Prostacyclin (PGI) Receptor Binding and Cyclic AMP Synthesis Activities of PGI\(_{1}\) Analogues, SM-10906 and Its Methyl Ester, SM-10902, in Mastocyteoma P-815 Cells

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The prostacyclin I\(_1\) (PGI\(_1\)) analogue, SM-10906 and its methyl ester, SM-10902, have been compared with the PGI\(_1\) analogue, iloprost, with respect to binding to the PGI\(_1\) receptor, stimulation of adenylyl cyclase activity and inhibition of thrombin-induced Ca\(^{2+}\) mobilization in mastocyteoma P-815 cells. SM-10906 displaced [\(^3\)H]iloprost binding to the membrane fraction, the IC\(_{50}\) value being 20 nM, but showed very low affinity for the prostaglandin E (PGE) receptor. SM-10906 dose-dependently stimulated GTP-dependent adenylyl cyclase activity in the membrane fraction, the EC\(_{50}\) value being 35 nM. Furthermore, SM-10906 prevented a thrombin-induced increase in the intracellular Ca\(^{2+}\) concentration, the IC\(_{50}\) value being 100 nM. These IC\(_{50}\) and EC\(_{50}\) values are much lower than those of SM-10902. These results demonstrate that SM-10906, a stable PGI\(_1\) derivative, is an agonist for the [\(^3\)H]iloprost-binding (PGI\(_1\)) receptor, and that it prevents thrombin-induced Ca\(^{2+}\) mobilization through stimulation of the adenylyl cyclase system in mastocyteoma cells. On the other hand, a methyl ester derivative of PGI\(_1\), SM-10902, was inactive in the binding assay, but it seems to be a partial agonist for adenylyl cyclase activity.

Keywords: prostacyclin (PGI) receptor; stable PGI\(_1\) derivative; mastocyteoma cell; binding activity; adenylyl cyclase; calcium ion mobilization.

The function of mast cells is to secrete histamine and other mediators of inflammation in response to various stimuli such as IgE and thrombin. IgE-induced histamine release from rat peritoneal mast cells has been inhibited by an increase in the intracellular cAMP level.\(^{11}\) We recently demonstrated in mouse mastocyteoma P-815 cells that prostacyclin I\(_1\) (PGI\(_1\)) strongly stimulates cAMP synthesis\(^{35}\) and that a highly specific PGI\(_1\) receptor was coupled to the adenylyl cyclase system via a stimulatory GTP-binding protein\(^{36}\); also that TEI-9063, a stable agonist for the PGI\(_2\) receptor, prevented thrombin-induced Ca\(^{2+}\) mobilization through stimulation of the adenylyl cyclase system.\(^{44}\) Furthermore, we have characterized a PGI\(_1\) binding protein with a molecular weight of 45 kDa from mastocyteoma cell membrane by photoaffinity labeling with the stable PGI\(_1\) analogue, [\(^{15}\)H]15-19-(3-azidophenyl)-20-norisorcarbacyclin ([\(^{3}\)H]APNIC), which is used as a potent photoaffinity probe for the receptor.\(^{55}\)

Because of the chemical instability of PGI\(_2\), various stable PGI\(_1\) analogues, but almost exclusively 2-series prostacyclins, have been developed. Therefore, information about the biological and pharmacological activities of 1-series prostacyclins is almost nonexistent.\(^{69}\) With respect to the distinct activity between 1-series and 2-series prostaglandins, PGE\(_1\), but no PGE\(_2\), has been well known to inhibit platelet aggregation via an increased cAMP level. Thus, it is pharmacologically important to examine the mode of receptor binding and cAMP synthesis of 1 series prostacyclins, as compared those of the 2 series, in mastocyteoma cells.

SM-10906 and SM-10902, chemically stable PGI\(_1\) analogues, have unique structural features different from those of natural PGI\(_1\) (Fig. 1). SM-10906 is a free acid and SM-10902 is a methyl derivative of the free acid. So, using these analogues we can evaluate the effects of PGI\(_1\), as compared with the PGI\(_1\) analogue, iloprost, on binding to the PGI\(_1\) receptor, stimulation of adenylyl cyclase activity and inhibition of thrombin-induced Ca\(^{2+}\) mobilization in mastocyteoma P-815 cells. We report here that SM-10906 is a biologically active form with respect to binding to the PGI\(_1\) receptor, stimulation of GTP-

![Chemical Structures of SM-10906 and SM-10902](image_url)

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dependent adenylate cyclase activity and the prevention of thrombin-induced Ca\(^{2+}\) mobilization in mastocytoma cells, and that the methyl derivative of the \(\alpha\)-chain carboxyl group of a PGI\(_1\) analogue is essentially inactive in the binding assay, but has partial agonist activity.

**MATERIALS AND METHODS**

**Materials** The PGI\(_1\) analogues, SM-10906 and SM-10902, were prepared at the Research Laboratories of Sumitomo Pharmaceuticals Co., Ltd. (Osaka). The purity of the PGI\(_1\) analogues was found to be 99\% on thin layer chromatography. The materials were obtained from the following sources: \(^{[3H]}\)iloprost (14.1 Ci/mmol) and the cAMP \(^{[125I]}\) assay system from Amersham Corp.; [5,6,8,11,12,14,15-\(^{3}H\)]PGE\(_2\) (185 Ci/mmol) from Du Pont- New England Nuclear; PGE\(_2\) from Funakoshi Pharmaceuticals (Tokyo); GTP from Boehringer Mannheim; fura-2/AM from Dojindo Laboratories (Kumamoto, Japan); and thrombin from Mochida Pharmaceutical Co., (Tokyo). All other chemicals were of reagent grade.

Mastocytoma P-815 cells were maintained in an ascitic form in BDF1 mice and harvested from the ascitic fluid of inoculated mice as described previously. The crude membrane fraction (100000 \(\times\) g pellet) were prepared from the cells as described previously. \(^{[3]}\)

\(^{[3H]}\)Illoprost or \(^{[3H]}\)PGE\(_2\) Binding Assay The standard assay mixture was comprised of either 20 nM \(^{[3H]}\)iloprost (28.2 nCi) or 5 nM \(^{[3H]}\)PGE\(_2\) (95.2 nCi), and 200 \(\mu\)g of the membrane fraction in 100 \(\mu\)l of 10 mM potassium phosphate, p\(H\) 6.0, containing 1 mM EDTA and 10 mM MgCl\(_2\) (buffer A). After incubation for 1 h at 37 °C, the reaction was terminated by the addition of 2 ml of ice-cold buffer A, after which the mixture was rapidly filtered through a Whatman GF/C glass filter (i.d. 2.4 cm). The filter was then washed four times with 2 ml of ice-cold buffer A, and the radioactivity associated with the filter was measured in 5 ml of Clearsol (Nacalai Tesque, Kyoto, Japan). Nonspecific binding was determined using a 1000-fold excess of the respective unlabeled PG in the incubation mixture. The specific binding was calculated by subtracting the nonspecific binding from the total binding.

Adenylate Cyclase and cAMP Assays The standard mixture for assaying adenylate cyclase activity comprised the membrane fraction (18 \(\mu\)g of protein), 1 mM EDTA, 10 mM MgCl\(_2\), 1 mM dithiothreitol, 1 mM 3-isobutyl-1-methylxanthine (IBMX) and 1 mM ATP in 100 \(\mu\)l of 50 mM Hepes–NaOH, p\(H\) 8.0. Reactions were started by the addition of the membrane fraction, and were allowed to proceed for 10 min at 37 °C, they were then terminated by the addition of 100 \(\mu\)l of 10% trichloroacetic acid. cAMP formed was measured by radioimmunoassay with an Amersham cAMP \(^{[125I]}\) assay system.

**Measurement of Cytosolic Ca\(^{2+}\)** Cells suspended at a density of 10\(^{5}\) cells/ml were loaded with 3 \(\mu\)M fura-2/AM for 30 min at 37 °C. The cells were then washed twice with Hepes-buffered saline containing 0.5% bovine serum albumin and kept at 4 °C in the same solution until assayed. After centrifugation, the cells (2 \(\times\) 10\(^{5}\) cells) were resuspended in Hepes-buffered saline without bovine serum albumin, and the fluorescence intensity was measured, at an excitation wavelength of 340 or 380 nm and an emission wavelength of 510 nm, with a fluorescence spectrometer (Jasco, CAF-100). The cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) was calculated from cellular fura-2 fluorescence as described previously. \(^{[7]}\)

**RESULTS**

**Binding Affinities of the Prostacyclin Analogues for PGI\(_2\) and PGE Receptors** We previously reported the presence of PGI\(_1\) and PGE receptors in mouse mastocytoma P-815 cells. \(^{[2]}\) To compare the binding specificities of SM-10906 and its methyl ester, SM-10902, in reaction to both the PGI\(_2\) and PGE receptors, we examined the effects of SH-10906, SM-10902 and iloprost on both specific \(^{[3H]}\)iloprost and \(^{[3H]}\)PGE\(_2\) binding to the membrane fraction of mastocytoma P-815 cells. As shown in Fig. 2A, specific \(^{[3H]}\)iloprost binding was inhibited by the unlabeled PGs in the order of iloprost > SM-10902 > SM-
10906. The IC_{50} value of SM-10906 was 20 nm. In contrast, specific [^3]H]PGE₃ binding was inhibited by the unlabeled PGs in the order of PGE₃ > iloprost > SM-10906 > SM-10902 (Fig. 2B). These results indicated that SM-10906 is a specific ligand for the PGI₁ receptor, but not for the PGE receptor. On the other hand, SM-10902, a methyl ester of SM-10906, is about two order less effective than SM-10906 in binding for the PGI₁ receptor.

**Effects of SM-10906 and SM-10902 on Adenylate Cyclase Activity** We have reported that PGI₁[2] and one of its stable analogues[4] strongly stimulate cAMP formation in mastocytoma P-815 cells, and that the PGI₁ receptor is coupled to the adenylate cyclase system via a stimulatory G-protein.[3] Therefore, we here examined the ability of PGI₁ analogues to stimulate GTP-dependent adenylate cyclase activity in the membrane fraction of mastocytoma cells. SM-10906 dose-dependently stimulated adenylate cyclase activity in the presence of GTP, the half-maximal concentration being about 35 nm, which is roughly similar to that of iloprost. On the other hand, SM-10902 showed much lower activity than SM-10906 (Fig. 3). These results indicate that SM-10906 is a potent agonist for the PGI₁ receptor, but its methyl ester, SM-10902, is greatly reduced in this ability.

**Dose Dependencies of the Effects of PGI₁ Analogues on the Thrombin-Induced Increase in [Ca^{2+}]ᵢ** We recently reported that thrombin induces intracellular Ca^{2+} mobilization, and that PGI₁ analogues, iloprost and TEI-9063, suppress thrombin-induced Ca^{2+} mobilization through the elevation of the intracellular cAMP level in mastocytoma cells.[4,8] Thus, we compared the effects of PGI₁ analogues with iloprost on thrombin-induced Ca^{2+} mobilization (Fig. 4). Thrombin induced a rapid and transient increase in [Ca^{2+}]ᵢ, which was prevented by pretreatment for 5 min with iloprost in the presence of IBMX.[4,8] Iloprost dose-dependently inhibited the thrombin-induced increase in [Ca^{2+}]ᵢ. Pretreatment with SM-10906 and IBMX dose-dependently, but slightly less potently than iloprost, prevented the thrombin-induced increase in [Ca^{2+}]ᵢ, the reduced half-maximal concentrations being 300 nm. SM-10902 was less potent than SM-10906. Taken together, the results in Figs. 3 and 4 show that the modification of SM-10906 following the addition of a methyl ester to the a chain reduces its ability. The potency of PGI₁ analogues in terms of the prevention of a thrombin-induced increase in [Ca^{2+}]ᵢ, agreed well with the same prevention potency in displacing the specific binding of [^3]H]iloprost (Fig. 2A) and in stimulating GTP-dependent adenylate cyclase activity (Fig. 3).

**DISCUSSION**

Since PGI₁ is a chemically unstable compound, several stable derivatives of PGI₁ have been developed. Among them, iloprost, one of the carbacyclin derivatives, has been frequently used as a potent ligand for the PGI₁ receptor in human platelets.[9] On the other hand, very few stable PGI₁ analogues have been reported. As far as we know, one stable analogue, the 5,6-dihydro prostacyclin (6β-PGI₁), was developed to inhibit gastric secretion by histamine from canine parietal cells.[6] In this case, 6β-PGI₁ produced a dose-related elevation of cAMP levels, but this effect was only found above a concentration of 1 μm. However, a chemically stable PGI₁ analogue, SM-10906, has unique structural features different from those of 6β-PGI₁. We reported here that SM-10906 is a specific ligand for the PGI₁ receptor, but is slightly less potent than iloprost in terms of the displacement of [^3]H]iloprost binding (IC_{50} value being 20 nm, Fig. 2A) and stimulation of GTP-dependent adenylate cyclase activity in the membrane fraction of mastocytoma cells (EC_{50} value being 35 nm, Fig. 3), and in the prevention of thrombin-induced [Ca^{2+}]ᵢ in mastocytoma cells (EC_{50} value being 100 nm, Fig. 4). Yet, the PGI₁ analogue and iloprost both behave as a very weak ligand for the PGE receptor (Fig. 2B). These results indicate that the PGI₁ analogue specifically binds to PGI₁ receptors, but not to PGE.
receptors. We have recently cloned cDNAs for mouse mastocytoma PGE receptor subtypes, EP1, EP2 and EP3.\(^{10-12}\) The cloned PGE receptor subtypes expressed in cultured COS cells specifically bound \(^{3}H\)PGE\(_2\), and this \(^{3}H\)PGE\(_2\) binding was displaced by unlabeled PGs in the order of PGE\(_2\) > PGF\(_{2\alpha}\) > iloprost > PGF\(_{2\alpha}\) > PGD\(_2\). From these results, the PGI\(_2\) receptors are found to be distinct from the PGE receptors in mastocytoma cells. Recently we demonstrated the specific PGI\(_2\) binding protein in mastocytoma cell membranes by photoaffinity labeling with the stable PGI\(_2\) analogue, \(^{3}H\)APNIC.\(^5\) However, it is unclear whether the PGI receptor subtype specific to PGI\(_1\) is present in mastocytoma cells. On the other hand, SM-10902, a methyl ester of SM-10906, is almost inactive for the binding assay shown in Fig. 2A, but seems to be a partial agonist for the adenylate cyclase activity shown in Fig. 3. A similar discrepancy in the range of effective doses between binding assay and adenylate cyclase activity assay was also experienced in the action of a methyl ester of the stable PGI\(_2\) analogue, TEI-9063.\(^4\) These results suggest that methyl esterification of the \(\alpha\)-chain carboxyl group of a PGI\(_1\) analogue may possibly be important in the binding to its receptor site. As to the binding site of the ligand carboxyl group of prostaglandins, Hirata et al. suspected that a carboxyl group of the mouse TXA\(_2\)/PGH\(_2\) receptor interacts with Arg 295 in the transmembrane segment VII of an amino acid sequence deduced from its cloned cDNA.\(^{13}\) This suspicion is supported with the recent data of a TXA\(_2\) receptor model, which was constructed based on the \(\beta_2\) receptor model.\(^{14}\) Furthermore, the corresponding Arg residue in transmembrane segment VII is well conserved in every PGE receptor subtype.\(^{10-12}\) If the \(\alpha\)-chain carboxyl group of a PGI\(_1\) analogue is the key residue for binding to its receptor, it is likely that methyl esterification of the \(\alpha\)-chain carboxyl group render impossible the interaction with an Arg residue. In order to elucidate this subject, it is necessary to determine the amino acid sequence of the PGI\(_2\) receptor.

Methyl esterification of the \(\alpha\)-chain carboxyl group of PG has been thought to prevent the \(\beta\) oxidation of a carboxylic acid. However, it is not clear whether the methyl ester PG is a biologically active form or a prodrug form of its free acid. Recently, Tsai et al., through in vivo studies on animals and man after oral administration, showed that misoprostol was converted to misoprostol free acid through deesterification,\(^{15}\) and that the reduction of deesterification of misoprostol by paraoxon, an esterase inhibitor, decreased gastric antisecretory activity and receptor binding activity.\(^{16}\) From these findings, they suggested that misoprostol is a prodrug and that its antisecretory activity is expressed by its free acid. If we assume that the methyl residue of SM-10902 is subjected to hydrolysis resulting in its free acid under the experimental conditions, we can suspect the same situation as that of misoprostol in the distinct activity of SM-10902 in comparison to SM-10906. This is probable, because we detected the production of SM-10906 by incubating SM-10902 for several min with animal or human sera, followed by high-performance liquid chromatography. The half-life of the conversion of SM-10902 to SM-10906 was calculated to be about 10 min in human serum. Furthermore, we found that diisopropyl fluorophosphate (DFP) abolished most of the activity of SM-10902 in the prevention of adenosine diphosphate (ADP)-induced rabbit platelet aggregation in a serum-supplemented solution (T. Yamamoto et al., in preparation). Therefore, the activity of SM-10902 may be expressed by the PGI\(_1\) derivative free acid, SM-10906, which may be produced by enzymatic hydrolysis in tissue or serum. However, it is necessary to measure the percentage of conversion of SM-10902 to SM-10906 during incubation for the agonist activity assays shown in Figs. 3 and 4.

REFERENCES