Presynaptic BK type Ca^{2+} -activated K^{+} channels are involved in prostanoid TP receptor-mediated inhibition of noradrenaline release from the rat gastric sympathetic nerves

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Abstract

Previously, we reported that prostanoid TP receptor mediates the inhibition of electrically evoked noradrenaline release from gastric sympathetic nerves in rats. Prostanoid TP receptor has been shown to activate phospholipase C (PLC), which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-triphosphate (IP₃) and diacylglycerol; IP₃ triggers the release of Ca²⁺ from intracellular stores and diacylglycerol activates protein kinase C. In the present study, therefore, we examined whether these PLC-mediated mechanisms are involved in the TP receptor-mediated inhibition of gastric noradrenaline release using an isolated, vascularly perfused rat stomach. U-46619 (9,11-dideoxy-9 α ,11 α -methanoepoxy PGF_{2 α}) (a prostanoid TP receptor agonist)-induced inhibition of noradrenaline release from the stomach was reduced by U-73122 [1-[6-[[[(17 β)-3-methoxyestra-1,3,5(10)-trien-17-yl]-amino]hexyl]-1*H*-pyrrole-2,5-dione] (a PLC inhibitor) and ET-18-OCH₃ (1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphorylcholine) (a phosphatidylinositol-specific PLC inhibitor), respectively. 2-APB (2-aminoethyldiphenyl borate) (a putative IP₃ receptor antagonist) also abolished the U-46619-induced inhibition of noradrenaline release, but Ro 31-8220 [2-{1-[3-(amidinothio)propyl]-1*H*-indol-3-yl}-3-(1-methylindol-3-yl)-maleimide] (a protein kinase C inhibitor) had no effect. Furthermore, a small dose of tetraethylammonium and charybdotoxin [blockers of BK-type Ca²⁺-activated K⁺ channel] abolished the U-46619-induced inhibition, but apamin (a blocker of SK-type Ca²⁺-activated K⁺ channel) had no effect. These results suggest that BK type Ca²⁺-activated K⁺ channels are involved in prostanoid TP receptor-mediated inhibition

of electrically evoked noradrenaline release from the gastric sympathetic nerve terminals in rats.

Keywords: TP receptor; IP₃-induced Ca²⁺ release; BK channel; inhibition of noradrenaline release

1. Introduction

Cytosolic Ca^{2+} plays an important role in regulating a variety of neuronal processes such as regulation of cell membrane excitability, exocytosis of neurotransmitters, cell growth and gene expression (Berridge, 1998). Increases in cytosolic Ca^{2+} can result from Ca^{2+} influx through the plasma membrane and Ca^{2+} release from an intracellular Ca^{2+} store through intracellular Ca^{2+} release channels. Two types of intracellular Ca^{2+} release channels, ryanodine receptors and inositol 1,4,5-triphosphate (IP_3) receptors, exist on the neuronal endoplasmic reticulum, which is a specialized Ca^{2+} storing organelle and makes a continuous network distributed throughout neurons including synaptic terminals (Verkhratsky, 2005). Ca^{2+} release via ryanodine receptors, termed Ca^{2+} -induced Ca^{2+} release, is activated by an increase in intracellular Ca^{2+} from Ca^{2+} influx through the plasma membrane voltage- or ligand-gated channels (Verkhratsky and Shmigol, 1996), while Ca^{2+} release through IP_3 receptors, termed IP_3 -induced Ca^{2+} release, is activated by an increase in intracellular IP_3 , which is generated through cleavage of phosphatidylinositide lipids by phospholipase C (PLC) coupled to cell-surface receptors (Berridge, 1993).

Both types of intracellular Ca^{2+} release channels are heterogeneously distributed on neuronal cell bodies and synaptic terminals (Llano et al., 2000; Bouchard et al., 2003) and their activation triggers cytoplasmic Ca^{2+} signals to evoke neurotransmitter release in the central and peripheral neurons (Smith and Cunnane, 1996; Kubota et al., 2005; Mathew and Hablitz, 2008). In several types of neurons, however, elevation of intracellular Ca^{2+} has been shown to activate Ca^{2+} -activated K^+ channels, thereby producing a long-lasting after-hyperpolarization of an action potential to reduce

neurotransmitter release from synaptic terminals (Cassell and McLachlan, 1987; Kawai and Watanabe, 1989; Marrion and Tavalin, 1998). The IP₃-evoked activation of Ca²⁺-activated K⁺ channels plays a role in regulating the excitability of rat midbrain dopamine neurons (Morikawa et al., 2000). Furthermore, ryanodine receptors have been shown to form a functional triad with Ca²⁺-activated K⁺ channels and voltage-gated Ca²⁺ channels located on the cell membrane, thereby directly shaping the spike repolarization of an action potential in bullfrog sympathetic neurons (Akita and Kuba, 2000).

Prostanoids have been shown to modulate noradrenaline release from central and peripheral noradrenergic nerves (Starke et al., 1989; Malik and Sehic, 1990). In a previous study, we reported that prostanoid TP receptors located on the sympathetic nerve terminals mediate the inhibition of electrically evoked noradrenaline release from the rat stomach (Yokotani et al., 2003). TP receptors have been shown to couple with PLC-mediated Ca²⁺ signaling in the guinea pig airway smooth muscle (Shiraki et al., 2009), rat pulmonary artery (McKenzie et al., 2009) and cultured rat myenteric neurons (Rehn and Diener, 2006). Therefore, in the present study, we tried to clarify the mechanisms involved in prostanoid TP receptor-mediated inhibition of noradrenaline release from the rat stomach with regard to PLC-mediated Ca²⁺ signaling.

2. Materials and methods

2.1. *Perfusion experiments*

The present experiments were conducted in compliance with the guiding principles for care and use of laboratory animals approved by Kochi University. All efforts were made to minimize animal suffering and the number of animals used.

Male Wistar rats (Japan SLC, Inc., Hamamatsu, Japan) weighing about 400 g were fasted overnight before experiments. The isolated, vascularly perfused stomach preparations were made as described previously (Yokotani et al., 1992; Nakamura et al., 2003). Briefly, under urethane anesthesia, the abdomen was opened with a midline incision. After ligation of the abdominal aorta just above the branching of the celiac artery, the cannula was inserted into the celiac artery via an incision placed on the aorta and modified Krebs-Ringer solution (pH 7.4) bubbled with a mixture of 95% O₂ and 5% CO₂ was perfused with a constant flow rate of 2.5 ml per min. Modified Krebs-Ringer solution was composed of 117.5 mM NaCl, 4.7 mM KCl, 2.4 mM CaCl₂, 1.1 mM MgCl₂, 1.1 mM NaH₂ PO₄, 25 mM NaHCO₃, 11.1 mM glucose, 0.05% bovine serum albumin, 3 μM indomethacin, 10 μM pargyline and 1 μM phentolamine. A tube was inserted into the lumen of the stomach via a pylorus ring to drain the contents of the stomach throughout the experiment. The esophagus, duodenum, spleen and pancreas were dissected after ligation of the vessels, and the vascularly perfused stomach was kept in a chamber prewarmed at 37°C. Each 2-min effluent from the portal vein was collected in chilled tubes containing 0.5 ml of 4 M perchloric acid, 1 ng of

3,4-dihydroxybenzylamine (DHBA) as an internal standard, and 50 µl of 2% sodium pyrosulfite solution.

After an equilibration period of 60 min, perfusion medium was changed to the new medium containing none or one blocking reagent such as PLC inhibitors, a protein kinase C (PKC) inhibitor, an inositol 1,4,5-triphosphate (IP₃) receptor antagonist or K⁺ channel blocker. The first electrical stimulation [consisting of square-wave pulses at 1.0 Hz, 2 ms duration, 15 mA (a supramaximal intensity) for 1 min] was applied to periarterial nerves around the left gastric artery, which contain the postganglionic sympathetic nerves, with bipolar electrodes, at 14 min after the start of new medium. The second stimulation was carried out 26 min after the first stimulation. Perfusion of U-46619 (a prostanoid TP receptor agonist) was started at 14 min before the second stimulation and continued until the end of experiment with or without one blocking reagent described above.

2.2. Noradrenaline assay in the medium and the stomach

At the end of each experiment, the stomach was homogenized in 20 ml of 0.4 M perchloric acid containing 18.6 mg of disodium EDTA dihydrate, 200 µl of 2% sodium pyrosulfite solution and 500 ng of DHBA as an internal standard. The homogenate was centrifuged for 10 min at 14,000 g at 4°C. The supernatant was analyzed to determine the tissue level of noradrenaline.

Catecholamines in the effluent and the supernatant of tissue homogenate were extracted by the method of Anton and Sayre (1962) with a slight modification, and were assayed electrochemically with high-performance liquid chromatography (HPLC)

(Yokotani et al., 1992; Nakamura et al., 2003). Specifically, 2 ml of effluent or an aliquot (0.1 ml) of supernatant was transferred to a centrifuge tube containing 30 mg of activated alumina and 3 ml of 1.5 M Tris Buffer (pH 8.6) containing 0.1 M disodium EDTA dihydrate, then the preparations were shaken for 10 min. The supernatant was discarded and the alumina was washed three times with chilled twice-deionized water. Then, noradrenaline adsorbed onto the alumina was eluted with 500 μ l of 4% acetic acid containing 0.1 mM disodium EDTA dihydrate. The recovery of catecholamines by these procedures was about 85%.

The HPLC-electrochemical detection system consisted of a pump (PU-980: JASCO, Tokyo, Japan), a sample injector (AS-2050: JASCO) and an electrochemical detector (ECD-300: Eicom, Kyoto, Japan) equipped with a graphite electrode. Analytical conditions were as follows: detector, +450 mV potential against a Ag/AgCl reference electrode; column, Cosmosil 5C₁₈-PAQ, 4.6 x 150 mm (Nacalai Tesque, Kyoto, Japan); column temperature, 35.0°C; mobile phase, 0.1 M NaH₂PO₄-Na₂HPO₄ buffer (pH 6.0) containing 50 mg/l disodium EDTA dihydrate, 3.45 g/l sodium 1-octanesulfonate (Nacalai Tesque) and 18% methanol at a flow rate of 0.5 ml/min. The amount of noradrenaline in each sample was calculated using the peak height ratio relative to that of DHBA. This assay could accurately determine 2 pg of noradrenaline.

2.3. Evaluation and statistical analysis

Released noradrenaline from the stomach is expressed as percentage of its tissue content per 2 min. Basal release is the value just before the electrical stimulation. The amounts of released noradrenaline above the basal during 12 min after the first and

second stimulation are expressed as S_1 and S_2 , respectively. The effect of prostanoid TP receptor agonist (U-46619) is expressed as the ratio of S_2 to S_1 determined in the absence (the control) or presence of this reagent during the second stimulation. All values are expressed as the means $\pm$ S.E.M.

Data were analyzed by repeated-measures analysis of variance (ANOVA), followed by *post-hoc* analysis with the Bonferroni method for comparing the control to all other means (Fig. 1B). When only two means were compared, the data were analyzed by an unpaired Student's *t*-test or Welch's *t*-test (Figs. 1A, 2-4). *P* values of less than 0.05 were taken to indicate statistical significance.

2.4. Compounds

The following drugs were used: 2-aminoethoxydiphenyl borate (2-APB), indomethacin, pargyline hydrochloride, phentolamine hydrochloride, tetraethylammonium (TEA) chloride (Sigma-Aldrich Fine Chemicals, St. Louis, MO, U.S.A.); apamin, charybdotoxin (Peptide Institute Inc. Osaka, Japan); 9,11-dideoxy-9 α ,11 α -methanoepoxy PGF_{2 α} (U-46619) (Cayman Chemical Co., Ann Arbor, MI, U.S.A.); 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphorylcholine (ET-18-OCH₃), 2-{1-[3-(amidinothio)propyl]-1*H*-indol-3-yl}-3-(1-methylindol-3-yl)-maleimide (Ro 31-8220), 1-[6-[[[(17 β)-3-methoxyestra-1,3,5(10)-trien-17-yl]-amino]hexyl]-1*H*-pyrrole-2,5-dione] (U-73122) (Biomol Research Lab., Plymouth Meeting, PA, U.S.A.); 3,4-dihydroxybenzylamine (DHBA) (Alfa Aesar, Ward Hill, MA, U.S.A.); activated alumina (Wako Pure Chemical Industries, Ltd.). All other reagents

were of the highest grade available (Nacalai Tesque). Indomethacin, U-46619 and ET-18-OCH₃ were dissolved in 100% ethanol, then the final concentration of ethanol in the perfusion medium was max. 0.09%. U-73122 and 2-APB were dissolved in 100% N,N-dimethylformamide (DMF) and the final concentration of DMF was max. 0.1%. In all controls, ethanol and/or DMF were added at the same concentration(s) as in the corresponding experiments.

3. Results

3.1. Effect of U-46619 on the electrically evoked release of noradrenaline from the isolated rat stomach

In the whole cohort of rats used in the present study, the amount of noradrenaline remaining in the stomach at the end of each experiment was 635.7 ± 10.0 ng (n=107).

The basal release of noradrenaline before the first electrical stimulation of gastric sympathetic nerves was $0.047 \pm 0.002\%$ of its tissue content per 2 min (n=18) (Fig. 1A). The first stimulation at 1.0 Hz for 1 min evoked an increase of noradrenaline release and this increase rapidly declined toward their basal level ($S_1 = 0.353 \pm 0.032\%$ of its tissue content per 12 min, n=18). In the control experiment, the second electrical stimulation evoked a consistent and reproducible noradrenaline release (S_2/S_1 ratio = 0.986 ± 0.011 , n=6) (Fig. 1, A and B).

After the first stimulation, the perfusion medium was changed to the next one containing U-46619 (an agonist of the prostanoid TP receptors) (0.1 and 0.3 μ M). U-46619 (0.1 and 0.3 μ M) significantly inhibited the evoked noradrenaline release to the same extent [S_2/S_1 ratio was 0.635 ± 0.069 for 0.1 μ M (n=6) and 0.638 ± 0.077 for 0.3 μ M (n=6), respectively] (Fig. 1, A and B), as shown in a previous paper in which U-46619 (0.01, 0.1 and 0.3 μ M) inhibited the evoked noradrenaline release from the rat stomach with a maximal inhibition at 0.1 μ M (Yokotani et al., 2003). Thus, 0.1 μ M of U-46619 was used in the following experiments.

3.2. Effects of U-73122 and ET-18-OCH₃ on the U-46619-induced inhibition of the

evoked noradrenaline release from the isolated rat stomach

U-73122 (3 μ M) (an inhibitor of PLC) or ET-18-OCH₃ (3 μ M) (an inhibitor of phosphatidylinositol-specific PLC) was added throughout the experiments. ET-18-OCH₃ (3 μ M), but not U-73122 (3 μ M), slightly but significantly ($p < 0.05$; compared to that of non-treated group in Fig. 1) elevated the basal noradrenaline release before the first stimulation [$0.057 \pm 0.004\%$ of its tissue content per 2 min for U-73122 (3 μ M)-treated group ($n=10$) and $0.076 \pm 0.004\%$ for ET-18-OCH₃ (3 μ M)-treated group ($n=9$), respectively].

U-73122 (3 μ M) and ET-18-OCH₃ (3 μ M) had no effect on the first stimulation-induced increase of noradrenaline release from the stomach [$S_1 = 0.314 \pm 0.030\%$ of its tissue content per 12 min for U-73122 (3 μ M)-treated group ($n=10$), and $0.303 \pm 0.018\%$ for ET-18-OCH₃ (3 μ M)-treated group ($n=9$), respectively]. Under the presence of these reagents, the second stimulation of the gastric sympathetic nerves evoked a consistent and reproducible noradrenaline release [S_2/S_1 ratios were 0.980 ± 0.017 for U-73122 (3 μ M)-treated group ($n=5$) and 1.096 ± 0.086 for ET-18-OCH₃ (3 μ M)-treated group ($n=5$), respectively] (Fig. 2, A and B).

In the presence of U-73122 (3 μ M) or ET-18-OCH₃ (3 μ M), the U-46619 (0.1 μ M)-induced inhibition of the evoked noradrenaline release disappeared [S_2/S_1 ratios were 0.903 ± 0.033 for U-73122 (3 μ M)-treated group ($n=5$) and 1.025 ± 0.037 for ET-18-OCH₃ (3 μ M)-treated group ($n=4$), respectively] (Fig. 2, A and B).

3.3. Effects of Ro 31-8220 and 2-APB on the U-46619-induced inhibition of the evoked noradrenaline release from the isolated rat stomach

Ro 31-8220 (1 or 3 μM) (an inhibitor of PKC) or 2-APB (75 μM) (a putative antagonist of IP_3 receptor) was added throughout the experiments. Ro 31-8220 (1 μM) and 2-APB (75 μM), but not Ro 31-8220 (3 μM), slightly but significantly ($p < 0.05$; compared to that of non-treated group in Fig. 1) elevated the basal noradrenaline release before the first stimulation [$0.077 \pm 0.004\%$ of its tissue content per 2 min for Ro 31-8220 (1 μM)-treated group ($n=13$), $0.066 \pm 0.006\%$ for Ro 31-8220 (3 μM)-treated group ($n=8$), and $0.105 \pm 0.010\%$ for 2-APB (75 μM)-treated group ($n=10$), respectively].

Ro 31-8220 (1 and 3 μM) and 2-APB (75 μM) had no effect on the first stimulation-induced increase of noradrenaline release from the stomach [$S_1 = 0.344 \pm 0.016\%$ of its tissue content per 12 min for Ro 31-8220 (1 μM)-treated group ($n=13$), and $0.311 \pm 0.025\%$ for Ro 31-8220 (3 μM)-treated group ($n=8$), and $0.316 \pm 0.014\%$ for 2-APB (75 μM)-treated group ($n=10$), respectively]. Under the presence of these reagents, the second stimulation evoked a consistent and reproducible noradrenaline release (Fig. 3, A and B) [S_2/S_1 ratio = 0.988 ± 0.041 for Ro 31-8220 (1 μM)-treated group ($n=7$), 0.880 ± 0.028 for Ro 31-8220 (3 μM)-treated group ($n=4$), and 0.935 ± 0.043 for 2-APB (75 μM)-treated group ($n=6$), respectively].

In the presence of Ro 31-8220 (1 and 3 μM), U-46619 (0.1 μM) still effectively inhibited the evoked noradrenaline release from the stomach (Fig. 3A) [S_2/S_1 ratio = 0.708 ± 0.067 for Ro 31-8220 (1 μM)-treated group ($n=6$), 0.702 ± 0.033 for Ro 31-8220 (3 μM)-treated group ($n=4$), respectively]. However, the U-46619-induced inhibition was abolished by 2-APB (75 μM) (Fig. 3B) [S_2/S_1 ratio = 0.943 ± 0.057 for 2-APB (75 μM)-treated group ($n=4$)].

3.4. Effects of TEA, charybdotoxin and apamin on the U-46619-induced inhibition of the evoked noradrenaline release from the isolated rat stomach

TEA (1.0 mM), charybdotoxin (0.01 or 0.03 μ M) or apamin (0.3 μ M) was added throughout the experiments. TEA (1.0 mM) and charybdotoxin (0.01 μ M), but not charybdotoxin (0.03 μ M) and apamin (0.3 μ M), slightly but significantly ($p < 0.05$; compared to that of non-treated group in Fig. 1) elevated the basal noradrenaline release before the first stimulation [$0.066 \pm 0.004\%$ of its tissue content per 2 min for TEA (1.0 mM)-treated group ($n=12$), $0.063 \pm 0.005\%$ for charybdotoxin (0.01 μ M)-treated group ($n=10$), $0.058 \pm 0.004\%$ for charybdotoxin (0.03 μ M)-treated group ($n=9$), and $0.052 \pm 0.003\%$ for apamin (0.3 μ M)-treated group ($n=8$), respectively].

TEA (1.0 mM) significantly ($p < 0.05$; compared to that of non-treated group in Fig. 1) elevated the first stimulation-induced increase of noradrenaline release from the stomach [$S_1 = 0.681 \pm 0.052\%$ of its tissue content per 12 min for TEA (1.0 mM)-treated group ($n=12$)], although charybdotoxin (0.01 or 0.03 μ M) and apamin (0.3 μ M) had no effect on the first stimulation-induced increase of noradrenaline release [$S_1 = 0.448 \pm 0.043\%$ for charybdotoxin (0.01 μ M)-treated group ($n=10$), $0.458 \pm 0.022\%$ for charybdotoxin (0.03 μ M)-treated group ($n=9$), and $0.291 \pm 0.049\%$ for apamin (0.3 μ M)-treated group ($n=8$), respectively]. Under the presence of these reagents, the second stimulation evoked a consistent and reproducible noradrenaline release (Fig. 4, A, B and C) [S_2/S_1 ratios were 1.082 ± 0.026 for TEA (1.0 mM)-treated group ($n=8$), 1.034 ± 0.036 for charybdotoxin (0.01 μ M)-treated group ($n=6$), 0.971 ± 0.101 for charybdotoxin (0.03 μ M)-treated group ($n=4$), and 0.988 ± 0.026 for apamin (0.3

μM)-treated group (n=4), respectively].

In the presence of TEA (1.0 mM), U-46619 (0.1 μM)-induced inhibition of the evoked noradrenaline release from the stomach disappeared (Fig. 4A) [S_2/S_1 ratio= 1.138 ± 0.139 , n=4]. Charybdotoxin (0.01 μM) had no effect on the U-46619-induced inhibition of the evoked noradrenaline release, however at 0.03 μM the peptide significantly ($p < 0.05$) attenuated the U-46619-induced inhibition of the noradrenaline release (Fig. 4B) [S_2/S_1 ratios were 0.502 ± 0.059 for charybdotoxin (0.01 μM)-treated group (n=4) and 0.833 ± 0.039 for charybdotoxin (0.03 μM)-treated group (n=5), respectively]. On the other hand, a large dose of apamin (0.3 μM) had no effect on the U-46619-induced inhibitory responses (Fig. 4C) (S_2/S_1 ratio was 0.783 ± 0.037 , n=4).

4. Discussion

Stimulation of prostanoid TP receptors has been shown to cause diverse biological effects via activation of several effectors such as PLC, adenylyl cyclase and nucleotide exchange factor of Rho (Huang et al., 2004; Nakahata, 2008). In the present study, U-46619, a prostanoid TP receptor agonist, effectively attenuated the electrically evoked noradrenaline release from the isolated rat stomach, as show in a previous paper (Yokotani et al., 2003). Thus, we attempted to clarify the mechanism involved in the prostanoid TP receptor-mediated inhibition of gastric noradrenaline release with regard to PLC. U-73122 has been shown to be a selective inhibitor of PLC in human platelets and polymorphonuclear neutrophils (Bleasdale et al., 1990; Smith et al., 1990) and to antagonize the hyperalgesia evoked by histamine H₁ receptor agonist [2-(3-trifluoromethylphenyl)histamine]-induced activation of brain PLC in mice (Galeotti et al., 2004). In the present experiment, U-73122 effectively attenuated the prostanoid TP receptor-mediated inhibitory response, suggesting the involvement of PLC in the prostanoid TP receptor-mediated inhibition of noradrenaline release from the rat stomach.

To date, PLC has pharmacologically been divided into two types, phosphatidylinositol-specific PLC and phosphatidylcholine-specific PLC. The former hydrolyzes phosphatidylinositol phosphate(s) while the latter hydrolyzes phosphatidylcholine, thereby both generating diacylglycerol. The source of early production of diacylglycerol has been shown to be exclusively mediated by phosphatidylinositol-specific PLC, while phosphatidylcholine is the source of sustained production of diacylglycerol by phosphatidylcholine-specific PLC (Exton, 1994; Rebecchi and Pentylala, 2000). Then, we examined which PLC is involved in the

prostanoid TP receptor-mediated inhibition of gastric noradrenaline release using ET-18-OCH₃, an inhibitor of phosphatidylinositol-specific PLC (Powis et al., 1992). In the present experiment, ET-18-OCH₃ effectively attenuated the prostanoid TP receptor-mediated inhibitory response, suggesting the involvement of the neuronal phosphatidylinositol-specific PLC in the prostanoid TP receptor-mediated inhibition of gastric noradrenaline release in rats.

Phosphatidylinositol-specific PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce diacylglycerol in addition to IP₃. Diacylglycerol functions as an intracellular second messenger to activate PKC (Nishizuka, 1992). PKC-induced inhibition of voltage-sensitive Ca²⁺ channels has been shown to reduce catecholamine secretion from adrenal chromaffin cells (Sena et al., 1995). Ro 31-8220, a selective inhibitor of PKC (Dieter and Fitzke, 1991), effectively attenuated the PKC-dependent [³H]-dopamine release from rat striatal synaptosomes at 1 μM (Soliakov and Wonnacott, 2001) and the phorbol ester-induced activation of PKC in the rat calyx of Held synapse at 3 μM (Korogod et al., 2007; Lou et al., 2008). In the present experiment, Ro 31-8220 even at 3 μM had no effect on the prostanoid TP receptor-mediated response, suggesting that the neuronal PKC is not involved in the prostanoid TP receptor-mediated inhibition of gastric noradrenaline release in rats.

IP₃, the other product of phosphatidylinositol-specific PLC, is water-soluble and diffuses rapidly within the cytosol to interact with IP₃ receptors on the endoplasmic reticulum in neurons, including synaptic terminals (McGraw et al., 1980; Verkhratsky, 2005). The IP₃ receptors located on the endoplasmic reticulum function as intracellular Ca²⁺ channels to release luminal stored Ca²⁺ of endoplasmic reticulum and generate the initial Ca²⁺ signal phase (Wang and Thompson, 1995). IP₃-induced Ca²⁺ release is

elicited by activation of PLC-coupled receptors such as metabotropic glutamate receptors in the rat hippocampal CA1 pyramidal neurons (Nakamura et al., 1999), muscarinic ACh receptors in the rat hippocampal neurons and rat cerebellar granule neurons (Simpson et al., 1996; Irving and Collingridge, 1998) and bradykinin receptors in the rat sympathetic neurons (Cruzblanca et al., 1998; Liu et al., 2007). In addition, the depletion of Ca^{2+} stored within the endoplasmic reticulum by IP_3 -induced Ca^{2+} release serves as a primary trigger for a message which is returned to the plasma membrane resulting in the activation of a “store-operated channel” (SOC) which allows the entry of external Ca^{2+} (Parekh and Penner, 1997). In the present experiment, we examined whether a rise of intracellular Ca^{2+} concentration is involved in the prostanoid TP receptor-mediated inhibition of gastric noradrenaline release with 2-APB, which inhibits both IP_3 receptor and SOC at high concentrations (50-100 μM), thereby suppressing the elevation of the intracellular Ca^{2+} concentration at the nerve terminal (Wu et al., 2000; Prakriya and Lewis, 2001; Ma et al., 2002). In the present experiment, 75 μM of 2-APB abolished the prostanoid TP receptor-mediated inhibitory response. The result suggests a possibility that IP_3 -induced Ca^{2+} release from the endoplasmic reticulum is involved in the prostanoid TP receptor-mediated inhibition of gastric noradrenaline release from the sympathetic nerve terminals in rats.

IP_3 -induced Ca^{2+} release results in the activation of Ca^{2+} -activated K^+ channels in neurons (Hoesch et al., 2004). The activation of K^+ channels negatively influences neuronal excitability and neurotransmitter release (Hu et al., 2001; Raffaelli et al., 2004). Ca^{2+} -activated K^+ channels are classified into large-conductance Ca^{2+} -activated K^+ channels (BK channels) and small-conductance Ca^{2+} -activated K^+ channels (SK channels). In neurons, Ca^{2+} -activated K^+ channels mediate action potential

repolarization and after-hyperpolarization following action potentials; the former is mediated by BK channel activation and the latter by SK channel activation (Storm, 1987; Sah, 1996). BK channels are selectively inhibited by a small dose of TEA (Storm, 1987), charybdotoxin (Gimenez-Gallego et al., 1988) and iberiotoxin (Galvez et al., 1990), while SK channels are selectively inhibited by apamin (Blatz and Magleby, 1986). In the present experiment, a small dose of TEA (1.0 mM) and charybdotoxin (0.03 μ M) effectively attenuated the prostanoid TP receptor-mediated inhibition of gastric noradrenaline release, while apamin (0.3 μ M) had no effect. These results suggest the involvement of Ca^{2+} -activated BK channels in the prostanoid TP receptor-mediated inhibition of the evoked gastric noradrenaline release from the sympathetic nerve terminals in rats. In accordance with these results, Rehn and Diener (2006) also demonstrated that in cultured rat myenteric neurons, carbocyclic thromboxane A_2 , a stable thromboxane A_2 derivative, elicits the elevation of neuronal cytosolic Ca^{2+} concentration by PLC-mediated mechanisms, thereby hyperpolarizing the plasma membrane via activation of BK channels.

Using rat hippocampal or olfactory bulb slices, numerous electrophysiological studies have been shown an implication of postsynaptic BK channels to repolarization of somatodendritic action potentials, generation of fast after-hyperpolarization, spike broadening during repetitive firing and a close association with glutaminergic NMDA receptors (Shao et al., 1999; Isaacson and Murphy, 2001). Presynaptic expression of BK channels also suggests their pivotal role for regulating neurotransmitter release as an “emergency brake”, which protects against hyperactivity, particularly in pathological conditions such as brain ischemia and epilepsy (Hu et al., 2001; Runden-Pran et al., 2002; Raffaelli et al., 2004). The present study demonstrates the possibility that

presynaptic BK channels are also involved in some presynaptic receptor-mediated inhibition of neurotransmitter from nerve terminals.

In conclusion, we propose that the activation of prostanoid TP receptors located on gastric sympathetic nerve terminals elicits IP₃-induced Ca²⁺ release from the endoplasmic reticulum via PLC-mediated mechanisms, which leads to the activation of BK channels resulting in the suppression of membrane potential, thereby inhibiting the electrically evoked noradrenaline release from gastric sympathetic nerve terminals in rats.

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Legends to figures

Fig. 1. Effect of U-46619 on the electrically evoked release of noradrenaline from the isolated rat stomach. Periarterial nerves around the left gastric artery, which contain the gastric postganglionic sympathetic nerves, were electrically stimulated twice at a stimulus frequency of 1.0 Hz for 1 min. The first stimulation was carried out in normal medium and the second stimulation was carried out in medium containing U-46619 (a prostanoid TP receptor agonist) (0.1 or 0.3 μ M). (A) noradrenaline (NA) release is expressed as a percentage of its tissue content per 2 min. E.S., electrical stimulation of the gastric sympathetic nerves. Values are the means $\pm$ S.E.M. *Significant difference ($P < 0.05$) from the corresponding value. (B) Effects of U-46619 are expressed as the S_2/S_1 ratio. Numbers in parenthesis represent the number of experiments. *Significant difference ($P < 0.05$) from the control value.

Fig. 2. Effects of U-73122 and ET-18-OCH₃ on the U-46619-induced inhibition of noradrenaline release from the isolated rat stomach. The gastric sympathetic nerves were electrically stimulated twice at a stimulus frequency of 1.0 Hz for 1 min. (A) U-73122 (an inhibitor of PLC) (3 μ M) or (B) ET-18-OCH₃ (an inhibitor of phosphatidylinositol-specific PLC) (3 μ M) was added throughout experiments. U-46619 (a prostanoid TP receptor agonist) (0.1 μ M) was added during the second electrical stimulation in (A) and (B). The effect of U-46619 is expressed as the S_2/S_1 ratio. The values of control and U-46619-treated experiments without PLC inhibitors in (A) and (B) were cited from Fig. 1B. *Significant difference ($P < 0.05$) from the control value. Other conditions were the same as those in Fig. 1B.

Fig. 3. Effects of Ro 31-8220 and 2-APB on the U-46619-induced inhibition of noradrenaline release from the isolated rat stomach. The gastric sympathetic nerves were electrically stimulated twice at a stimulus frequency of 1.0 Hz for 1 min. (A) Ro 31-8220 (a PKC inhibitor) (1 or 3 μ M) or (B) 2-APB (a putative IP_3 receptor antagonist) (75 μ M) was added throughout experiments. U-46619 (a prostanoid TP receptor agonist) (0.1 μ M) was added during the second electrical stimulation in (A) and (B). The effect of U-46619 is expressed as the S_2/S_1 ratio. The values of control and U-46619-treated experiments without Ro 31-8220 and 2-APB in (A) and (B) were cited from Fig. 1B. *Significant difference ($P<0.05$) from the control value. Other conditions were the same as those in Figs. 1 and 2.

Fig. 4. Effects of TEA, charybdotoxin and apamin on the U-46619-induced inhibition of noradrenaline release from the isolated rat stomach. The gastric sympathetic nerves were electrically stimulated twice at a stimulus frequency of 1.0 Hz for 1 min. Tetraethylammonium (TEA) (1.0 mM) and charybdotoxin (ChTX) (0.01 or 0.03 mM) (selective blockers of BK type Ca^{2+} -activated K^+ channel) or apamin (0.3 mM) (a selective blocker of SK type Ca^{2+} -activated K^+ channel) was added throughout experiments. U-46619 (a prostanoid TP receptor agonist) (0.1 mM) was added during the second electrical stimulation. The effects of these reagents are expressed as the S_2/S_1 ratio. The values of control and U-46619-treated experiments without TEA, charybdotoxin and apamin in (A), (B) and (C) were cited from Fig. 1B. *Significant difference ($P<0.05$) from each control. Other conditions were the same as those in Figs. 1-3.

Figure-1

Figure-2

Figure-3

Figure-4