Two Isoforms of the Prostaglandin E Receptor EP3 Subtype Different in Agonist-independent Constitutive Activity* 

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Hiroshi Hasegawa, Manabu Negishi, and Atsushi Ichikawa
From the Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606, Japan

We previously identified two isoforms of the mouse prostaglandin E receptor EP3 subtype, EP3α and EP3β, with different carboxyl-terminal tails, produced through alternative splicing and showing different efficiency of inhibition of adenylate cyclase (Sugimoto, Y., Negishi, M., Hayashi, Y., Namba, T., Honda, A., Watabe, A., Hirata, M., Narumiya, S., and Ichikawa, A. (1993) J. Biol. Chem. 268, 2712-2718). To assess the role of the carboxyl-terminal tails in the G protein coupling properties of the EP3 receptor, we examined the G activities of EP3α, EP3β, and the mutant receptor, in which the carboxyl-terminal tail was truncated at the splicing site. The EP3α receptor showed marked agonist-independent constitutive inhibition of adenylate cyclase, while EP3β receptor had no agonist-independent inhibition. On the other hand, the truncated receptor showed only agonist-independent constitutive inhibition. The constitutive activity of these receptors on the stimulation of GTPase activity of G, was also observed. Thus, alternative splicing produced two isoforms with different carboxyl-terminal tails and with different constitutive activity, and the truncation of the carboxyl-terminal tail caused full constitutive activity.

Prostaglandin E₂ (PGE₂) produces a broad range of biological actions in diverse tissues through its binding to specific receptors on plasma membranes (1, 2). PGE receptors are pharmacologically subdivided into four subtypes, EP1, EP2, EP3, and EP4, on the basis of their responses to various agonists and antagonists (3, 4). Among these subtypes, the EP3 receptor has been well characterized and has been suggested to be involved in such PGE₂ actions as contraction of the uterus (5), inhibition of gastric acid secretion (6), modulation of the neurotransmitter release (7), lipolysis in adipose tissue (8), and sodium and water reabsorption in the kidney tubuli (9, 10). Although EP3 receptor-mediated actions are believed to be mediated by inhibition of adenylate cyclase, the dose-response curve and potency of PGE₂ vary with tissue, implying heterogeneity of EP3 receptors (11, 12).

We have recently cloned the mouse EP3 receptor and demonstrated that this receptor is a G protein-coupled rhodopsin-type receptor that engages in inhibition of adenylate cyclase (13). Furthermore, we identified the two isoforms of the mouse EP3 receptor with different COOH-terminal tails, which are produced through alternative splicing and show different efficiency in inhibition of adenylate cyclase (14). Therefore, the COOH-terminal tails of the EP3 receptor may play an important role in the receptor-G protein coupling. To assess the role of the COOH-terminal tails of the EP3 receptor in coupling to G proteins, we constructed a mutated EP3 receptor, T-335, in which the COOH-terminal tail was truncated at the alternative splicing site, and showed that the truncated receptor retained identical agonist binding activity and ability to associate with G₁₃α, G₁₃β, and G₁₃γ (15). We studied in more detail the G protein coupling properties of EP3α, EP3β, and T-335 receptors. We report here that the two isoforms of EP3 receptor differ in agonist-independent constitutive activity, and the mutant receptor without the COOH-terminal tail is a fully constitutive active receptor.

EXPERIMENTAL PROCEDURES

Materials—M&B 28767 was a generous gift from Dr. M. P. L. Caton of Rhone-Poulenc Ltd. [γ³²P]GTP (6,000 Ci/mmol) and the 125I-labelled cAMP assay system were obtained from Amersham Corp. Pertussis toxin (PT) was obtained from Seikagaku Kogyo (Tokyo, Japan); forskolin was from Sigma, and rabbit antisera against G₁₃α, and G₁₃β (AS/7) was from DuPont NEN.

Chinese hamster ovary (CHO) cells stably expressing EP3α, EP3β, or the truncated receptor, T-335 (15), were cultured in the α-modification of Eagle’s medium lacking bicarbonate and deoxyribonucleosides, with 10% dialyzed fetal bovine serum under humidified air containing 5% CO₂ at 37°C.

Measurement of cAMP Formation—Cyclic AMP levels in CHO cells were determined as reported previously (16). The receptor-expressing CHO cells cultured in 24-well plates (5 × 10⁵ cells/well) were washed with Hepes-buffered saline containing 140 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 11 mM glucose, 15 mM HEPES, pH 7.4, and preincubated for 5 min. Reactions were started by the 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 in the cells was measured by radiomunnoassay with an Amersham cAMP assay system.

GTPase Activity—The harvested CHO cells were homogenized using a Potter-Evahjem homogenizer in 20 mM Tris-HCl, pH 7.5, containing 10 mM MgCl₂, 1 mM EDTA, 20 μM indomethacin, and 0.1 mM phenylmethylsulfonyl fluoride. After centrifugation at 250,000 × g for 20 min, the membrane pellet was washed, suspended in 20 mM Hepes-NaOH, pH 7.5, containing 2 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.1 mM NaCl (assay buffer). GTPase activity was assayed by incubating the membrane at 37°C for 5 min in a total volume of 100 μl of assay buffer, containing 1 mM GTP, 0.2 mM ATP, and 0.1 mM [γ³²P]GTP (0.5 μCi). The reactions were initiated by the addition of the membrane (10 μg) and stopped by the addition of 0.9 ml of ice-cold 5% Norit A and 0.1% bovine serum albumin in 20 mM sodium phosphate, pH 7.0. The mixtures were centrifuged (2000 × g, 5 min, 4°C), and the radioactivity of [³²P]P, released into the supernatant (150 μl) was determined. Nonspecific GTP hydrolysis was determined using a 1000-fold excess of unlabeled GTP in the incubation mixture. The specific low Km GTPase activity was calculated by subtracting the

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‡To whom correspondence should be addressed: Dept. of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Sakyoku-ku, Kyoto 606, Japan. Tel.: 81-75-753-4527; Fax: 81-75-753-4557; E-mail: aichikaw@pharmsun.pharm.kyoto-u.ac.jp.

The abbreviations used are: PG, prostaglandin; G protein, heterotrimeric GTP-binding protein; CHO, Chinese hamster ovary; AppNHp, adenylyl-5′-yl β,γ-imidodiphosphate; PT, pertussis toxin.

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Constitutive Activity of EP3 Isoforms

**RESULTS**

Inhibition of Adenylate Cyclase by EP3α, EP3β, and T-335 Receptors—To evaluate the inhibition of adenylate cyclase by the two EP3 receptor isoforms, EP3α and EP3β, and the truncated receptor, T-335, we chose a cell clone expressing each receptor with almost similar receptor number (EP3α, 1.23; EP3β, 1.11; T-335, 1.08 pmol/mg of the membrane protein). The ability of these receptors to inhibit the forskolin-activated adenylate cyclase was tested in the absence or presence of EP3 agonist, M&B 28767 (Fig. 1). In the absence of the agonist, forskolin increased the intracellular cAMP levels in the order of EP3β > EP3α > T-335, the cAMP level in the EP3β receptor-expressing cells (11.6 pmol/10^5 cells) being almost the same as that of forskolin-stimulated cAMP formation in the mock-transfected cells (12.2 pmol/10^5 cells). M&B 28767 concentration-dependently inhibited the forskolin-stimulated cAMP formation in the EP3α receptor- and EP3β receptor-expressing cells, the levels reaching the value of the forskolin-stimulated formation in the T-335-expressing cells. On the other hand, the agonist-induced inhibition was not observed in the T-335. These findings suggest that these receptors differed in constitutive inhibition of adenylate cyclase.

To assess the constitutive activities of the receptors, we examined the effect of PT on the G_i activities of these receptors. As shown in Fig. 2A, PT treatment concentration-dependently increased the forskolin-stimulated cAMP formation in the absence of the agonist in the EP3α receptor-expressing cells. PT treatment attenuated the M&B 28767-induced inhibition of the forskolin-stimulated cAMP formation, the cAMP level reaching the same value as that in the absence of the agonist. Although PT treatment completely attenuated the agonist-induced inhibition of the forskolin-stimulated cAMP formation in the EP3β receptor, this treatment did not increase the forskolin-stimulated cAMP formation in the absence of the agonist, and the treatment rather slightly suppressed the cAMP level at higher concentrations of PT (Fig. 2B). This suppression at higher concentrations of PT was also observed in the mock-transfected cells (data not shown). In the T-335-expressing cells, PT treatment increased the cAMP level of the forskolin-stimulated formation in the absence and presence of the agonist with the same concentration-dependent curve, the level reaching maximally the value of PT-treated EP3α- and EP3β-expressing cells (Fig. 2C). To confirm the effect of the toxin, membrane fractions exposed to the toxin were incubated with the activated toxin and [α-32P]NAD. The ADP-ribosylation of G_i was decreased progressively as the concentration of the toxin used for the pretreatment was increased (data not shown). These findings indicate that the truncated receptor is fully constitutively active and two isoforms differ in constitutive activity.

Activation of G_i by EP3α, EP3β, and T-335 Receptors—We next examined the ability of two EP3 isoforms and the truncated receptor to activate G_i in the presence or absence of the agonist (Fig. 3). The basal GTPase activity in the membrane of each receptor-expressing cell increased in the order of T-335 > EP3α > EP3β in the absence of the agonist. The T-335 showed the highest GTPase activity, and M&B 28767 did not change the value of the GTPase activity. On the other hand, M&B 28767 concentration-dependently stimulated the activities in the EP3α and EP3β receptors, the levels reaching maximally the value in the T-335. Pretreatment of the membrane expressing each receptor with anti-serum against G_i decreased the GTPase activities of the membranes expressing these receptors in the presence of the agonist to the level in the EP3β receptor in the absence of the agonist. These findings indicate that T-335 constitutively activates G_i, and the EP3α and EP3β receptors differ in the constitutive activation of G_i.

To confirm the constitutive activation of G_i in these receptors, we examined the effect of PT on the agonist-dependent or independent activation of G_i in the membrane expressing each receptor (Fig. 4). In the EP3β receptor, PT treatment suppressed the agonist-induced stimulation of GTPase activity without any change of the basal activity. On the other hand, PT treatment decreased both basal activity and agonist-induced stimulation of GTPase activity in the EP3α receptor to the level of the basal activity in the EP3β receptor. PT treatment also decreased the GTPase activity in the T-335 receptor in the absence and presence of the agonist to the level of the basal activity in the EP3β receptor.

**DISCUSSION**

Alternative splicing in transcription from a single gene produces related protein isoforms with distinct primary structures and adds different functional properties to the protein. We have already demonstrated that alternative splicing generates two EP3 receptor isoforms with different COOH-terminal tails, which are different in the sensitivity to agonists in the adenylate cyclase inhibition (14). We here revealed that the two isoforms differ in constitutive activity; the EP3α receptor has high constitutive activity, whereas the EP3β receptor has no constitutive activity. The EP3α and EP3β receptors are the
first example of isoforms produced through alternative splicing, showing different constitutive activity. Several reports have been made on endogenous receptors, having some levels of constitutive activity (17, 18), and receptors having greater constitutive activities have been speculated to show a reduced fold stimulation due to the high basal activity and receptors with lower or no constitutive activities to reflect a broader spectrum of agonist responses (19). The EP3 receptor isoforms might, therefore, underlie the diverse dose-response curves of PGE2 and provide a variety of the receptor responses in inhibition of adenylate cyclase. In addition to the EP3α and EP3β receptors, which are exclusively coupled to Gi, we identified a third EP3 receptor isoform, EP3γ, with a different COOH-terminal tail, which is also produced through alternative splicing, and we demonstrated that the EP3γ receptor is coupled to multiple G proteins, Gα1 and Gα5, suggesting that the COOH-terminal tails of EP3 receptor participate in determination of G protein specificity (20). The truncated EP3 receptor showed neither elevation of basal adenylate cyclase activity nor its agonist-dependent stimulation, indicating that the receptor did not show constitutive Gαi activity.2 Lefkowitz and co-workers have proposed a two-state model in which receptors are in equilibrium between the inactive conformation and the active conformation that can associate and activate G protein, and classical agonists increase the concentration of the latter conformation of the receptors (19). Our findings demonstrate that most of the EP3β receptors have the inactive conformation in the absence of the agonist, while about half of the EP3α receptors already have the active conformation in the absence of the agonist, and the agonist shifts all of the EP3β receptor and the EP3α receptor with an inactive form from inactive conformation to fully active conformation.

The domains of the receptors, which interact with and activate G proteins, have been studied extensively (22). These studies showed that synthetic peptides derived from specific regions in the second and third intracellular loops in various receptors directly activate G proteins. Therefore, the receptors in the inactive conformation may prevent the activating domains from association and activation of G proteins, and an agonist-promoted conformational change of the receptors may release the constraint, allowing the domains to associate with G proteins. Because the two EP3 receptor isoforms with different constitutive activities are different only in the COOH-terminal tail, and the truncated receptor exhibited agonist-independent constitutive activity, the COOH-terminal tails after the alternative splicing site may suppress activation of Gi by the EP3 receptor to a different extent dependent on the structure of the COOH-terminal tail. The COOH-terminal tail of the EP3β receptor completely suppresses the activity, while that of the EP3α receptor partially suppresses it, and agonists release the suppression by the COOH-terminal tail. Constitutively active receptors with mutations in the COOH-terminal domain of the third intracellular loops have been reported (22, 23). Recently, truncation of the COOH-terminal tail has been shown to cause constitutive activity in the thyrotropin-releasing hormone receptor (24). Thus, our finding also supports the idea that the COOH-terminal tail is the site involved in the constraint of the receptor in its inactive conformation in various receptors.

In summary, we present here that two EP3 receptor isoforms differ in constitutive activity and that the COOH-terminal tails play an important role in the constraint of the EP3 receptor in its inactive conformation. This study will contribute not only to the understanding heterogeneity of PGE2 actions but will also help to elucidate the molecular mechanism of G protein activation induced by receptors.

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