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Short Communication

Contribution of TRPA1 as a Downstream Signal of Proteinase-Activated Receptor-2 to Pancreatic Pain

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Abstract. We examined if TRPA1, like TRPV1, contributes to pancreatic nociceptor excitation following proteinase-activated receptor-2 (PAR2) stimulation and to pancreatitis-related pain in mice. A PAR2-activating peptide, infused into the pancreatic duct, caused spinal Fos expression, which was prevented by AP18, a TRPA1 inhibitor. Repeated administration of cerulein caused referred hyperalgesia accompanying pancreatitis, which was reversed by SB366791, a TRPV1 inhibitor, but not AP18. AP18, administered in combination with a subeffective dose of SB366791, significantly suppressed the referred hyperalgesia. Our findings suggest that TRPA1, like TRPV1, mediates PAR2-triggered pancreatic nociception and that TRPA1 in collaboration with TRPV1 latently contributes to pancreatitis-related pain.

Keywords: transient receptor potential ankyrin-1 (TRPA1), proteinase-activated receptor-2 (PAR2), pancreatic pain

Acute pancreatitis involves excessive activation of digestive enzymes including trypsinogen within the pancreas. Proteinase-activated receptor-2 (PAR2), a G protein–coupled receptor activated by certain enzymes including trypsin and tryptase, plays roles in the pathophysiology of the pancreatitis (1). PAR2 is expressed in the pancreatic duct, acini, and also primary afferents, and plays dual roles in acute pancreatitis, being pro- and anti-inflammatory (1, 2). PAR2 also mediates pancreatic pain signaling (1, 3 – 5). The trans-activation of transient receptor potential vanilloid-1 (TRPV1) by PAR2 activation is involved in the development of somatic pain (6 – 8) and also pancreatitis-related pain (3, 4). On the other hand, trans-activation of transient receptor potential ankyrin-1 (TRPA1) by PAR2 activation contributes to inflammatory pain (9). Nonetheless, it remains unclear whether TRPA1, like TRPV1, mediates pancreatic pain as a downstream signal of PAR2 during pancreatitis. Interestingly, TRPA1 as well as TRPV1 appears to mediate the continuation of acute pancreatitis or its transition to chronic pancreatitis (10, 11). However, it is unclear if TRPA1 and TRPV1 play a cooperative role in the maintenance of pancreatic pain after the establishment of acute pancreatitis. In the present study, we thus examined if blockade of TRPA1 attenuates nociceptive signals following pancreatic ductal administration of a PAR2-activating peptide in healthy mice and then asked whether blockade of TRPA1 reverses the referred hyperalgesia after the establishment of cerulein-induced acute pancreatitis in mice.

Male ddY mice weighing 12 – 25 g were purchased from Kiwa Laboratory Animals Co., Ltd. (Wakayama). All experimental protocols were approved by the Committee for the Care and Use of Laboratory Animals at Kinki University and were in accordance with the Guiding Principles approved by The Japanese Pharmacological Society. As described previously (12), in mice under anesthesia with i.p. urethane (1.35 – 1.5 g/kg), the PAR2-activating peptide, SLIGRL-NH_2 (a gift from Fuso Pharmaceutical Industries, Ltd., Osaka), at 15 nmol/mouse was infused into the pancreatic duct. The mice were perfused transcardially with physiological saline in a volume of 10 ml/10 g body weight and subsequently with 4% paraformaldehyde in a phosphate

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buffer (pH 7.4) in a volume of 20 ml/10 g body weight, 2 h after administration of SLIGRL-NH_2. The spinal cord was removed, postfixed in 4% paraformaldehyde at 4°C for 24 h and then cryoprotected in a phosphate-buffered 30% sucrose solution overnight at 4°C. The T8 – T10 spinal segments were serially sectioned at 30-μm thickness using a freezing microtome. The sections were collected in 0.1 M phosphate-buffered saline (PBS) and processed for Fos immunostaining. The consecutive sections were incubated in 1% normal goat serum for 30 min, and then reacted with a rabbit polyclonal antibody against human Fos p62 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:7000 for 16 h at 4°C. The sections were then incubated with a biotinylated goat antiserum against rabbit immunoglobulin G (IgG) for 1 h and subsequently treated with a peroxidase-conjugated avidin–biotin complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA, USA) for 30 min at 4°C. To develop the ABC reaction, the sections were incubated in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.05% 3,3′-diaminobenzidine-tetra HCl, 0.4% nickel ammonium sulfate, and 0.035% hydrogen peroxide for 10 min at 24°C. The sections were then rinsed three times with the Tris-HCl buffer for 10 min. The number of Fos-positive cells was determined bilaterally in laminae I-II, III-IV, and V-VI of the T8-T10 spinal sections. In conscious mice, acute pancreatitis was created by 6 i.p. administrations of cerulein (Bachem, Bubendorf, Swizerland) at 50 μg/kg at 1-h intervals, as described previously (12). Referred hyperalgesia in the upper abdomen was assessed 30 min after the final dose of cerulein. Briefly, mice were placed on a raised wire mesh, under a clear plastic box (23.5 × 16.6 × 12.4 cm), and acclimated to the experimental environment for 20 – 30 min. The upper abdomen of each mouse was stimulated using three distinct von Frey filaments with strengths of 0.02, 0.16, and 1.0 g, in ascending order of their strength, at intervals of 5 – 10 s, 10 times for each filament. Scoring of nociceptive behavior was defined according to the previous report (12). For evaluation of the severity of the pancreatitis, blood samples were collected from the abdominal aorta in the mice under urethane anesthesia, and the pancreas was excised and weighed. Plasma amylase activity was determined using an automatic analyzer (Dri-Chem 3500i; Fujifilm, Tokyo) with its exclusive colorimetric assay kit (AMYL-P) (Fujifilm). SLIGRL-NH_2 and cerulein was dissolved in saline. AP18 (Enzo Life Science, Farmingdale, NY, USA), a TRPA1 inhibitor, and SB366791 (Sigma Ltd., St Louis, MO, USA), a TRPV1 inhibitor, were dissolved in a solution containing 7.5% DMSO, 92% PBS, and 0.5% Tween 80 and in a saline solution containing 2% DMSO and 1% cremophol, respectively. AP18 at 10 mg/kg was administered i.p. 30 min before infusion of SLIGRL-NH_2 into the pancreatic duct. In order to examine possible involvement of TRPA1 in pancreatic pain, but not

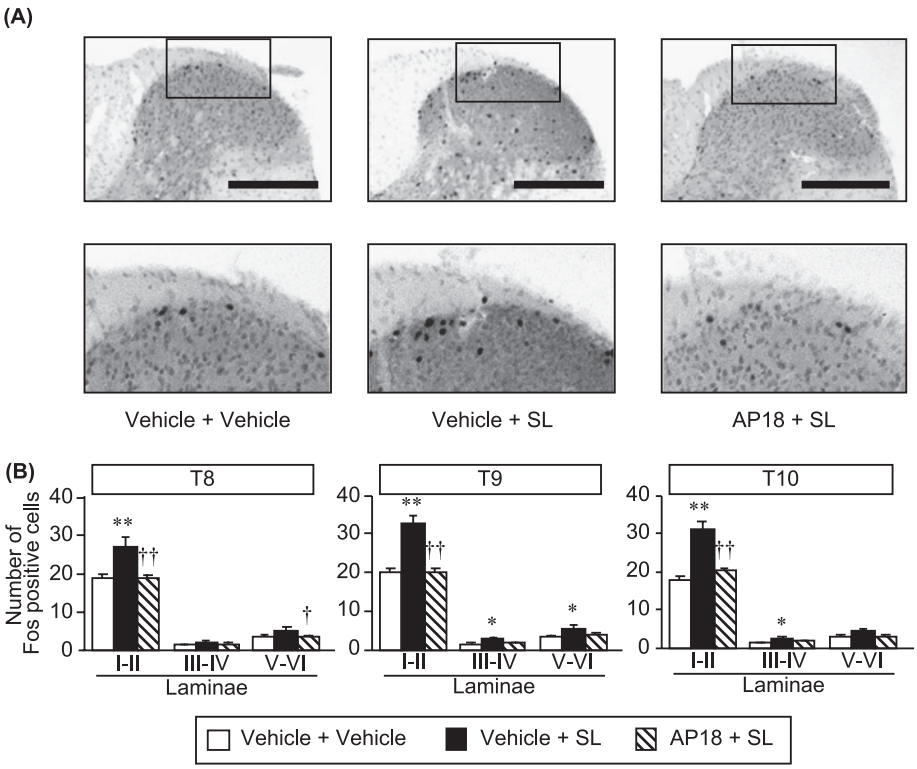

Fig. 1. Effect of AP18, a TRPA1 inhibitor, on the increased number of cells expressing Fos following ductal injection of SLIGRL-NH_2 (SL), a PAR2-activating peptide, in mice. AP18 at 10 mg/kg or vehicle was administered i.p. 30 min before infusion of SL at 15 nmol/mouse or vehicle (saline) in a volume of 25 μl into the pancreatic duct of mice. The spinal cord was transcardially perfused and fixed 2 h after the infusion. A) Typical microphotographs for immunostaing of Fos in the spinal dorsal horn at a T9 level. Scale bars indicate 100 μm. The bottom photographs correspond to the square areas in the top photographs. B) The number of Fos-positive cells in laminae I-II, III-IV and V-VI of the spinal dorsal horn at T8 – T10 levels. Data show the mean with S.E.M. from 20 slices (5 mice). $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle + vehicle. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. vehicle + SL.

development of inflammation, in the acute pancreatitis model, AP18 at 10 mg/kg was administered i.p. 10 min after the final dose of cerulein. Similarly, SB366791 at 0.1 or 0.5 mg/kg was administered i.p. 10 min after the final dose of cerulein. Data are shown as means with S.E.M. Statistical analyses for parametric data were performed by analysis of variance followed by Tukey's test for multiple comparisons. For non-parametric analysis, the Kruskal-Wallis H test followed by a least significant difference (LSD)-type test was employed for multiple comparisons. Significance was set at a level of $P < 0.05$.

SLIGRL-NH_2, the PAR2-activating peptide, infused into the pancreatic duct at 15 nmol/mouse, caused Fos expression in the superficial layers of the T9 spinal dorsal horn segments, 2 h later (Fig. 1A), and significant increase in the number of Fos-positive cells, most clearly in laminae I-II of T8 – T10 spinal segments (Fig. 1B), in agreement with the previous report (4). AP18, the TRPA1 inhibitor, preadministered i.p. at 10 mg/kg, inhibited the spinal Fos expression (Fig. 1A) and increase in the number of Fos-positive cells caused by SLIGRL-NH_2 (Fig. 1B), although AP18 alone did not cause Fos expression (data not shown). Repeated administrations of cerulein induced referred hyperalgesia accompanying acute pancreatitis characterized by increases in pancreatic weight and plasma amylase activity (Fig. 2). AP18, when given i.p. at 10 mg/kg alone after the final dose of cerulein, did not significantly suppress the referred hyperalgesia (Fig. 2A), the increased pancreatic weight, or the elevated plasma amylase activity (Fig. 2B). On the other hand, SB366791, a TRPV1 inhibitor, when given i.p. at 0.5 mg/kg, but not 0.1 mg/kg, in the same administration schedule, strongly suppressed the referred hyperalgesia (Fig. 2A), without affecting the pancreatitis-related parameters (Fig. 2B). AP18 at 10 mg/kg, when administered in combination with SB366791 at 0.1 mg/kg, a sub-effective dose, significantly reversed the referred hyperalgesia (Fig. 2A), without affecting the pancreatitis-related parameters (Fig. 2B).

Our findings that inhibition of TRPA1 by AP18 prevented the ductal SLIGRL-NH_2–evoked spinal Fos

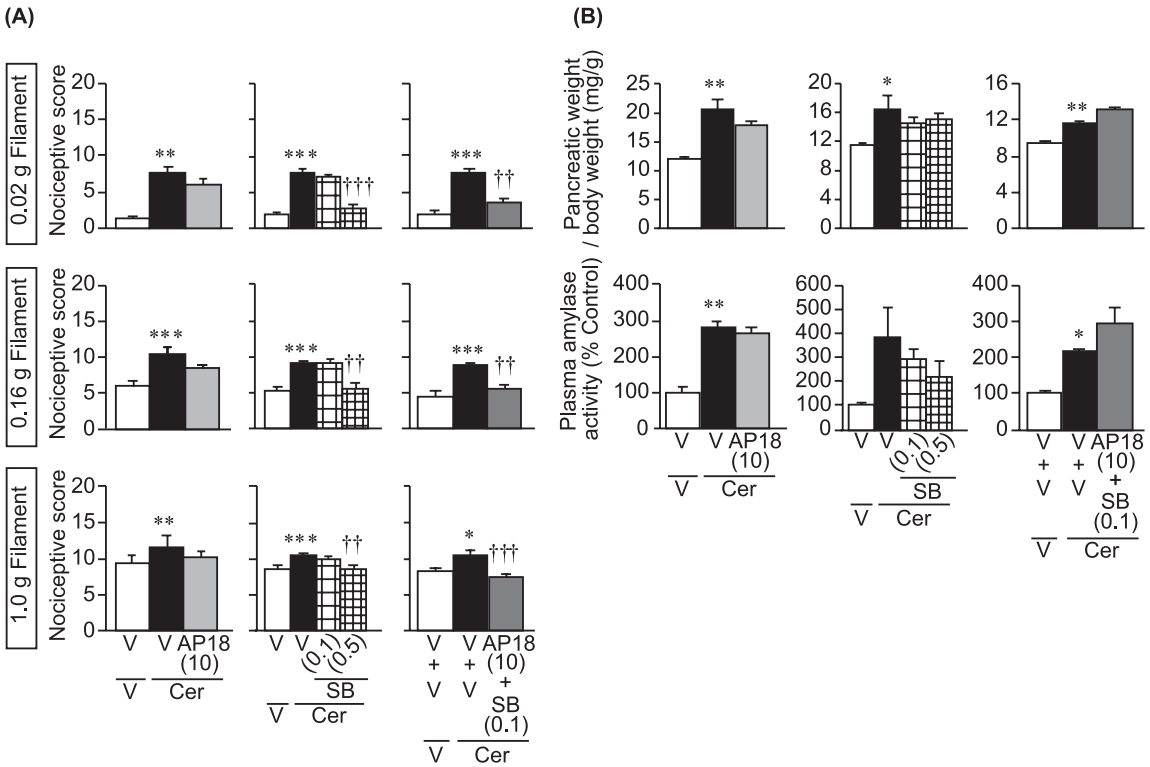

Fig. 2. Effects of AP18, a TRPA1 inhibitor, and/or SB366791 (SB), a TRPV1 inhibitor, on cerulein-induced referred hyperalgesia and increases in pancreatic weight and plasma amylase activity in mice. Cerulein (Cer) at 50 μg/kg was administered i.p. to mice at 1-h intervals, 6 times in total. AP18 at 10 mg/kg or SB at 0.1 – 0.5 mg/kg alone was administered i.p. to mice 10 min after the final administration of cerulein. AP18 at 10 mg/kg and SB at 0.1 mg/kg, a subeffective dose, were co-administered i.p. to mice 10 min after the final administration of cerulein. Referred hyperalgesia was evaluated by the von Frey test 30 min after the final administration of cerulein (A), and the mice were sacrificed for measurement of pancreatic weight and plasma amylase activity 1 h after the final administration of cerulein (B). V, vehicle. Values in parentheses show the doses (mg/kg). Data show the mean with S.E.M. for 6 – 12 mice. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. vehicle + vehicle. $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ vs. cerulein + vehicle.

expression suggest the pro-nociceptive role of TRPA1, in addition to TRPV1 (3, 4), as downstream signals of PAR2 activation in the pancreas. This is consistent with evidence for trans-activation of TRPA1 as well as TRPV1 following PAR2 stimulation (6 – 9). PAR2 stimulation causes trans-activation of TRPA1 through phospholipase C (PLC)-catalyzed hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) that constitutively suppresses TRPA1 functions (9), while TRPV1 activation by PAR2 involves direct phosphorylation of TRPV1 by protein kinase C (PKC) (6, 7). We thus expected that both TRPA1 and TRPV1 would contribute to the pancreatic pain accompanying cerulein-evoked pancreatitis in which PAR2 activation by endogenous proteinases is involved (1, 4, 13). Nonetheless, AP18 alone did not significantly suppress the referred hyperalgesia accompanying cerulein-evoked acute pancreatitis, being in contrast to the previous evidence that inhibition of TRPV1 significantly attenuates the pancreatitis-related pain (4). However, our finding that AP18 facilitated the effect of the TRPV1 inhibitor SB366791 at the subeffective dose, implies the latent pro-nociceptive role of TRPA1 during pancreatitis. The reason for the distinct extent of contribution of TRPA1 to excitation of nociceptive neurons following pancreatic PAR2 stimulation in healthy mice and after the development of pancreatitis is still open to question. It is likely that TRPV1 may be functionally upregulated by G protein–coupled receptors other than PAR2 during pancreatitis or that expression levels of TRPA1 and TRPV1 might decrease and increase, respectively, following the development of pancreatitis. It is noteworthy that both TRPV1 and TRPA1 appear to contribute to the transition of acute to chronic pancreatic inflammation and pain (11). We did not determine the effect of TRP inhibitors on cerulein-induced Fos expression in the present study because 6 hourly repeated administrations of cerulein caused only slight spinal Fos expression in the preliminary experiments. In addition, the TRP inhibitors, administered after the establishment of acute pancreatitis, would not affect the Fos expression, a delayed marker of persistent pain, if any, in response to nociceptive input in the developmental process of acute pancreatitis. In conclusion, our study implies that TRPA1, like TRPV1, participates in excitation of pancreatic nociceptors following PAR2 stimulation and plays a latent pro-nociceptive role during acute pancreatitis, contributing to pancreatic pain in collaboration with TRPV1 possibly as downstream signals of PAR2.

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