SP in the airway that are mediated by the NK1-R strongly desensitize in intact animals. The importance of this process in the regulation of endothelial responses to SP and the mechanism by which it occurs have not been previously studied in the pancreas. In theory, the desensitization of SP-induced plasma extravasation seen in our experiments could be due to nonspecific “plugging” of endothelial pores by plasma proteins, as has been shown (1) to occur with the synthetic peptide mystixin in the rat airway. In our experiments, however, desensitization was specific for NK1-R-mediated extravasation because the response to the agonist BK was unchanged. These results suggest that continuous exposure of the pancreatic endo-

![Fig. 7. Mean arterial pressure was measured continuously in anesthetized rats. Saline (○) or SP (8 nmol·kg⁻¹·h⁻¹; ■) was infused continuously for 30 min. Some animals were infused with Nipride (160 µg·kg⁻¹·min⁻¹; △) or Neo-Synephrine (16 µg·kg⁻¹·min⁻¹; ×) for 30 min, as positive controls. SP had no effect on mean arterial pressure.

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neutral endopeptidase (NEP) activity could contribute to uncontrolled pancreatitis. If a similar phenomenon were to occur in pancreatitis, then impaired neutral endopeptidase (NEP) activity could contribute to uncontrolled plasma extravasation. We measured the level of plasma extravasation at the end of the SP infusion period. Alternatively, one could measure the cumulative effect of SP over the entire study period, by injecting Evans blue at the start of SP infusion. In preliminary experiments, cumulative Evans blue levels paralleled those in the present study. To minimize the potential confounding effects of lymphatic clearance, redistribution, or metabolism of Evans blue, we chose to assess extravasation at a constant time interval after injection of Evans blue.

The cell surface enzyme NEP degrades SP in the extracellular fluid (28). The effects of NEP depend on coexpression of the enzyme with the NK1-R in the same cells (24). We used pharmacological inhibition of NEP with phosphoramidon to modulate the local availability of SP. In the absence of exogenous SP, phosphoramidon induced a nearly twofold increase in both pancreatic SP levels and NK1-R-mediated plasma extravasation in the pancreas. Phosphoramidon also potentiated the effect of exogenous SP. In fact, peak extravasation after phosphoramidon plus SP (6 nmol·kg⁻¹·h⁻¹) exceeded maximal extravasation induced by exogenous SP alone (8 nmol·kg⁻¹·h⁻¹). This finding underscores the importance of NEP in regulating the local concentration of SP available to interact with the NK1-R. The proinflammatory effects of SP in the pancreas are attenuated by both 1) desensitization of NK1-receptors on endothelial cells and 2) degradation of SP in extracellular fluid by NEP. These mechanisms may protect the pancreas from uncontrolled sensory nerve stimulation. Marked reductions in NEP activity accompany the development of inflammation in rat intestine (13). These changes are associated with a concomitant increase in SP immunoreactivity (13). If a similar phenomenon were to occur in pancreatitis, then impaired NEP activity could contribute to uncontrolled pancreatic inflammation.

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