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Augmentation of Receptor-Mediated Adenylyl Cyclase Activity by Gi-Coupled Prostaglandin Receptor Subtype EP3 in a G$\beta\gamma$ Subunit-Independent Manner

Noriyuki Hatae,* Kumiko Yamaoka,* Yukihiko Sugimoto,*
Manabu Negishi,† and Atsushi Ichikawa*,[1]

**Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, and †Department of Molecular Neurobiology, Graduate School of Bioscience, Kyoto University, Yoshida, Sakyo-ku, Kyoto 606, Japan*

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We previously demonstrated that the mouse EP3β receptor and its C-terminal tail-truncated receptor (abbreviated T-335) expressed in Chinese hamster ovary cells showed agonist-dependent and fully constitutive Gi activity in forskolin-stimulated cAMP accumulation, respectively. Here we examined the effect of the EP3β receptor or T-335 receptor on adenylyl cyclase activity stimulated by the Gs-coupled EP2 subtype receptor in COS-7 cells. As a result, sulprostone, a selective EP3 agonist, dose dependently augmented butaprost-stimulated adenylyl cyclase activity in EP3β receptor- or T-335 receptor-expressing COS-7 cells. However, such adenylyl cyclase augmentation was not attenuated by either pertussis toxin treatment or expression of the PH domain of rat βARK1, which serves as a scavenger of G$\beta\gamma$ subunits, but was partially attenuated by treatment with either 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetra(acetoxymethyl)ester, an intracellular Ca^{2+} chelator, or W-7, a calmodulin inhibitor. These findings suggest that the C-terminal tail of the EP3β receptor is not essentially involved in activation of EP2 receptor-stimulated adenylyl cyclase in a Ca^{2+}/calmodulin-dependent but G$\beta\gamma$ subunit-independent manner.

Key Words: **PGE_2 receptor; EP3 subtype; EP2 subtype; adenylyl cyclase; Gi protein; G$\beta\gamma$ subunits; calmodulin; COS-7 cells.**

The interaction of cell surface hormone receptors with heterotrimeric G proteins is crucial for hormonal action (1). Most G protein coupled receptors (GPCRs) share conserved structural features, consisting of seven-transmembrane-spanning domains and three intracellular loops and one C-terminal tail (2). In a number of GPCRs, several regions in the cytoplasmic domains were reported to contribute directly or indirectly to G protein coupling (3). The specific regions in the second and third intracellular loops were reported to function in G protein coupling (4). However, the precise molecular mechanism for regulation of the receptor-induced G-protein activation is still poorly understood.

We previously cloned the mouse prostaglandin (PG) EP3 receptor and demonstrated that this receptor is a G protein coupled rhodopsin-type receptor that engages in inhibition of adenylyl cyclase (5). Furthermore, we identified the three isoforms of the EP3 receptor, EP3α, β, and γ, with different C-terminal tails, which were produced through alternative splicing (6, 7) and differed in agonist-independent constitutive Gi activity (8, 9). The EP3β receptor exhibits a full agonist-dependent Gi activity. In addition, the C-terminal tail–truncated receptor, T-335, showed only agonist-independent constitutive Gi activity. This suggests that the core of the EP3 receptor has an ability to associate with and activate Gi, and then the C-terminal tails of the EP3 receptor can suppress the activation of Gi (8).

Although Gi/o-coupled receptors generally act to inhibit adenylyl cyclase, it was also reported that long-term activation of the inhibitory receptors with agonists leads to an increase in adenylyl cyclase activity in a time- and dose-dependent manner (10, 11). This phenomenon is termed adenylyl cyclase superactivation or sensitization, which is mediated by adenylyl cyclase isozymes such as adenylyl cyclase II, directly being activated by G$\beta\gamma$ subunits derived from activation of Gi/o protein (12).

Abbreviations used: PG, prostaglandin; G protein, heterotrimeric GTP-binding protein; CHO, Chinese hamster ovary; COOH, carboxyl; PT, pertussis toxin; BAPTA/AM, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetra(acetoxymethyl)ester.

[1] To whom correspondence and reprint requests should be addressed at Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606, Japan. Fax: +81-75-753-4557. E-mail: aichikaw@pharm.kyoto-u.ac.jp.

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To assess the role of the C-terminal tail of the EP3β receptor in regulation of receptor-medicated adenylyl cyclase activity, we examined the effect of EP3 receptor on adenylyl cyclase activity in EP2-receptor expressing COS-7 cells. As a result, the adenylyl cyclase activity of the EP2 receptor was augmented EP3 agonist-dependently in T-335 expressing cells, and augmentation was closely related to the activity of Ca^{2+}/calmodulin but not the Gi/o-derived G$\beta\gamma$ subunits.

MATERIALS AND METHODS

Materials. The cDNAs for the mouse EP2 receptor (13) and EP3 receptor (5) were cloned in our laboratory. The truncated T-335 receptor was constructed as described previously (14). The cDNA for the rat LH/CG-R (15) was from Dr. D. L. Segaroff of the Department of Physiology and Biophysics, University of Iowa College of Medicine. LipofectAMINE PLUS was obtained from Life Technologies, Inc. M & B 28767, butaprost, and sulprostone were from Dr. M. P. L. Caton of Rhone-Poulenc Ltd. The ^{125}I-labeled cAMP assay system was obtained from Amersham Corp. Pertussis toxin (PT) was obtained from Seikagaku Kogyo (Tokyo, Japan). Rabbit polyclonal anti-GRK2 (C-15) and rabbit polyclonal anti-A cyclase III (C-20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

DNA construction. The peptide-encoding cDNA fragment for the PH domain of rat βARK1 (Gly^{495}-Leu^{689}) (16) were constructed by means of a polymerase chain reaction-mediated mutagenesis technique. The cDNAs encoding mouse EP2 receptor, rat LH/CG-receptor, EP3 receptor, T-335 receptor and the PH domain of rat βARK1 were subcloned into pcDNA3 (Invitrogen) eukaryotic expression vector.

Cell culture and transient expression of receptors in COS-7 cells. COS-7 cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% heat inactivated fetal bovine serum under humidified air containing 5% CO_2 at 37°C. For transfection using the LipofectAMINE PLUS reagent, cells in 60-mm tissue culture dishes were incubated at 37°C with a transfection mixture composed of 2.5 ml of serum-free DMEM containing 8 μg of DNA/dish, 10 μl of LipofectAMINE and 8 μl of PLUS reagent. After 3 h, the medium was changed to 3 ml of DMEM containing 10% heat inactivated fetal bovine serum. In cAMP assay COS-7 monolayers were trypsinized 24 h after transfection, and aliquots of recovered cells were transferred to 24-well tissue culture plates.

Expression of the βARK1 PH domain and adenylyl cyclase III. COS-7 cells were transiently cotransfected with increasing amounts of plasmid DNA encoding the PH domain of the rat βARK1. Expression of the βARK1 PH domain was determined by immunoblot analysis of whole cell detergent lysates using rabbit polyclonal anti-GRK2 (C-15). The PH domain expressing cells were harvested and washed twice in PBS. The cell pellet was suspended in 1 ml of RIPA buffer containing 30 mM Hepes–NaOH (pH 7.3), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate and 0.1% SDS and incubated for 1 h at 4°C. For protection against proteolytic degradation, a mixture of protease inhibitors (0.2 mM PMSF, 100 μM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml E-64, and 1 μg/ml pepstatin A) was added. The mixture was then centrifuged at 100,000*g* for 10 min at 4°C. The resulting supernatant was dissolved in Laemmli buffer and heated for 5 min at 100°C. Aliquots (10 μg proteins) were then subjected to SDS–PAGE (15%) as described by Laemmli (17), and the separated proteins were transferred electrophoretically to a PVDF membrane in 25 mM Tris base containing 40 mM 6-aminohexanoic acid, 0.02% SDS and 20% methanol at room temperature for 30 min at 15 V. The membrane was rinsed in Tris-buffered saline (TBS) containing 20 mM Tris–HCl (pH 7.5) and 150 mM NaCl, and then preincubated overnight in TBS containing 5% non-fat milk at 4°C. The membrane was then incubated with anti-GRK2 (C-15) (1:200) antibody in TBS containing 5% nonfat milk for 1 h at 37°C. The membrane was washed three times with TTBS (TBS containing 0.05% Tween 20) at room temperature. The membrane was incubated with peroxidase-conjugated anti-rabbit IgG in TTBS for 1 h at room temperature, and then stained with the ECL Western blot detection reagent.

Expression of the adenylyl cyclase III was determined by immunoblot analysis of whole-cell detergent lysates using rabbit polyclonal anti-A cyclase III (C-20).

Measurement of cAMP formation. Cyclic AMP levels in COS-7 cells were determined as reported previously (18). The receptor-expressing COS-7 cells were pretreated with or without W-7 for 10 min in Hepes-buffered saline [140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose, and 15 mM Hepes (pH 7.4)], or BAPT/AM for 30 min in Ca^{2+}-free Hepes-buffered saline containing 5 mM EGTA in place of 2.2 mM $CaCl_2$. The cells were washed and preincubated for 10 min with 10 μM indomethacine in Hepes-buffered saline. Reactions were started by addition of test agents along with 100 μM Ro-20-1724. After incubation for 10 min at 37°C, reactions were terminated by the addition of 10% trichloroacetic acid. The content of cAMP was measured by radioimmunoassay with an Amersham cAMP assay system.

ADP ribosylation of membrane with PT. The preparation and ADP-ribosylation assay of crude membrane fractions were conducted by the method of Ui *et al.* (19). The COS-7 cells were treated with various doses of PT for 15 h. The treated cells were harvested and washed twice in PBS. The cell pellet was homogenized in 1 ml of Tris-buffered saline containing 20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 10 mM $MgCl_2$, 1 mM DTT, 0.2 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 100 μM benzamidine using a Potter-type Teflon glass homogenizer, and then the mixture was centrifuged at 100,000 $\times$ g for 10 min at 4°C. The resulting pellets were used as the crude membrane fractions. The crude membrane fractions (20 μg of protein each) were incubated with 10 μg/ml of PT for 40 min at 30°C in 50 μl of 50 mM Tris–HCl (pH 7.5) containing 1 mM EDTA, 10 mM thymidine, 2.5 mM $MgCl_2$, 1 mM ADP-ribose, 15 mM isonicotinohydrazide, 2.5 μM [α-^{32}P]NAD (30 Ci/mmol), and 0.05% digitonin. The radiolabeled membranes were dissolved in Laemmli buffer and heated for 5 min at 100°C. Aliquots (6 μg each) were then subjected to SDS–PAGE (10%), as described by Laemmli (17). The gel was dried in a gel drier and exposed to X-ray film for 6 h at room temperature.

RESULTS

Sulprostone-Induced Augmentation of Butaprost-Stimulated cAMP Formation in EP3β Receptor- or T-335 Receptor-Expressing COS-7 Cells

In COS-7 cells expressing the EP3β receptor together with the EP2 receptor, 10^{-6} M butaprost, an EP2-specific agonist, elicited a significant cAMP accumulation. 10^{-7} M sulprostone, an EP3 agonist, further augmented butaprost-induced cAMP accumulation (Fig. 1A). We previously reported that a mutant of EP3 receptor with a deletion in the variable C-terminal tail, named T-335, exhibited constitutive Gi activity in CHO cells (8). However, in COS-7 cells expressing T-335 receptor together with EP2 receptor, butaprost increased cAMP accumulation, whose level was similar to that of cells expressing EP3β together with EP2 receptor. Sulprostone treatment resulted in enhancement of butaprost-induced cAMP accumulation (Fig.

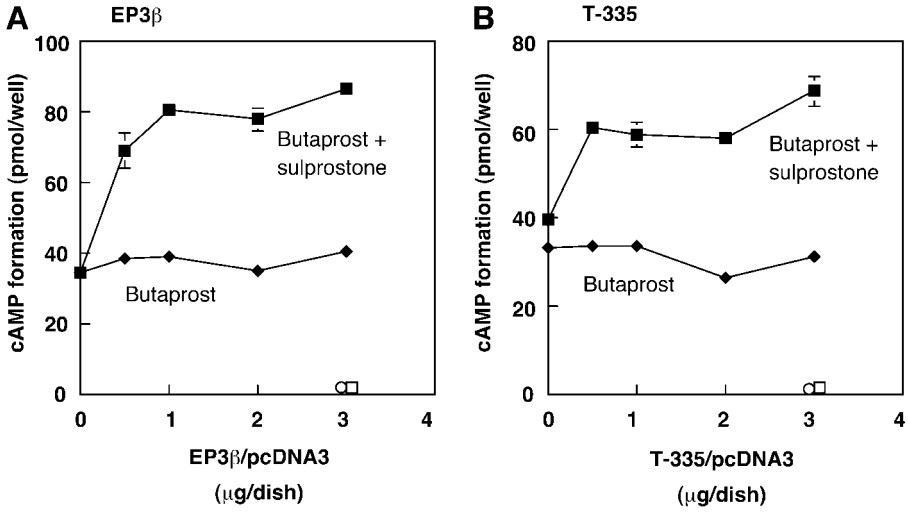

FIG. 1. Sulprostone-induced augmentation of butaprost-stimulated cAMP formation in EP3β receptor- or T-335 receptor-expressing COS-7 cells. COS-7 cells were transiently cotransfected with increasing amounts of plasmid DNA encoding EP3β receptor (A) or T-335 receptor (B) (0–3 μg/dish) with or without EP2/pcDNA3 (1 μg/dish). They were incubated at 37°C for 10 min with 1 μM butaprost in the absence (closed diamonds) or presence (closed squares) of 0.1 μM sulprostone. cAMP formations were determined as described under Materials and Methods. The control cells transfected the plasmid DNA encoding EP3 receptor (3 μg/dish) were incubated at 37°C for 10 min with 0.1 μM sulprostone in the absence (open circles) or presence (open squares) of 1 μM butaprost. Values are means ± SE of triplicate experiments.

1B). In the preliminary experiment, sulprostone dose-dependently enhanced the butaprost-stimulated cAMP accumulation in EP3β- or T-335- transfected COS-7 cells, but the half maximal concentration for EP3β mediated augmentation (1×10^{-8} M) were 2 orders of magnitude higher than for EP3β mediated Gi activity (1×10^{-10} M) (data not shown). Sulprostone alone did not stimulate adenylyl cyclase activity in COS-7 cells expressing the EP3β receptor or T-335 receptor alone (open circle in Fig. 1), suggesting that the adenylyl cyclase augmentation did not result from the direct activation of Gs protein via the EP3 receptor. Similar results were obtained when another EP3-agonist, M & B 28767, was used instead of sulprostone. Furthermore, the effects of EP3 agonists were obtained when LH/CG receptor-cDNA was used instead of the EP2-cDNA, followed by stimulation of LH (data not shown). These results suggested that stimulation of adenylyl cyclase by the EP3 receptor is irrespective of the Gs activation input signals.

Effects of Pertussis Toxin and PH Domain of Rat βARK1 on Sulprostone-Induced Augmentation of Butaprost-Stimulated cAMP Formation in EP3β Receptor- or T-335 Receptor-Expressing COS-7 Cells

Recently, it was reported that augmentation of Gs activity was achieved through G$\beta\gamma$ subunits resulting from the Gi/o protein activation in COS-7 cells (12). Therefore, we investigated whether the current findings included this machinery. Interestingly, it was found that PT failed to reverse the augmentation in adenylyl cyclase activity in COS-7 cells expressing the EP3β receptor or T-335 receptors (Fig. 2). These results suggest that EP3 receptor-involved adenylyl cyclase activation appears to be independent of Gi activation. Furthermore, when we transfected COS-7 cells with a minigene containing PH domain of rat βARK1 as a "decoy" of G$\beta\gamma$ dimmers in combination with EP3 and EP2 receptors, the treatment did not affect the levels of sulprostone-induced augmentation (Fig. 3). These findings suggested that the adenylyl cyclase augmentation was not due to G$\beta\gamma$ subunits resulting from the Gi activation by the EP3β receptor.

Effect of BAPTA/AM, Intracellular Ca^{2+} Chelator, and W-7, Calmodulin Inhibitor, on Sulprostone-Induced Augmentation of Butaprost-Stimulated cAMP Formation in EP3β Receptor- or T-335 Receptor-Expressing COS-7 Cells

To clarify which signaling pathways are involved in EP3 receptor-elicited adenylyl cyclase superactivation, we examined the effects of intracellular Ca^{2+} chelator, BAPTA/AM (20), and calmodulin inhibitor, W-7. As a result, it was found that sulprostone-induced augmentation was lost by treatment of each receptor-expressing COS-7 cells with BAPTA/AM, although this treatment slightly suppressed the basal activity by butaprost-induced adenylyl cyclase (Fig. 4). Recently, it was reported that the some isozymes of adenylyl cyclase, such as types I, III, and VIII, are stimulated by a high concentration of Ca^{2+}/calmodulin in the pres-

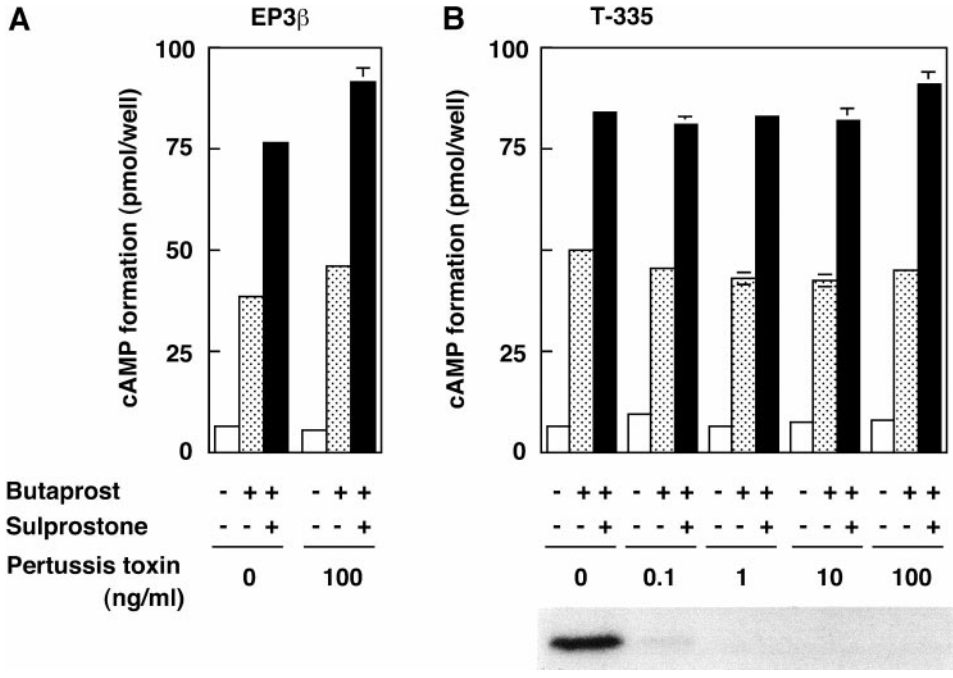

FIG. 2. Effect of pertussis toxin on sulprostone-induced augmentation of butaprost-stimulated cAMP formation in EP3β receptor- or T-335 receptor-expressing COS-7 cells. COS-7 cells were transiently cotransfected with plasmid DNA encoding EP3β receptor (A) or T-335 receptor (B) (1 μg/dish) with EP2/pcDNA3 (1 μg/dish). COS-7 cells expressing each receptor were pretreated with the indicated doses of PT (0–100 ng/ml) for 15 h. Then the treated cells were incubated at 37°C for 10 min with 1 μM butaprost in the absence or presence of 1 μM sulprostone. cAMP formations were determined as described under Materials and Methods. Values are shown as means ± SE of triplicate experiments. The lower panel shows the ADP-ribosylation of Gi protein. The crude membrane fractions from the cells pretreated with the indicated doses of PT as described above were incubated with [α-^{32}P]NAD and PT, and then subjected to SDS–PAGE. The band of the 41-kDa protein on an autoradiogram is shown. Autoradiogram was obtained after 6 h exposure to X-ray film.

ence of Gαs (21, 22). When we treated the each receptor-expressing COS-7 cells with W-7, the treatment attenuated the sulprostone-induced augmentation of butaprost-stimulated cAMP formation (Fig. 5). Furthermore, since W-7 itself had no effects on basal activity of butaprost-induced adenylyl cyclase, stimulation of adenylyl cyclase is unlikely to result from activation of Ca^{2+}/calmodulin-sensitive adenylyl cyclase.

DISCUSSION

One of the most significant findings in the present study was that the mouse EP3 receptor could be exclusively coupled to augmentation of receptor-mediated adenylyl cyclase activity, when expressed in COS-7 cells. Augmentation of adenylyl cyclase in COS-7 cells was previously reported in various Gi-coupled receptors such as α2 adrenoceptor, and its mechanism was suspected to be an increase of adenylyl cyclase type II activity by direct interaction of G$\beta\gamma$ subunits resulting from activation of Gi/o proteins (12). Such phenomena are termed adenylyl cyclase superactivation. However, the current superactivation of adenylyl cyclase was insensitive to PT treatment and the PH domain overexpression (Figs. 2 and 3), indicating that adenylyl

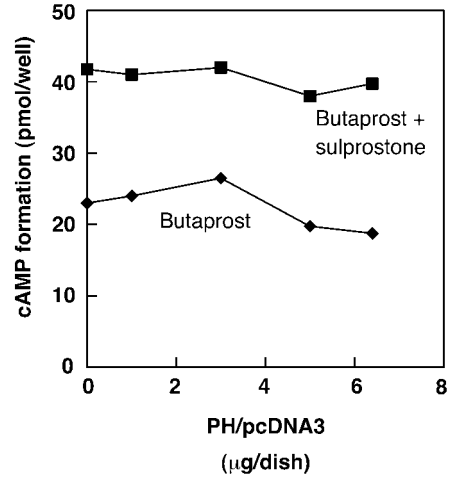

FIG. 3. Effect of the PH domain of rat βARK1 on sulprostone-induced augmentation of butaprost-stimulated cAMP formation in T-335 receptor-expressing COS-7 cells. COS-7 cells were transiently cotransfected with increasing amounts of plasmid DNA encoding the PH domain of rat βARK1 (0–6.4 μg/dish) with EP2/pcDNA3 (0.8 μg/dish) and T-335/pcDNA3 (0.8 μg/dish). They were incubated at 37°C for 10 min with 1 μM butaprost in the absence (closed diamonds) or presence (closed squares) of 1 μM sulprostone, and cAMP formations were determined as described under Materials and Methods. Values are means ± SE of triplicate experiments.

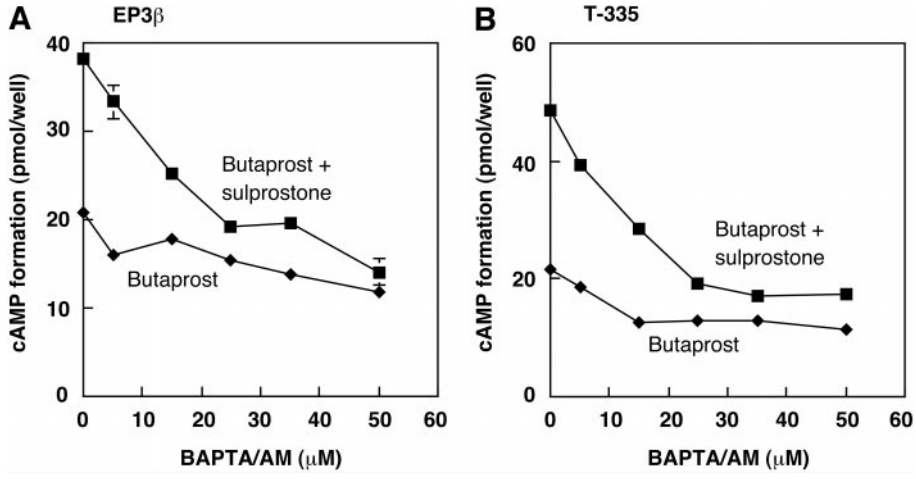

FIG. 4. Effect of BAPTA/AM on sulprostone-induced augmentation of butaprost-stimulated cAMP formation in EP3β receptor- or T-335 receptor-expressing COS-7 cells. COS-7 cells were transiently cotransfected with plasmid DNA encoding EP3β receptor (A) or T-335 receptor (B) (1 μg/dish) with EP2/pcDNA3 (1 μg/dish). After cells were pretreated with the indicated concentrations of BAPTA/AM in Ca^{2+}-free buffered saline for 30 min, they were incubated with 1 μM butaprost with (closed squares) or without (closed diamonds) 1 μM sulprostone. cAMP formations were determined as described under Materials and Methods. Values are means ± SE of triplicate experiments.

cyclase superactivation by the EP3β receptor appears to be mediated via a novel signal pathway without the involvement of G$\beta\gamma$ subunits. The inhibitory effects of BAPTA/AM and W-7 support the hypothesis that adenylyl cyclase superactivation via the EP3 receptor should be achieved via a signaling pathway relating to the Ca^{2+}/calmodulin-involved reactions (Figs. 4 and 5). We previously reported that the activation of mouse EP3α and EP3β receptors leads to Ca^{2+} mobilization in a PT-sensitive manner in COS-7 cells (23). Since the current adenylyl cyclase superactivation by the EP3β receptor was conducted Ca^{2+}/calmodulin pathway in a PT-insensitive manner, indicating that EP3β receptor could be linked to Ca^{2+} mobilization via activation of any PT-insensitive G proteins. Recently, nine distinct isozymes of adenylyl cyclase have been cloned. These isozymes differ in their properties, including their capacity to be inhibited or stimulated by G protein αi, αs, and $\beta\gamma$ subunits, as well as by protein kinase C, and Ca^{2+}/calmodulin (20, 21). Furthermore, the adenylyl cyclase III has been reported to be stimulated by a high concentration of Ca^{2+}/calmodulin in the presence of Gαs, and to be unaffected by G$\beta\gamma$ subunits (24). Indeed the adenylyl cyclase III was expressed in COS-7 cells (data not shown), then it can be that the activation of EP3β receptor leads to superactivation of this cyclase.

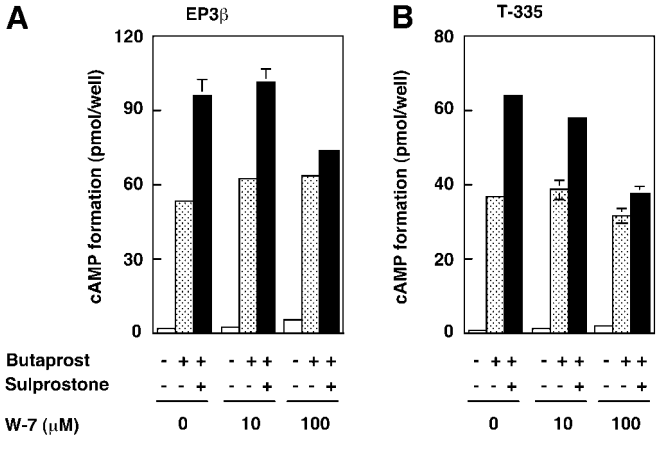

FIG. 5. Effect of W-7 on sulprostone-induced augmentation of butaprost-stimulated cAMP formation in EP3β receptor- or T-335 receptor-expressing COS-7 cells. COS-7 cells were transiently cotransfected with plasmid DNA encoding EP3β receptor (A) or T-335 receptor (B) (1 μg/dish) with EP2/pcDNA3 (1 μg/dish). After cells were pretreated with the indicated concentrations of W-7 for 10 min, they were incubated with 1 μM butaprost with or without 1 μM sulprostone. cAMP formations were determined as described under Materials and Methods. Values are means ± SE of triplicate experiments.

We previously demonstrated that EP3β receptor is entirely coupled to Gi activation resulting in inhibition of forskolin-stimulated adenylyl cyclase in CHO cells (6). Furthermore, we showed that removal of the C-terminal tail of EP3β receptor resulted in agonist-independent constitutive Gi activity in CHO cells (8). Indeed, similar results were obtained when we transiently introduced EP3β receptor cDNA or T-335 receptor cDNA together with EP2 receptor cDNA into HEK293 cells; EP3β and T-335 receptors showed Gi activity against butaprost-stimulated adenylyl cyclase in agonist-dependent and agonist-independent manners, respectively, and these activities were PT-sensitive (data not shown). From these results together with the previous our findings that EP3α and EP3γ receptors have different constitutive activity, it is suggested that the "common" structure of EP3 receptor contains domains necessary for the association and activation of Gi proteins, and the C-terminal tails have

a role for inhibition of leakage of constitutive activity. However, in the present study, EP3β and T-335 receptors raised similar profiles in the expression of adenylyl cyclase superactivation in COS-7 cells. Subsequently, the common structure in EP3 receptors has potential sites for eliciting two signaling pathways in an agonist dependent manner; one is Gi activity and the other is adenylyl cyclase superactivation, the latter effect is unlikely to be elicited by the direct involvement of Gs protein and adenylyl cyclase. Since C-terminal tails of EP3 receptors are dispensable in the latter signaling pathway, the conformational feature of the common structure of EP3β receptor may be quite different at the expression of adenylyl cyclase augmentation from the Gi activation.

Recently, Audoly *et al.* reported that the rabbit EP3 receptors can couple to activation of cAMP response element (CRE)-mediated gene transcription, which is PT-insensitive in HEK 293 cells (25). They also showed that the C-terminus-truncated isoform of the rabbit EP3 receptor can elicit this activation in an agonist-dependent manner although their EC_{50} values are 15-fold higher than that in Gi activity. Furthermore, based on the increase in intracellular Ca^{2+}, they suspected that CRE activation is mediated, in part, by a Ca^{2+}-dependent kinase pathway. It is currently unknown whether the signaling pathway they found is associated with the adenylyl cyclase superactivation in COS-7 cells, although they failed to detect cAMP accumulation by sulprostone alone in HEK 293 cells. Thus, the signal transduction properties of the EP3 receptor appear to be dependent on the cellular background in which they are expressed. However, it remains to be clarified which elements of cellular background determine the signaling pathway of EP3 receptors.

In summary, the C-terminal tail of the EP3β receptor is not essentially involved in activation of EP2 receptor-stimulated adenylyl cyclase in a Ca^{2+}/calmodulin-dependent manner, while these C-terminal regions of EP3 receptors play an important role in the inhibition of the activation of Gi. This study will contribute not only to the understanding of the heterogeneity of PGE_2 actions but also to elucidate the molecular mechanism of G protein activation induced by EP3β receptor.

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