Expression of NK-1 and NK-3 tachykinin receptors in pancreatic acinar cells after acute experimental pancreatitis in rats

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To date, few reports exist on the role of the TKs in regulating exocrine pancreatic secretion. TKs acting on NK-1 and NK-2 receptors have been reported to stimulate in vitro pancreatic amylase secretion (12–14, 16, 20, 33), and NK-1 and NK-2 receptors have been detected in the pancreatic acinar cells of guinea pig and mouse (6, 11, 29, 30, 32). No information is available on whether acinar cells in the pancreas of these and other animal species express the NK-3 receptor, and little is known on the effects of NK-3 receptor agonists on pancreatic exocrine secretion (7, 12, 18, 20, 33). In our recent studies, using in vitro functional (19) and immunocytochemical (4) assays, we reported that guinea pig pancreatic acinar cells contain homogeneously distributed NK-1 and NK-3 receptors that mediate a direct stimulatory action on amylase release. We also showed that in rat pancreatic acinar cells, the hitherto unidentified NK-1 receptors are widely expressed and mediate a secretagogue action on exocrine pancreas, whereas acinar NK-3 receptors are scarcely expressed and apparently do not mediate amylase release. These differences in expression and efficacy suggested that NK-1 receptors play a physiological role in pancreatic secretion in rats, whereas NK-3 receptors do not.

TKs in general, and in particular the NK-1 receptor agonist SP, mediate a variety of inflammatory processes including asthma (8, 21), inflammatory bowel disease (38), arthritis (36), and also pancreatitis (3). Information is lacking on the exact events during pancreatitis. A useful tool to define the underlying cellular and specific organ mechanisms is secretagogue-induced experimental pancreatitis. In vitro and in vivo data (34) indicate that the earliest and most important factor responsible for this experimental model of acute pancreatitis is the increase and the activation of intraacinar digestive enzymes. After this initial insult, other components (ischemia, pancreatic glutathione deficiency, oxygen free radicals, cell death, inflammatory mediators) intervene to worsen pancreatitis. Increasing evidence indicates that neurogenic proinflammatory factors, such as SP, can play important roles in determining the severity of acute pancreatitis (27), both in mice and rats. Previous data (2) show that SP levels and NK-1 receptors in pancreatic acinar cells are upregulated during experimental pancreatitis in mice. In addition, NK-1 receptor deletion or antagonism protects mice against pancreatitis (3, 9, 15). SP-induced activation of the NK-1 receptor is an important proinflammatory step also in rat acute pancreatitis. Caerulein (CK)-induced plasma extravasation, hyperamylasemia, and histological damage were blocked by antagonism of the NK-1 receptor in rats (9). Hence,
CK-induced, NK-1 receptor-mediated plasma extravasation in acute pancreatitis arises from intrapancreatic release of SP. The peptide’s precise etiopathogenetic mechanism nevertheless remains unclear. No study has tried to determine the possible relationship between NK-1 receptor expression in acinar cells, amylase secretion, and the severity of inflammation during pancreatitis in rats.

Despite the predominant interest in SP as a proinflammatory mediator in acute pancreatitis, current knowledge merely shows that NK-3 receptor agonists have no effect on plasma extravasation in the pancreas (9), and this receptor type, scarcely expressed on acinar cells, apparently plays no role in rat exocrine pancreatic secretion under basal conditions (4). Hence, whether NK-3 receptor agonists and NK-3 receptors are involved in this condition in rats remains unknown.

Seeking to extend the knowledge of the biological and molecular alterations linked to acute pancreatitis and the underlyng regulatory events, in this study, we therefore investigated NK-1 and NK-3 receptor expression in rat pancreatic acinar cells. Using an immunofluorescence assay and Western blot analysis, we determined the expression of both receptor subtypes under normal conditions and after acute pancreatitis induced by stimulating pancreatic secretion with CK. To give evidence of pancreatic injury, we also evaluated plasma amylase levels, pancreatic water content, and morphological alterations in the pancreatic tissue.

**MATERIALS AND METHODS**

**CK-induced experimental acute mild pancreatitis.** Acute pancreatic inflammation was induced in male rats (200–250 g, Morini, RE, Italy) as previously described (5) by three intraperitoneal injections of CK (Bachem, Germany) at the dose of 10 μg/kg with a 1-h interval between injections. Normal control rats were intraperitoneally injected with a 0.9% NaCl solution. One hour after the last injection, rats were euthanized by CO2 and blood was obtained for serum amylase determinations (see *Serum amylase assay*). The pancreas was rapidly removed, and samples were resected for morphological evaluation and for use as isolated acini preparations. Dispersed acini were then processed for immunofluorescence, Western blot procedures and functional secretory assay.

The specific indexes evaluated to assess the degree of pancreatic inflammation included serum amylase levels, pancreatic edema, and morphological examination of pancreatic tissue.

**Serum amylase assay.** Amylase levels in serum were determined with the colorimetric quantitative kinetic method according to the manufacturer’s instructions (Sigma). An enzyme unit was defined as the amount of enzyme that produces an increase of one absorbance unit per minute.

**Pancreatic edema.** To assess the development of pancreatic edema, samples of pancreas were weighed and then dried for 48–72 h at 60°C and reweighed to determine pancreatic water content. The results were calculated as (wet weight − dry weight)/wet weight and are expressed as a percentage (26).

**Morphological examination.** Random cross sections of the head, body, and tail of the pancreas (multiple specimens of pancreas tissue) were fixed with 4% neutral formalin and routinely paraffin embedded for histological study. Five-micrometer-thick sections were stained with hematoxylin-eosin and acid-periodic Schiff (PAS).

For transmission electron microscopy, small samples of pancreatic tissue were fixed in a 2.5% solution of purified glutaraldehyde in 0.1 M cacodylate buffer at pH 7.3, then postfixed in 1.33% osmium tetroxide. The tissues were then dehydrated in increasing concentrations of ethanol and embedded in epoxy resin (Epon 812) (37).

Semithin sections were stained with azure II methylene blue to select appropriate fields. Ultrathin sections obtained with an LKB Ultratome III were stained with uranyl acetate and lead citrate and observed under a Zeiss EM 10 electron microscope (25). Semithin section 0.5- to 1-μm thick were observed under a Zeiss photo microscope.

**Rat-isolated pancreatic acini preparation.** Male Wistar rats from Morini (RE, Italy), weighing 200–250 g were euthanized (70% CO2) after an overnight fast. The pancreas was harvested, and fat and lymphatic tissue was removed. Pancreatic acini were prepared by collagenase digestion as described by Peikin et al. (24). In brief, the pancreas was injected with 5 ml of digestive solution (standard buffer containing collagenase 0.2 mg/ml) and digested twice for 15 min at 37°C in a Dubnoff shaking bath (120 oscillations/min). After being hand shaken, fragments of tissue were removed and acini were dispersed by trituration with pipettes of decreasing diameter and washed twice with a standard solution containing 2% (wt/vol) albumin and 2 mM CaCl2. Acini were put into 25 ml of standard solution and centrifuged for 10 s at 150 g (at 4°C). Krebs-Ringer-HEPES buffer (pH:7.4) was used as standard solution containing (in mM) 103 NaCl, 8 KCl, 1.2 KH2PO4, 2 glucose, 5 glycine, 25 HEPES, 1.3 CaCl2, 0.6 MgSO4 with 1% (vol/vol) amino acid supplement, 1% (wt/vol) bovine albumin, 0.1% (wt/vol) trypsin inhibitor, and aprogin (600 KIU/ml). Acini from the pancreas of one rat were suspended in 100 ml of standard solution and preincubated for 30 min at 37°C in a Dubnoff shaking bath. After being shaken, 10-ml aliquots of this acini suspension were rapidly distributed into vials for immunocytochemistry and Western blot procedures (see **Immunocytochemistry**).

**Immunocytochemistry.** Isolated pancreatic acini of rat were spun down on glass slides using a cytocentrifuge (Shandon) and then incubated in PBS for 1 h at 4°C with a rabbit polyclonal anti-NK-1 receptor (NB 300–101, Novus Biologicals) or anti-NK-3 receptor antibody (NB 300–102, Novus Biologicals) diluted 1:400, cross-reacting with guinea pig and rat receptors. Cells were then washed again with PBS before being incubated with a goat anti-rabbit rhodamine-conjugated secondary antibody (Sigma) for 30 min at room temperature. Cells were fixed in 4% (wt/vol) paraformaldehyde for 10 min at room temperature on immunofluorescence-labeled coverslips, then incubated with Hoechst 33258 (0.25 μg/ml) for 5 min at room temperature, then visualized with an LAICA fluorescent microscope, and counted using a ×60 objective and a ×10 eyepiece on an Olympus BX51 photomicroscope. Acinar cells were scored by counting 30–50 microscopic fields per coverslip in four coverslips from four to six experiments. In control experiments to assess primary antibody specificity, the primary antibodies were omitted. In these samples, no immunostaining was detected (data not shown).

**Western blotting procedures.** Samples of rat dispersed pancreatic acini were homogenized in ice-cold Tris-buffered saline containing 40 mM Tris·HCl (pH 7.5), 2% SDS, protease inhibitor cocktail (in mM: 0.08 aprotinin, 104 AEBSF, 1.4 E-64, 4 bestatin, 1.5 pepstatin-A, and 2 leupeptin; Sigma), 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1 mM EDTA. Homogenates were centrifuged at 10,000 g for 10 min, and the supernatants were collected and stored at −70°C. Protein concentration of the homogenates was determined using the BCA protein assay kit (Pierce, Rockford, IL). For gel loading, the homogenates were heated for 4 min at 95°C in an equal volume of sample buffer (100 mM Tris, pH 6.9, 2% SDS, 2% 2-mercaptoethanol, 0.001% bromophenol blue, 20% glycerol). The samples were loaded onto 10% SDS-PAGE gels in equal protein amounts (30 mg/lane). Samples were separated by electrophoresis in Tris-glycine buffer (25 mM Tris, 250 mM glycine, 0.1% SDS) at 100 V for 120 min. Proteins were transferred overnight (12–14 h) to a polyvinylidene difluoride membrane at 30 V in transfer buffer containing 20% MeOH, 20 mM Tris, 150 mM glycine, pH 8.0. Membranes were incubated for 1 h at room temperature in blocking buffer containing 5% no-fat powdered milk in TBS-Tween (20 mM Tris, 137 mM NaCl, 0.1% Tween-20), then washed three times for 10 min each.
in TBS-Tween. Membranes were incubated overnight (12–14 h) with primary antibodies directed against the NK-1 receptor protein (COOH-terminus, rat NK-1 protein 393–407, Novus Biologicals) and against the NK-3 receptor protein COOH-terminus, rat NK-3 receptor protein 438–452, Novus Biologicals) in blocking-buffer at a dilution of 1:1,000 with β-actin (1:5,000, Sigma) used as loading control. After being washed three times for 15 min each with TBS-Tween, membranes were incubated with rabbit horseradish peroxidase-conjugated secondary antibody, diluted 1:2,000 in blocking buffer for 1 h, then washed in TBS for 15 min. Peroxidase activity was detected using the Pierce SuperSignal West Femto Maximum Sensitivity Substrate kit (Pierce Chemicals, Rockford, IL) and quantified using ScanAnalysis software.

Functional secretory assay and amylase determination. Two-milliliter aliquots of the dispersed acini suspension were distributed in 25-ml flasks, incubated with the appropriate agent (SP, 10^{-7} M; PG-KII, 10^{-7} M) for 30 min at 37°C, and tested for their functional secretory activity (amylase determination). Two control flasks were prepared for each experiment. When incubation ended, the total content of the flasks was transferred into chilled tubes and centrifuged at 3,000 g for 4 min. The supernatant was collected, and acini were lysed with a buffer (pH 7.8) containing 0.01 M NaH2PO4·H2O, 0.01 M NaH2PO4·2H2O, 0.1% SDS, and 0.1% (wt/vol) albumin. Amylase activity was determined in duplicate for the media and for acini preparations using a colorimetric test (Ultimate Amylase EPS, Roche) and expressed as percentage of total cellular content. Mean percent of total amylase release from duplicate incubations for each animal was used for statistical analysis.

Drugs. SP was purchased from Peninsula Laboratories (San Carlos, CA), and PG-KII (pGlu-Pro-Asn-Glu-Val-Gly-Leu-Met-NH2) was purchased from Neosystem (Strasbourg, France).

Statistics. Data are expressed as means ± SE. The significance of changes was evaluated by using Student’s t-test, where necessary. For analysis of the Western blotting data, density for β-actin control values obtained using the ScanAnalysis software was defined as 100%; experimental densities are reported as a percentage of control density.

RESULTS

About 4 h after rats received three intraperitoneal injections with hyperstimulating doses of the secretagogue CK (10 \mu g/kg with a 1-h interval between injections), serum amylase concentrations, concentrations, and ultrastructural morphology (the three indexes indicating the severity of pancreatitis) all changed. The serum amylase concentrations were about fivefold higher in CK-treated rats than in control rats (Fig. 1). CK treatment also caused a significantly larger increase in pancreatic water content (a measure of pancreatic edema) in treated rats than in normal control rats (P < 0.05; Fig. 1). In CK-hyperstimulated rats, light microscopy disclosed no evident pancreatic lesions (data not reported). Conversely, transmis-

ion electron microscopy disclosed marked ultrastructural alterations of the pancreatic tissue (Fig. 2). The acinar cells showed changes in the plasma membrane with a reduction in the number of microvilli and density of intracytoplasmic zymogen granule, formation of cytoplasmic vacuoles, dilatation of the rough endoplasmic reticulum cisternae, and mitochondrial damage. Acinar-cell nuclei had minimal lesions and showed prominent nucleoli.

In samples of dispersed pancreatic acinar cells prepared from normal control rats, immunofluorescent staining of NK-1 and NK-3 receptors showed a typical punctuate membrane localization, but they differed markedly in density (Fig. 3, A and C). Cell counts indicated a larger number of acinar NK-1 immunoreactive cells than NK-3 immunoreactive cells (51 ± 6 vs. 8 ± 2%; Table 1). Photomicrographs of pancreatic acinar cells after CK-induced acute pancreatitis disclosed a similar staining density for NK-1 receptors and a larger density of NK-3 receptors in stimulated than in control unstimulated pancreatic samples (Fig. 3, B and D). Cell counts revealed an equal percentage of acinar NK-1 immunoreactive cells after CK-induced pancreatitis and in normal pancreas (50% ± 8 vs. 51% ± 6; Table 1) but a larger percentage of NK-3 immunoreactive cells in CK-induced pancreatitis than in normal pancreas (85 ± 3 vs. 8.0 ± 2; Table 1). The lack of detectable staining when the secondary antibodies were used alone confirmed the specificity of the antibodies used in this study.

Western blotting analysis of NK-1 and NK-3 receptor protein levels (Fig. 4A) showed no change in NK-1 receptor protein expression in pancreatic acini after CK pancreatitis compared with control unstimulated acini. In contrast, CK-hyperstimulated pancreatic acini exhibited a more intense signal for NK-3 receptor protein expression than did normal acini. The densitometric analysis demonstrated about a threefold larger increase in NK-3 receptor protein levels in pancreatic acini hyperstimulated with CK than in unstimulated acini (Fig. 4B). Equal amounts of proteins were loaded for each sample, as demonstrated by the comparable density levels for β-actin, used as internal control, confirming that the differences in immunoreactivity observed in the different bands did not depend on the different amounts of proteins loaded.

Functional secretory assays indicated that the basal amylase secretion by pancreatic acinar cells after acute experimental pancreatitis in rats was significantly higher than that obtained in pancreatic acinar cells under normal conditions (16.5 ± 3.1 vs. 6.5 ± 0.5). The NK-1 receptor agonist SP (10^{-7} M), when
SP is found in the majority of fibers innervating the pancreas. The NK-1 receptor agonist SP has been considered the neurogenic factor playing an important role in the inflammatory cascade in experimental pancreatitis in rats (23). Studies using immunohistochemistry and Western blotting analysis show NK-1 receptor upregulation in pancreatic tissues of rats (35) and also in humans (31) during pancreatitis. CK hyperstimulation in mice causes biochemical and histological evidence of acute pancreatitis via capsaicin VR1 receptors on primary sensory neurons, which release the endogenous SP responsible for the inflammatory cascade (23). Similarly, during experimental pancreatitis in mice, pancreatic levels of SP undergo several-fold upregulation after three hourly injections of CK, and NK-1 receptor antagonists protect against acute pancreatitis (17).

Notwithstanding this knowledge, precisely how SP contributes to the severity of pancreatitis is not clear. Recent observations that the expression of NK-1 receptors is markedly increased on acinar cells in samples prepared from mice with pancreatitis induced by 12 hourly injections of CK (2) suggest that SP may exert its proinflammatory effect directly on the acinar cells (17). Hence, CK-stimulated amylase release could be considered the earliest and sensory neuron-independent event in the induction of CK pancreatitis in mice. In line with this hypothesis, our previous results in rats (19) have demonstrated that under normal conditions, by directly activating acinar NK-1 receptors, SP stimulates pancreatic secretion, thus indicating that also in rats, SP-mediated functions within the pancreas could go beyond sensory signals of neurogenic inflammation alone.

Our new findings on the expression of NK-1 receptors on the pancreatic acini during CK-induced pancreatitis in rats showed that the already dense distribution of NK-1 receptors under normal conditions remains quantitatively unchanged after CK pancreatitis. Furthermore, functional data, showing that SP evoked a similar increase in amylase release under normal and pathological conditions, give a further confirm that after pancreatitis in rats the NK-1 receptors do not change their expression. This finding differs substantially from what has been previously reported in mice acinar cells expressing an increased number of NK-1 receptors after pancreatitis (2). Because our study in rats investigated mild acute pancreatitis, whereas others investigating mice studied severe pancreatitis, the severity of pancreatitis could explain the different NK-1 receptor expression obtained in rats and mice. However, our preliminary but not reported immunocytochemistry and Western blot results, indicating that rat acinar cells express the same number of NK-1 receptors after mild and severe pancreatitis, remove this possibility. Even if further investigation are required, beside the different severity of pancreatitis, the only alternative we can put forward is a species difference.

A further distinctive finding in this study is that the TK receptor subtype NK-3, which has a very low density in rat pancreatic acinar cells under basal conditions, become upregulated after secretagogue-induced pancreatitis. In addition, NK-3 receptor agonists, which are completely inactive on exocrine pancreatic secretion under normal conditions, significantly stimulate amylase secretion in rat acinar cells after pancreatitis. Overall, these findings to date indicate that NK-3 receptors, which apparently have no physiological role in pancreatic secretion in rats, under CK stimulation become

**DISCUSSION**

To our knowledge, this is the first study reporting the expression of NK-1 and NK-3 receptors in pancreatic acinar cells after acute experimental pancreatitis in rats. Whereas CK-induced pancreatitis left NK-1 receptor expression pancreatic acini apparently unchanged compared with normal pancreas, it caused NK-3 receptor overexpression. In this secretagogue-induced model of acute pancreatitis, hyperamylasemia, increased pancreatic water content, and ultrastructural alterations in the pancreatic tissue provided clear evidence of mild pancreatic injury.

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**REFERENCES**

The detailed references are not included here for brevity, but they are available in the full publication. The full text document can be accessed at the provided URL.
activated, alter cell function, and probably intervene in mediating acute pancreatitis.

Differences in the function and expression of the two TK receptor classes depend closely on the regulatory processes they trigger. NK-1 and NK-3 receptors exhibit marked differences in the rate of resensitization and in their ability to colocalize with isoforms of β-arrestins (determined by domains in intracellular loop 3 and the COOH tail), and these differences could have important functional implications. CK-induced SP release activates subsequent NK-1 receptor endocytosis in acinar cells (22). After activation, NK-1 receptors interact with β-arrestins 1 and 2 with high affinity, internalize rapidly, but are slowly resensitized (28). The NK-1 receptor desensitization in the continued presence of agonist presumably occurs via mechanisms that implicate G protein-coupled receptor kinase and β-arrestins. Biochemical and electrophysiological studies on cells expressing NK-1 receptors indicate that NK-1 receptor signaling diminishes within seconds after agonist exposure (1). Conversely, NK-3 receptors transiently interact with β-arrestin (preferentially β-arrestin 2), are largely excluded from endosomes, and rapidly resensitized (28). In pancreatic acinar cells coexpressing NK-1 and NK-3 receptors such as enteric neurons and endothelial cells, the activation of the NK-1 receptor, by sequestering β-arrestins and NK-1 receptors in endosomes, contributes to desensitization by depleting NK-1 receptors from the cell surface and by impeding endocytosis of NK-3 receptors causes their retention on the cell surface. Such regulation could allow the NK-3 receptor to become the major receptor responding to SP at a time when NK-1 receptors are desensitized and internalized. Rapid and increased resensitization of the NK-3 receptor would permit cells to respond quickly to SP stimulation. Hence NK-1 and NK-3 receptors might both be involved in the stimulatory

Table 1. *Immunofluorescent cell counts*

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Coverslips, N°</th>
<th>Microscopic fields, N°</th>
<th>N° Total cells (Hoechst +)</th>
<th>N° Positive Cells</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>Anti-NK-1 4</td>
<td>42</td>
<td>384</td>
<td>198</td>
<td>51 ± 6</td>
</tr>
<tr>
<td></td>
<td>Anti-NK-3 4</td>
<td>38</td>
<td>334</td>
<td>27</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>Treated</td>
<td>Anti-NK-1 4</td>
<td>44</td>
<td>390</td>
<td>195</td>
<td>50 ± 8</td>
</tr>
<tr>
<td></td>
<td>Anti-NK-3 4</td>
<td>42</td>
<td>344</td>
<td>283</td>
<td>85 ± 3</td>
</tr>
</tbody>
</table>

Cells were counted on immunofluorescence-labeled coverslips, using a ×60 objective and a ×10 eye-piece on a Olympus BX51 photomicroscope. Cells were scored by counting 30–50 microscopic fields per coverslip in 4 coverslips from 4–6 experiments.

Fig. 3. Neurokinin (NK)-1 receptor (NK-1R) and NK-3 receptor (NK-3R) immunofluorescent staining in pancreatic acinar cells in control rats (no pancreatitis; A and C) and after CK-induced pancreatitis (B and D).

Fig. 4. Lane 1, top: representative Western blot analysis of NK-1 and NK-3 receptor proteins (50 kDa) from control pancreatic acini; bottom: levels of internal control protein β-actin (42 kDa). Lane 2, top: levels of NK-1 receptor and NK-3 receptor; bottom: β-actin protein in CK-induced pancreatitis. Quantitation of the density of the Western blotting bands. The levels of NK-1 receptor and NK-3 receptor proteins in control rats and in CK-induced pancreatitis are expressed as percentages of the density in β-actin controls.
secretory pathway participating in the pathogenesis of acute pancreatitis in rats.

In conclusion, this study suggests that SP could mediate the inflammatory response to acute pancreatitis in rats directly by activating TK acinar cell receptors. Whether the interaction of SP with pancreatic acinar cells and the resulting increase in enzyme release or the production of other inflammatory mediators contribute to its proinflammatory role in experimental pancreatitis in rats requires elucidation.

An extended knowledge of the biological and molecular alterations linked to acute pancreatitis and the underlying regulatory events will open the way for new therapies for this life-threatening condition. Once the responsible receptors are identified, selective antagonists able to block these receptors could be useful therapeutic remedies.

ACKNOWLEDGMENTS

The authors thank C. Munari for expert technical assistance in performing the experiments.

GRANTS

This work was supported by grants from the Italian Ministry of University and Scientific and Technological Research.

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28. Schmidlin F, Dery O, Bunnett NW, and Grady EF. Heterologous regulation of trafficking and signaling of G protein-coupled receptors: Table 2. Effects of SP and PG-KII on amylase release from rat dispersed pancreatic acini under normal condition and after pancreatitis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Amylase Release (percentage of total/30 min)</th>
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<tbody>
<tr>
<td></td>
<td>Unstimulated pancreatic acini (normal condition)</td>
</tr>
<tr>
<td>Saline</td>
<td>6.5±0.5</td>
</tr>
<tr>
<td>SP 10−7</td>
<td>12.0±1.5*</td>
</tr>
<tr>
<td>PG-KII 10−7</td>
<td>6.3±1.2</td>
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</table>

Data are expressed as the mean ± SE of duplicate determinations from 5 separate experiments. *P < 0.05 and †P < 0.01 vs. own saline value. CK, caerulein; SP, substance P.


