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A Prostacyclin Receptor Antagonist Inhibits the Sensitized Release of Substance P from Rat Sensory Neurons

Koichi Nakae, Kiyoko Saito, Takashi Iino, Noriyuki Yamamoto, Mayo Wakabayashi, Satoru Yoshikawa, Satoshi Matsushima, Hiroshi Miyashita, Hiromi Sugimoto, Atushi Kiba, and Jung Gupta

Urology, Research Center Kyoto, Bayer Yakuhin Ltd., Kyoto, Japan

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ABSTRACT

Prostacyclin, one of the cyclooxygenase metabolites, causes various biological effects, including vasodilation and antithrombogenicity, and is also involved in several pathophysiological effects, such as inflammatory pain and bladder disorders. The prostacyclin receptor (IP receptor) agonists iloprost, cicaprost, and carbacyclin have been useful for clarifying the role of the IP receptor signaling, since the endogenous ligand, prostacyclin, is very unstable. On the other hand, only a few IP receptor antagonists have been reported to date. Here, we characterized the biological activities of 2-[4-(1*H*-indol-4-yloxymethyl)-benzyloxycarbonylamino]-3-phenyl-propionic acid (compound A) in various in vitro systems. Compound A inhibited the accumulation of the second messenger cyclic AMP in the UMR-108 rat osteosarcoma cell line and primary cultured rat dorsal root ganglion (DRG) neurons in a concentration-dependent manner up to 10 μM, without affecting other eicosanoid receptors. Functionally, the IP receptor plays an important role in DRG neuron sensitization, which is measured by release of the neurotransmitter substance P. Although the effects of iloprost or Lys-bradykinin, an inflammatory peptide, alone on substance P release were limited, stimulation of the neurons with both these ligands induced substantial amounts of substance P release. This synergistic effect was suppressed by compound A. Collectively, these results suggest that compound A is a highly selective IP receptor antagonist that inhibits iloprost-induced sensitization of sensory neurons. Furthermore, these findings suggest that IP receptor antagonist administration may be effective for abnormal neural activities of unmyelinated sensory afferents. Compound A should prove useful for further investigations of the IP receptor in various biological processes.

Prostanoids (PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane A_2) are bioactive lipid mediators generated from membrane phospholipids. They are formed from unsaturated 20-carbon fatty acids and activate second messengers via specific G protein-coupled receptors (prostaglandin D_2 receptor, CRTH2, EP_1, EP_2, EP_3, EP_4, prostaglandin F receptor, IP, and TP) (Breyer et al., 2001; Samad et al., 2002). EP_1, prostaglandin F receptor, and TP are known to be G_q G protein-coupled receptors and increase the intracellular Ca^{2+} concentration, whereas prostaglandin D_2 receptor, EP_2, EP_4, and IP are G_s G protein-coupled receptors and mediate adenylyl cyclase activation. CRTH2 is a G_i G protein-coupled receptor, and EP_3 is a multiple G protein-coupled receptor that shows different properties in various tissues or cells (Breyer et al., 2001; Hirai et al., 2001).

PGI_2 is known to have various biological effects (Breyer et al., 2001; Samad et al., 2002), and IP receptor mRNA expression has been identified in the vascular tissues of various organs, including the aorta, arteries, lungs, thymus, and spleen as well as neurons, such as dorsal root ganglion (DRG) neurons, by in situ hybridization (Oida et al., 1995). These properties led to the consideration of PGI_2 as a therapeutic target molecule (Bley et al., 1998; Samad et al., 2002). Continuous intravenous infusion of prostacyclin or aerosolized iloprost, a stable IP receptor agonist, has been used to treat primary pulmonary hypertension by inducing vasodilation (Bunting et al., 1983; Hoeper et al., 2000). Studies on IP-

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ABBREVIATIONS: PG, prostaglandin; CRTH2, chemoattractant receptor homologous molecule expressed on T-helper 2 cells; EP, prostanoid EP receptor; TP, thromboxane A_2 receptor; IP, prostacyclin receptor; DRG, dorsal root ganglion; PKA, protein kinase A; compound A, 2-[4-(1*H*-indol-4-yloxymethyl)-benzyloxycarbonylamino]-3-phenyl-propionic acid ($C_{26}H_{24}N_2O_5$); compound B, (*R*)-2-(4-phenoxymethyl-benzyloxycarbonylamino)-3-phenyl-propionic acid ($C_{24}H_{23}NO_5$); H89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; LTB_4, leukotriene B4; BSA, bovine serum albumin; CHO, Chinese hamster ovary; BLT1, leukotriene B4 receptor 1; I CAY10441, (4,5-dihydro-1*H*-imidazol-2-yl)-[4-(4-isopropoxybenzyl)phenyl]amine; VR1, vanilloid receptor 1; SC-51322, 8-chlorodibenz[*b*,*f*][1,4]oxazepine-10(11*H*) carboxylic acid,2-[3-[(2-furanylmethyl)-thio]-1-oxopropyl]hydrazide; U-46619, 9,11-dideoxy-9,11-methanoepoxy-prostaglandin F_2; HEL, human erythroleukemia.

deficient mice clarified that susceptibility to thrombosis is increased and inflammatory pain responses are reduced to the levels observed in indomethacin-treated wild-type mice (Murata et al., 1997). PGI_2, which is also involved in bladder disorders (Anderson, 1993), is the major prostaglandin generated locally from the human bladder in response to pathophysiologic stimuli, such as stretching of the detrusor smooth muscle, injuries to the vesical mucosa and nerve stimulation (Anderson, 1993). For example, one of the inflammatory bladder disorders, cystitis, is associated with urgency, frequency, and pain with abnormal neural activity of unmyelinated sensory afferents (Sant and Theoharides, 1999; Oberpenning et al., 2002; Yoshimura et al., 2002). Therefore, IP receptor antagonists may be useful for the treatment of some types of bladder disorders.

In general, analyses of the membrane potential and measurements of second messengers, such as cAMP accumulation and intracellular Ca^{2+} influxes, are used to assess neural activity in vitro. In addition, the release of excitatory neuropeptides, such as substance P and calcitonin gene-related peptide from DRG neurons, has also been investigated (Hingtgen and Vasko, 1994). Excitatory neuropeptides were released in response to capsaicin, bradykinin, ATP, and other stimulatory agents (Smith et al., 2000; Nakatsuka et al., 2001), and their resulting excitation of neurons was mediated by protein kinase C and protein kinase A (PKA) (Adler and Walker, 2000; Huang et al., 2003). PGE_2 treatment was reported to sensitize sensory neurons, and the release of substance P induced by bradykinin was enhanced via the cAMP-PKA transduction pathway (Smith et al., 2000; Kopp et al., 2002). Similarly, PGI_2 and carbacyclin, a stable analog of PGI_2, also induced cAMP accumulation (Smith et al., 1998). These observations suggested that the IP receptor may sensitize sensory neurons (Pitchford and Levine, 1991) and, as a result, evoke hyperalgesia (Taiwo et al., 1989).

Previously, the stable IP receptor agonists iloprost, cicaprost, and carbacyclin have been used to clarify the function of the IP receptor pharmacologically (Vane and Botting, 1995). On the other hand, only a few IP receptor antagonists have been reported, although the antagonists of other prostanoid receptors were developed (Abramovitz et al., 2000; Cournoyer et al., 2001; Clark et al., 2004). These antagonists were analyzed for their antagonistic activities in vivo and found to possess anti-inflammatory/analgesic activities and affect the bladder contractions induced by isovolumetric bladder distension in rats (Cournoyer et al., 2001; Clark et al., 2004). In terms of in vitro data, only their affinities for the IP receptor were investigated, and their mechanisms of action and target tissues were not identified. In the present study, we investigated the detailed pharmacological activities of two IP receptor antagonists, namely 2-[4-(1*H*-indol-4-yloxymethyl)-benzyloxycarbonylamino]-3-phenyl-propionic acid (compound A) and (*R*)-2-(4-phenoxymethyl-benzyloxycarbonylamino)-3-phenyl-propionic acid (compound B) (Cournoyer et al., 2001), and further investigated the effects of compound A on unmyelinated sensory afferents using primary cultures of rat DRG neurons to clarify its mechanism in neuronal regulation.

Materials and Methods

Reagents. Compounds A and B (Fig. 1) were synthesized by Bayer Yakuhin Ltd. (Kyoto, Japan). ^{3}H-Labeled and nonlabeled iloprost were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). The rat UMR-108 osteosarcoma cell line and human erythroleukemia (HEL) cell line were obtained from the American Type Culture Collection (Manassas, VA). Lys-bradykinin was purchased from Peptide Institute Inc. (Osaka, Japan). SC-51322, H89, and leukotriene B_4 (LTB_4) were purchased from Sigma-Aldrich (St. Louis, MO). Sulprostone and U-46619 were purchased from Cayman Chemical (Ann Arbor, MI). Fluo-3AM and Pluronic F-127 were purchased from Invitrogen (Carlsbad, CA).

Assay for cAMP Accumulation in UMR-108 Cells. UMR-108 cells were plated in 96-well plates (1×10^5 cells/well) on the day before the experiment. The adherent cells were washed with assay buffer (Hanks' balanced salt solution, 17 mM HEPES, pH 7.6, 0.1% BSA, 250 μM 3-isobutyl-1-methylxanthine, and 1 mM sodium ascorbate). Various concentrations of the test compounds were then added, and the cells were incubated at 37°C for 30 min. Next, 100 nM iloprost was added, and the cells were incubated at 37°C for an additional 30 min. Whole-cell lysates were prepared by adding an equal volume of Lysis Buffer (cAMP enzyme-linked immunosorbent assay kit; Tropix, Bedford, MA) to each well, and the total cAMP contents were determined according to the manufacturer's instructions.

Ca^{2+} Mobilization Assay. Ca^{2+} loading buffer was prepared by mixing 2 μM Fluo-3AM and 0.02% Pluronic F-127 in Ca^{2+} assay buffer (17 mM HEPES, pH 7.6, 0.1% BSA, 1 mM probenecid, and Hanks' balanced salt solution). Cells were incubated in Ca^{2+} loading buffer at 37°C for 60 min and then washed with Ca^{2+} assay buffer. The drug concentrations and cells tested were 0.1 nM LTB_4 on BLT1-CHO cells, 30 nM PGE_2 on rat EP_1-CHO cells, 10 nM sulprostone on HEL cells, 10 nM PGD_2 on CRTH2-L1.2 cells, and 100 nM U-46619 on K562 cells. The fluorescence emission at 480 nm induced by 0.1 nM LTB_4 was measured using a FDSS6000 fluorimeter (Hamamatsu Photonics, Hamamatsu, Japan).

Receptor Binding Assay. HEL cells were grown in RPMI 1640 medium containing L-glutamine supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and glucose (4.5 mg/ml). For cell membrane preparation, cells were harvested by centrifugation at 500*g* for 5 min, and the cell pellets were washed once with PBS(−). The pellets were resuspended in ice-cold assay buffer (50 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 10 mM $MgCl_2$, and 1× protease inhibitor cocktail; Roche Diagnostics, Indianapolis, IN), homogenized, and centrifuged at 500*g* at 4°C for 10 min. The supernatants were then centrifuged at 100,000*g* at 4°C for 30 min, and the pellets were resuspended in assay buffer. The protein concentrations were measured using a protein assay kit (Pierce Chemical, Rockford, IL). The resulting HEL cell membranes were placed in polypropylene 96-well plates in binding buffer (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, and 0.1% BSA). Next, 20 nM ^{3}H-labeled iloprost and various concentrations of compound A were added and incubated at 37°C for 1 h. The binding assay mixtures were filtered through Multiscreen-MAFB (Millipore Corporation, Billerica, MA), and the filters were washed twice with the binding buffer. Each filter was transferred into a scintillation vial, and scintillant was added. The

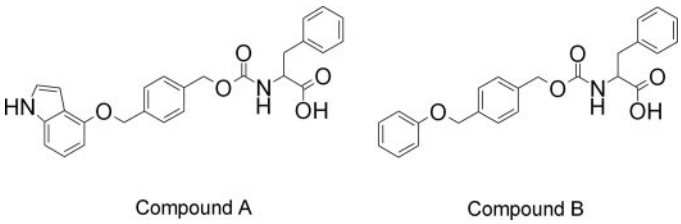

Fig. 1. Chemical structures of compounds A and B.

radioactivity on each filter was counted in a liquid scintillation counter (PerkinElmer Life and Analytical Sciences, Boston, MA). Nonspecific binding was measured using an excess of nonlabeled iloprost.

DRG Neuron Preparation. All animal handling procedures in this study were approved by the Animal Care and Use Committee of Bayer Yakuhin Ltd. Cultures of primary DRG neurons were prepared from newborn rats (Sprague Dawley strain). Dispersed single cells were obtained by mechanical dissociation in 1 mg/ml collagenase A solution (Hanks' balanced salt solution, pH 7.4) at 37°C for 60 min. The resulting DRG neurons were cultured in Ham's F-12 medium (Invitrogen, Paisley, Scotland) supplemented with 80 ng/ml nerve growth factor (Sigma-Aldrich), 0.1 mM 5-fluorouracil (Sigma-Aldrich), 7.5 mg/ml L-ascorbic acid, and 10% fetal calf serum in collagen type I-coated 96-well plates (BD Biosciences, San Jose, CA).

Assay for cAMP Accumulation in DRG Neurons. After a 3-day incubation, DRG neurons were washed with cAMP assay buffer and then incubated with 80 nM iloprost at 37°C for 30 min. Whole-cell lysates were prepared by removing the cAMP assay buffer and adding lysis buffer. The procedures for measuring cAMP accumulation in DRG neurons were basically the same as those described for assaying cAMP accumulation in UMR-108 cells.

Assay for Ca^{2+} Mobilization in DRG Neurons. DRG neurons were incubated with Ca^{2+} loading buffer at 37°C for 60 min, washed in Ca^{2+} assay buffer, and then treated with 200 nM iloprost at 25°C for 10 min. The fluorescence emission at 480 nm induced by various concentrations of Lys-bradykinin was measured using an FDSS6000 fluorimeter (Hamamatsu Photonics).

Substance P Release Assay. DRG neurons were washed with substance P assay buffer (Hanks' balanced salt solution, 17 mM HEPES, pH 7.4, and 0.1% BSA), incubated with various concentrations of the test compounds for 5 to 10 min at room temperature, and then treated with 200 nM iloprost at 25°C for 10 min. The substance P release into the assay buffer induced by treatment with various concentrations of Lys-bradykinin at 37°C for 30 min was quantified using an enzyme immunoassay kit (Cayman Chemical).

Statistics. Statistical significance was analyzed by Student's *t* test, and a p value of <0.05 was considered to indicate a significant difference.

Results

Effects of Compounds A and B on cAMP Accumulation in UMR-108 Cells. Rat UMR-106 osteosarcoma cells are known to have a functional IP receptor (Khanin et al., 1999). To determine whether UMR-108 cells also have a functional IP receptor, we monitored the cAMP accumulation induced by iloprost (Fig. 2A). Iloprost induced cAMP accumulation in a concentration-dependent manner with an EC_{50} value of 38 nM. Next, we examined whether compounds A and B showed functional antagonistic activities in UMR-108 cells. As shown in Fig. 2B, compounds A and B each inhibited cAMP accumulation in a concentration-dependent manner with IC_{50} values of 480 and 390 nM, respectively.

Effects of Compounds A and B on Ca^{2+} Mobilization in BLT1 Transfectants. To examine whether compounds A and B were selective IP receptor antagonists, their effects on Ca^{2+}mobilization in BLT1 transfectants were examined. LTB_4 stimulated Ca^{2+} mobilization in BLT1-CHO transfectants in a concentration-dependent manner with an EC_{50} value of 0.041 nM (Fig. 3A). As shown in Fig. 3B, the highest concentration of compound B inhibited the LTB_4-induced activity observed in BLT1 transfectants (IC_{50} = 4.5 μM), whereas compound A had no effect up to 10 μM.

Effects of Compounds A and B on Other Receptors. The selectivities of compounds A and B for EP_1, EP_3, CRTH2, and TP were further evaluated by PGE_2-induced Ca^{2+} mobilization in rat EP_1-CHO transfectants, sulprostone (EP_3 agonist)-induced Ca^{2+} mobilization in HEL cells, PGD_2-induced Ca^{2+} mobilization in CRTH2-L1.2 transfectants, and U-46619 (TP agonist)-induced Ca^{2+} mobilization in the K562 myelogenous leukemia cell line, respectively. Compounds A and B did not inhibit these receptor activities up to 10 μM (Table 1).

Compound A Antagonizes Iloprost Binding to HEL Cells. HEL cells are known to express a functional IP receptor (Murray et al., 1989). To analyze the binding of ^{3}H-labeled iloprost to HEL membranes, a Scatchard analysis was performed (Fig. 4A). ^{3}H-Labeled iloprost showed a single binding affinity for the HEL membranes (K_D = 3.7 nM, B_{max} = 210 pM). Nonlabeled iloprost and compound A each inhibited the binding of ^{3}H-labeled iloprost to HEL membranes in a concentration-dependent manner with IC_{50} values of 58 and 300 nM, respectively (Fig. 4B).

Effect of Compound A on cAMP Accumulation in DRG Neurons. It has been reported that DRG neurons express the IP receptor and that activation of these receptors induces cAMP accumulation (Smith et al., 1998). Iloprost induced cAMP accumulation in DRG neurons with an EC_{50} value of 22 nM (Fig. 5A). As shown in Fig. 5B, this accumulation was inhibited by compound A (IC_{50} = 1000 nM) but not by SC-51322 (EP_1 and EP_3 antagonist) up to 10 μM.

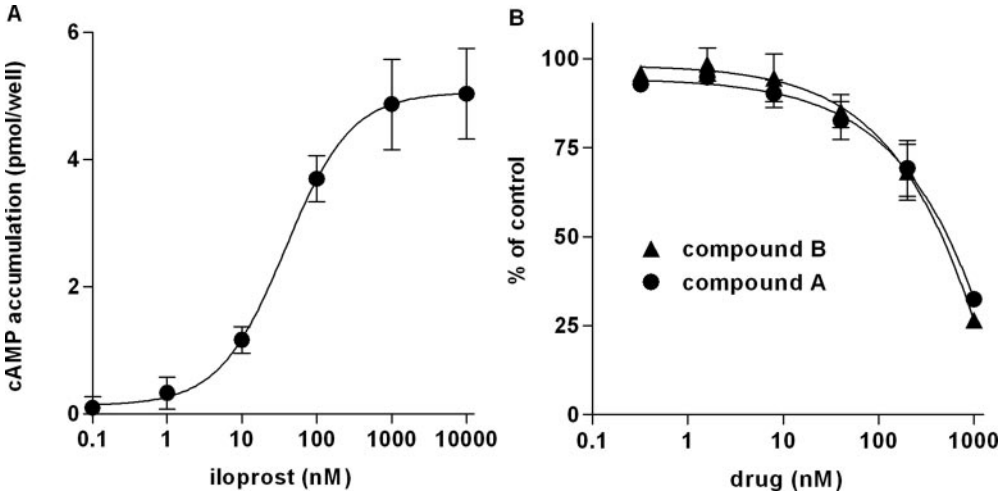

Fig. 2. Iloprost-induced cAMP accumulation in UMR-108 cells and the effects of compounds A and B on this response. A, concentration-response of cAMP accumulation in UMR-108 cells induced by iloprost. B, effects of compounds A and B on 100 nM iloprost-induced cAMP accumulation in UMR-108 cells. Data represent means ± S.E.M. of four independent experiments.

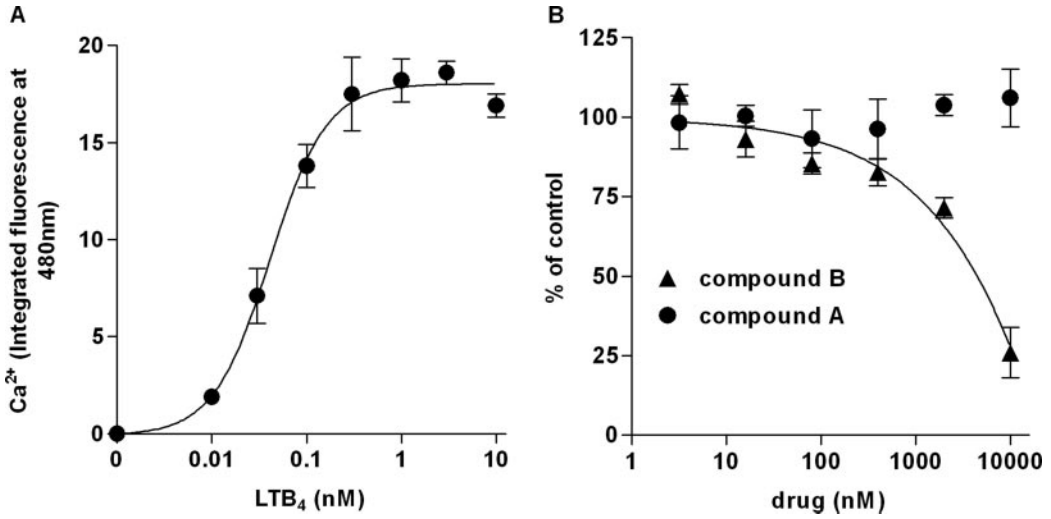

Fig. 3. LTB_4-induced Ca^{2+} mobilization in BLT1 transfectants and the effects of compounds A and B on this response. A, concentration-response of Ca^{2+} mobilization in BLT1 transfectants induced by LTB_4. B, effects of compounds A and B on 0.1 nM LTB_4-induced Ca^{2+} mobilization in BLT1 transfectants. Data represent means ± S.E.M. of four independent experiments.

TABLE 1
Effect of compound A and B on various stimulus

Ligand (Cell, Readout)	Compound A IC_{50}	Compound B IC_{50}
	nM	
Iloprost (UMR-108, cAMP)	480	390
LTB_4 (BLT1-CHO, Ca^{2+})	>10,000	4500
PGE_2 (rat EP1-CHO, Ca^{2+})	>10,000	>10,000
Sulprostone (HEL, Ca^{2+})	>10,000	>10,000
PGD_2 (CRTH2-L1.2, Ca^{2+})	>10,000	>10,000
U46619 (K562, Ca^{2+})	>10,000	>10,000

Effect of Compound A on Substance P Release from DRG Neurons. PGI_2 is known to sensitize DRG neurons (Hingtgen and Vasko, 1994). We investigated the effect of iloprost on Ca^{2+} mobilization in DRG neurons as a measure of their sensitization. As shown in Fig. 6A, Lys-bradykinin induced Ca^{2+} mobilization in a concentration-dependent manner, and the addition of 200 nM iloprost had no effect on this mobilization. Therefore, we examined the substance P release from DRG neurons induced by iloprost plus Lys-bradykinin. Iloprost alone induced substance P release, but its effect was limited (Fig. 6B). Lys-bradykinin alone also induced little substance P release from DRG neurons (Fig. 6C). However, when the neurons were treated with 200 nM iloprost before the addition of Lys-bradykinin, the substance P release was dramatically increased (Fig. 6C). We also examined the effects of compound A, H89 (protein kinase A inhibitor) and capsazepine (VR1 antagonist) on substance P release from DRG neurons. Compound A and H89 each inhibited substance P release from DRG neurons with IC_{50} values of 6.4 and 3.8 μM, respectively, whereas capsazepine had no effect up to 10 μM (Fig. 6D).

Discussion

To investigate the pharmacological activities of IP receptor antagonists in vitro, we first examined several cell-based assays. Compounds A and B both inhibited functional IP receptor activity, as evaluated by iloprost-induced cAMP accumulation, in UMR-108 cells with almost the same potency (IC_{50} values of 480 and 390 nM, respectively). Although compound B also inhibited functional BLT1 receptor activity at a high concentration (IC_{50} = 4.5 μM), its selectivities differed by more than 10-fold between the IP and BLT1 receptors, whereas compound A did not inhibit BLT1 activity up to 10 μM. Iloprost has been reported to have affinities for not only the IP receptor but also the EP_1 and EP_3 receptors. The reported K_i values were 11, 11, and 56 nM for the IP receptor, EP_1 receptor, and EP_3 receptor, respectively (Abramovitz et al., 2000). Therefore, we examined the functional inhibitory activity toward the rat EP_1 and human EP_3 receptors. PGE_2-induced Ca^{2+} mobilization in rat EP_1 transfectants remained unaffected by either compound A or B. Sulprostone (selective EP_3 agonist)-induced Ca^{2+} mobilization in HEL cells (Schwaner et al., 1995; Abramovitz et al., 2000) was also unaffected by either compound A or B. In addition, compounds A and B retained their selectivities against the CRTH2 and TP receptors. We further confirmed an affinity of compound A for HEL cell membranes that express the IP receptor, as evaluated using ^{3}H-labeled iloprost. The calculated K_i value of compound A was 47 nM. Recently, a series of IP receptor antagonists was reported. We investigated the effect of the reported IP receptor antagonist CAY10441 (Clark et al., 2004) and CAY10441 inhibited cAMP accumulation with IC_{50} value of 16 nM; however, it was also mentioned that, although CAY10441 did not display significant affinity for more than 30 other receptors, CAY10441 displayed affinity for adrenergic α2A (320 nM) and imidazoline I_2 binding site (2.0 nM) because of possessing an imidazoline moiety (Clark et al., 2004). In addition, indomethacin was reported to have an affinity for IP receptor (Parfenova et al., 1995). Therefore, compound A was examined for selectivity against adrenergic α1A, α2A, β1, and β2; imidazoline I_2 binding site; and cyclooxygenase cyclooxygenase-1 and cyclooxygenase-2 together with other eight receptors, and it was revealed that compound A kept its selectivity up to 10 μM (data not shown). Our current results therefore reveal that compound A is a highly selective IP receptor antagonist.

Among the prostaglandins, PGI_2 was previously reported to induce the highest cAMP accumulation in DRG neurons (Smith et al., 1998), thereby indicating that the IP receptor may represent a potential therapeutic target for pain and bladder disorders, both of which are induced by afferent nerve abnormalities (Murata et al., 1997; Sant and Theoharides, 1999; Yoshimura et al., 2002). To evaluate whether compound A possessed functional antagonistic activities in sensory neurons, its effect on cAMP accumulation in primary

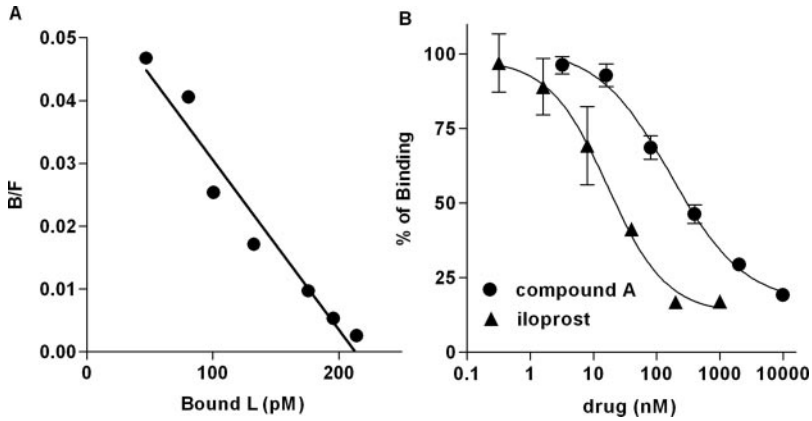

Fig. 4. Binding of ^{3}H-labeled iloprost to HEL membranes and the effects of compounds A and B on the binding. A, Scatchard plot of ^{3}H-labeled iloprost binding to HEL membranes. The means of data points determined in triplicate are shown. The graph represents data from one of three independently performed experiments that produced very similar results. B, homologous competitive binding of ^{3}H-labeled iloprost and HEL membranes ($n = 3$) and the effect of compound A on ^{3}H-labeled iloprost binding to HEL membranes ($n = 6$). Data represent means ± S.E.M.

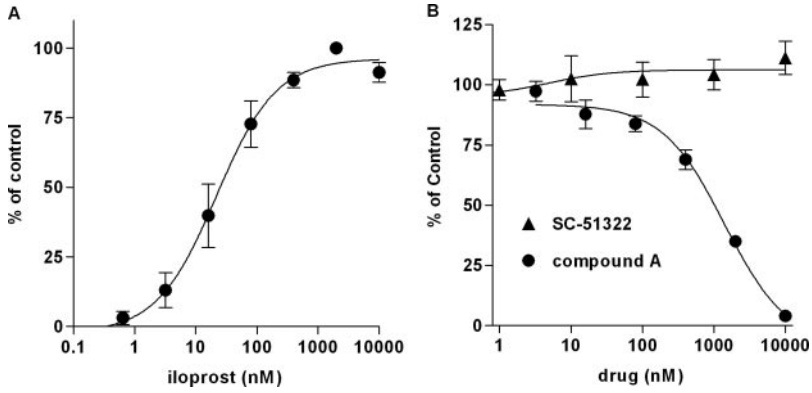

Fig. 5. Iloprost-induced cAMP accumulation in primary cultured DRG neurons and the effects of compound A and SC-51332 on this response. A, concentration-response of cAMP accumulation in primary cultured DRG neurons induced by iloprost. B, effects of compound A and SC-51332 on 80 nM iloprost-induced cAMP accumulation in primary cultured DRG neurons. Data represent means ± S.E.M. of five independent experiments.

cultured DRG neurons was evaluated. Compound A inhibited cAMP accumulation in the DRG neurons in a concentration-dependent manner. We further examined the effect of compound A on DRG neurons in more detail. It is known that PGI_2 sensitizes sensory neurons (Pitchford and Levine, 1991) and is generated in response to tissue injury or inflammation (Bombardieri et al., 1981). Moreover, the major plasma kinins bradykinin and Lys-bradykinin are produced from kininogen by kallikreins, and the responses of these kinins are mediated by the B2 receptor expressed on DRG neurons. Lys-bradykinin is also known to be produced after inflammatory insult or tissue injury (Proud and Kaplan, 1988; Bhoola et al., 1992). Our current experiments revealed a sensitization effect of iloprost on Lys-bradykinin-induced substance P release. Although some reports have described cAMP accumulation and the sensitization mechanism evoked by prostaglandins, these results remain controversial since changes in the intracellular Ca^{2+} concentration may be involved in the sensitization by prostaglandins (Lopshire and Nicol, 1998; De Petrocellis et al., 2001) and treatment with forskolin, which induces cAMP accumulation, enhanced the effect of capsaicin on the cytosolic Ca^{2+} concentration in VR1-expressing human embryonic kidney cells (De Petrocellis et al., 2001). On the other hand, it was reported that PKA activation failed to enhance the capsaicin-evoked inward current in *Xenopus laevis* oocytes or *Aplysia* neurons expressing the vanilloid receptor (VR1) (Lee et al., 2000). Therefore, we evaluated the Ca^{2+} mobilization induced by Lys-bradykinin. As a result, we found that Lys-bradykinin induced a Ca^{2+} influx in a concentration-dependent manner and that this mobilization remained unaffected by iloprost treatment. As an alternative method for assessing neuronal excitation in vitro, we detected the release of the excitatory neuropeptide substance P, which causes peripheral axonal reflexes, since its release was reported to be enhanced by prostaglandins (Hingtgen and Vasko, 1994; Smith et al., 2000). Although iloprost or Lys-bradykinin alone induced very limited substance P release, treatment with 200 nM iloprost plus Lys-bradykinin enhanced substance P release synergistically. In the presence of 200 nM iloprost plus 10 nM Lys-bradykinin, substance P release was enhanced by more than 300% compared with the basal level. These results suggest that IP receptor signaling sensitizes the release of substance P from DRG neurons and further indicate that iloprost sensitizes DRG neurons without an additional Ca^{2+} influx.

The iloprost-induced cAMP accumulation and subsequent signaling cascade were expected to cause PKA activation. It is reported that cAMP/protein kinase A signaling pathway increased the whole-cell currents elicited by capsaicin in rat sensory neurons (Lopshire and Nicol, 1998) and that VR1 was also suggested as a target molecule for phosphorylation by PKA (Augustine, 2001; Rathee et al., 2002). These reports indicate that VR1 may be one of the molecules involved in the substance P release pathway caused by the cAMP/PKA sensitization mechanism. Therefore, we examined the effects of compound A, H89, and capsazepine on the substance P release induced by treatment with iloprost plus Lys-bradykinin. Our results revealed that compound A and H89 inhibited substance P release, whereas capsazepine did not, suggesting that the sensitization signaling pathway induced by iloprost plus Lys-bradykinin may not involve the VR1 molecule.

Increases in the intracellular Ca^{2+} concentration have been reported to trigger neurotransmitter release, although a Ca^{2+}-independent neurotransmitter pathway has also been

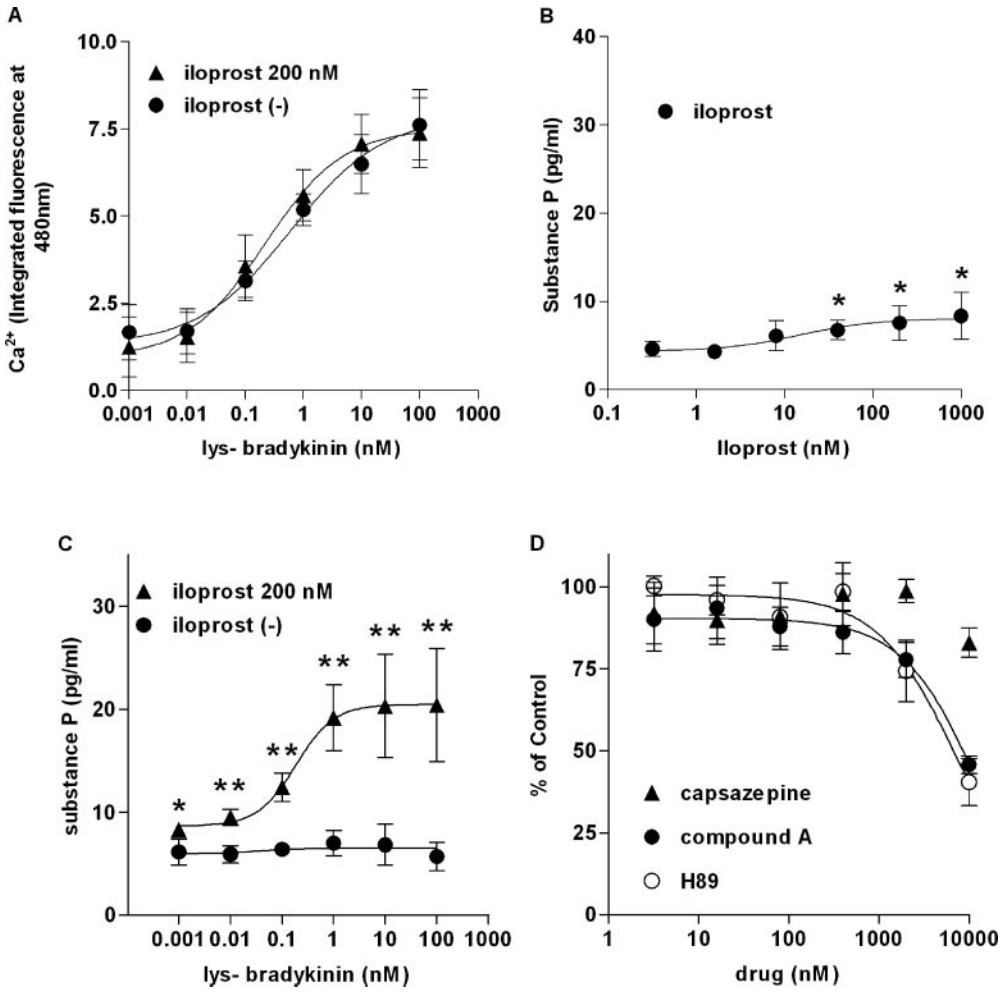

Fig. 6. Effects of iloprost on Ca^{2+} mobilization and substance P release from primary cultured DRG neurons. A, concentration-response of Ca^{2+} mobilization induced by Lys-bradykinin with or without treatment with iloprost for 10 min. B, concentration-response of substance P release from DRG neurons induced by 200 nM iloprost. C, concentration-response of substance P release from DRG neurons induced by Lys-bradykinin with or without treatment with 200 nM iloprost for 10 min. D, effects of compound A, capsazepine, and H89 on substance P release from DRG neurons induced by 1 nM Lys-bradykinin plus 200 nM iloprost. Data represent means ± S.E.M. of three independent experiments. Statistical significance was analyzed by Student's t test: *, $p < 0.05$; **, $p < 0.01$. A, versus iloprost (−); B, versus the control; and C, versus iloprost (−).

suggested (White, 1997; Augustine, 2001). Although we cannot exclude changes in the intracellular Ca^{2+} concentrations of local components in the cells during our experiments, our results suggest that the sensitization phenomenon of substance P release from DRG neurons was not proportional to the Ca^{2+} mobilization. Our experimental system using primary cultured DRG neurons is available for studying whole-cell events but not for investigating local or single molecule events. Considering that the IP receptor/cAMP/PKA pathway sensitizes various stimuli, such as capsaicin, KCl (Hingtgen and Vasko, 1994), and Lys-bradykinin, it is possible to speculate that a certain common regulatory molecule of the exocytosis mechanism is activated by this pathway. Moreover, a recent study also indicated that an exocytosis regulatory molecule was activated by PKA (Foletti et al., 2001).

Our present results demonstrate that compound A is a highly selective and potent IP receptor antagonist that inhibits iloprost-induced sensitization of DRG neurons. Therefore, compound A may prove to be a powerful tool for further investigations of IP receptor functions.

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Address correspondence to: Koichi Nakae, Microbial Chemistry Research Center, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141-0021, Japan. E-mail: nakaek@bikaken.or.jp