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Mouse Prostaglandin E Receptor EP_3 Subtype Mediates Calcium Signals via Gi in cDNA-transfected Chinese Hamster Ovary Cells

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Summary: We recently cloned the mouse prostaglandin (PG) E receptor EP_3 subtype that is coupled to adenylate cyclase inhibition through Gi and identified three isoforms which are produced through alternative splicing. In Chinese hamster ovary cells expressing each EP_3 isoform, PGE_2 induced an immediate increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) due to both Ca^{2+} mobilization from internal stores and influx from the extracellular medium. This increase was abolished by prior treatment with pertussis toxin (PT). PGE_2 also stimulated an accumulation of inositol trisphosphate (IP_3) in a PT-sensitive manner. Both the PGE_2-induced increase in $[Ca^{2+}]i$ and accumulation of IP_3 were blocked by the phospholipase C inhibitor U-73122. Thus, EP_3 is linked to phospholipase C activation via Gi, and this activation leads to Ca^{2+} mobilization from internal stores and influx from the extracellular medium.

Many receptors for hormones, autacoids and neurotransmitters transduce their biological signals via heterotrimeric GTP-binding proteins (G proteins) [1]. Gq-phospholipase C coupling is well-characterized, and Gq is responsible for Ca^{2+} mobilization, resulting from phospholipase C-β activation [2]. In contrast, Gi was initially believed to exclusively mediate inhibition of adenylate cyclase and not to be responsible for Ca^{2+} mobilization. However, recent studies showed that βγ-subunits of Gi, which is activated by the M_2 muscarinic receptor, stimulate phospholipase C-β2 [3], suggesting potential participation of Gi in the phospholipase C-Ca^{2+} mobilizing signal.

We have recently cloned the mouse prostaglandin (PG) E receptor EP_3 subtype, and demonstrated that it is a G protein-coupled rhodopsin-type receptor [4]. Furthermore, we identified three isoforms of the EP_3 receptor with different carboxy-terminal tails, $EP_{3\alpha}$, $EP_{3\beta}$ and $EP_{3\gamma}$, which are produced through alternative splicing,

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Abbreviations: G protein, heterotrimeric GTP-binding protein; PG, prostaglandin; PT, pertussis toxin; CHO, Chinese hamster ovary ; $[Ca^{2+}]i$, intracellular Ca^{2+} concentration; IP_3, inositol trisphosphate; IP_4, inositol tetrakisphosphate.

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and showed that $EP_{3\alpha}$ and $EP_{3\beta}$ were coupled to inhibition of adenylate cyclase [5], whereas $EP_{3\gamma}$ was coupled to both stimulation and inhibition of adenylate cyclase [6]. EP_3 mediates the diverse physiological actions of PGE_2, most of which occur through coupling of EP_3 to Gi and adenylate cyclase inhibition. However, coupling of EP_3 to Ca^{2+} signaling appears to mediate some actions of EP_3 such as contraction of rat myometrium [7], osmotic water flow in rabbit cortical collecting ducts [8], and histamine secretion from the mouse mast cell line BNu-2cl3 [9]. We then investigated Ca^{2+} signaling and phosphatidylinositol turnover in cDNA of mouse EP_3 transfected Chinese hamster ovary (CHO) cells. We report here that EP_3 causes Ca^{2+} release from intracellular stores and the influx from the extracellular medium due to Gi-phospholipase C coupling.

MATERIALS AND METHODS

Measurement of $[Ca^{2+}]i$ For measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]i$), Chinese hamster ovary cells (CHO-dhfr-) stably expressing each EP_3 isoform [6] were seeded at 4.0×10^4 cells/cm^2 on coverslips (circular; diameter, 13.2 mm), cultured for 48 h, and then loaded with 5 μM fura-2/AM (Dojindo Laboratories, Kumamoto, Japan) for 60 min at 37°C. The cells were then washed twice with HEPES-buffered saline solution containing 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). Each coverslip was placed diagonally in a cuvette holder with the cell monolayer facing the excitation beam. The fluorescence intensity was measured at an excitation wavelength of 340 nm or 380 nm and an emission wavelength of 510 nm with a fluorescence spectrophotometer (Jasco, CAF-100), as described previously [10].

Measurement of $[^3H]IP_3$ formation To measure [^{3}H]inositol trisphosphate (IP_3) formation, the CHO cells expressing each EP_3 isoform were seeded at 4.0×10^5/well in 6-well plates, cultured for 48 h, and then labeled with 1 μCi/ml myo-[2-^{3}H]inositol for 12 h [11]. Cells were washed twice with HEPES-buffered saline and preincubated in 2 ml of HEPES-buffered saline containing 10 mM LiCl for 10 min at 37°C. The solution was aspirated and the reaction was started by adding 2 ml of HEPES-buffered saline containing 10 mM LiCl and 1 μM PGE_2. After incubation at 37°C for 10 sec, the medium was quickly aspirated and 1 ml of 10% trichloroacetic acid was added. Measurement of [^{3}H]IP_3 formed was performed essentially as described by Berridge *et al.* [12].

RESULTS AND DISCUSSION

Fig. 1 shows PGE_2-induced $[Ca^{2+}]i$ change in $EP_{3\alpha}$-expressing CHO cells. In the presence of extracellular Ca^{2+}, PGE_2 induced a rapid increase in $[Ca^{2+}]i$ from 130 nM to 680 nM within a few seconds and then the level decreased quickly, regaining the basal level within 1 min (Fig. 1A). In Ca^{2+}-free medium, the PGE_2-induced $[Ca^{2+}]i$ increase was markedly reduced, but was still observed (Fig. 1B), indicating that the PGE_2-induced $[Ca^{2+}]i$ increase occurred via two distinct pathways, large extracellular Ca^{2+} influx (Ca^{2+} entry) and small Ca^{2+} release from internal stores (Ca^{2+} mobilization). Pretreatment of the cells with pertussis toxin (PT) at 20 ng/ml

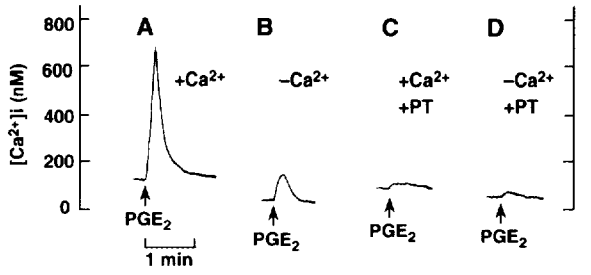

Fig. 1. Effects of PGE_2 on $[Ca^{2+}]i$ in cells expressing $EP_{3\alpha}$. CHO cells expressing $EP_{3\alpha}$ were treated without (A and B) or with (C and D) 20 ng/ml PT for 12 h. The cells were loaded with fura-2/AM, and then stimulated with 1 μM PGE_2 in HEPES-buffered saline ($+Ca^{2+}$) (A and C) or in Ca^{2+}-free buffer containing 1 mM EGTA ($-Ca^{2+}$) (B and D). PGE_2 was added at the time indicated by the arrow. The recordings shown are representatives of three independent experiments that yielded similar results.

for 12 h almost abolished both Ca^{2+} mobilization and Ca^{2+} entry (Fig. 1C and 1D), indicating that the PGE_2-induced $[Ca^{2+}]i$ change is mediated by a PT-sensitive G protein. PT-sensitive Ca^{2+} entry and Ca^{2+} mobilization were also observed in CHO cells expressing $EP_{3\beta}$ or $EP_{3\gamma}$ (Table I), suggesting that each EP_3 isoform exerts Ca^{2+} entry and mobilization via a PT-sensitive G protein.

It has been demonstrated that IP_3 induces Ca^{2+} release from endoplasmic reticulum [13], and that IP_3 or inositol tetrakisphosphate (IP_4) evoked Ca^{2+} influx via Ca^{2+}-permeable channels in the plasma membranes of olfactory cells [14, 15], T lymphocytes [16], CHO cells [17], sea urchin eggs [18] and endothelial cells [19]. We then examined whether PGE_2 stimulates IP_3 formation in CHO cells expressing

Table I. Effects of PGE_2 on $[Ca^{2+}]i$ in cells expressing each EP_3 isoform. CHO cells expressing each EP_3 isoform were treated with or without 20 ng/ml PT for 12 h. The cells were loaded with fura-2/AM, and then stimulated with 1 μM PGE_2 in HEPES-buffered saline ($+Ca^{2+}$) or in Ca^{2+}-free buffer containing 1 mM EGTA ($-Ca^{2+}$). The Peak $[Ca^{2+}]i$ levels induced by PGE_2 were determined as described in "Materials and Methods". Values are the means ± S.E.M. for three independent experiments.

	$\Delta[Ca^{2+}]i$ (nM)		
Treatment	$EP_{3\alpha}$	$EP_{3\beta}$	$EP_{3\gamma}$
None ($+Ca^{2+}$)	476 ± 61	306 ± 36	402 ± 39
None ($-Ca^{2+}$)	108 ± 22	125 ± 27	118 ± 24
PT ($+Ca^{2+}$)	27 ± 4	37 ± 7	37 ± 17
PT ($-Ca^{2+}$)	17 ± 7	11 ± 3	7 ± 4

Table II. Effects of PGE_2 and U-73122 on $[^3H]IP_3$ formation in cells expressing each EP_3 isoform. CHO cells expressing each EP_3 isoform were labeled with [^{3}H]inositol for 12 h. After preincubation with or without 10 μM U-73122 for 5 min, the cells were stimulated with 1 μM PGE_2. $[^3H]IP_3$ at 10 sec after stimulation was determined as described in "Materials and Methods". Values are expressed as percentages of the control and are the means ± S.E.M. for triplicate experiments. The radioactivity of the control in $EP_{3\alpha}$-, $EP_{3\beta}$- and $EP_{3\gamma}$-expressing cells was 897.2 ± 21.2, 968.8 ± 9.6 and 646.8 ± 20.8 dpm/10^6 cells, respectively.

Addition	$[^3H]IP_3$ formation (% of control)		
	$EP_{3\alpha}$	$EP_{3\beta}$	$EP_{3\gamma}$
None	100.0 ± 2.0	100.0 ± 1.0	100.0 ± 2.7
PGE_2	164.9 ± 21.4	149.7 ± 22.2	112.9 ± 5.8
U-73122	96.0 ± 3.9	95.6 ± 2.5	85.5 ± 1.9
U-73122 + PGE_2	110.3 ± 4.1	116.8 ± 3.2	98.6 ± 8.3

each EP_3 isoform. As shown in Table II, PGE_2 stimulated the accumulation of $[^3H]IP_3$ in the cells expressing each EP_3 isoform. Pretreatment for 5 min with the phospholipase C inhibitor U-73122 [20- 22] markedly prevented the accumulation of $[^3H]IP_3$ induced by PGE_2 (Table II). Moreover, pretreatment of the cells with PT also prevented the accumulation of $[^3H]IP_3$ induced by PGE_2 (Table III), suggesting that each EP_3 isoform causes phosphatidylinositol hydrolysis through activation of the PT-sensitive G protein by its coupling to phospholipase C as in the case of M_2 muscarinic receptors [3]. We next examined whether the PGE_2-induced $[Ca^{2+}]i$ increase is the result of activation of phospholipase C. As shown in Table IV, U-73122 abolished the PGE_2-induced $[Ca^{2+}]i$ increase both in the presence and absence

Table III. Effects of PT treatment on PGE_2-induced $[^3H]IP_3$ formation in cells expressing each EP_3 isoform. CHO cells expressing each EP_3 isoform were labeled with [^{3}H]inositol in the presence of 20 ng/ml PT for 12 h. The cells were stimulated with 1 μM PGE_2, and then $[^3H]IP_3$ at 10 sec after stimulation was determined as described in "Materials and Methods". Values are expressed as percentages of the control and are the means ± S.E.M. for triplicate experiments. The radioactivity of the control in $EP_{3\alpha}$-, $EP_{3\beta}$- and $EP_{3\gamma}$-expressing cells was 767.0 ± 56.0, 859.9 ± 29.8 and 371.8 ± 16.8 dpm/10^6 cells, respectively.

Addition	$[^3H]IP_3$ formation (% of control)		
	$EP_{3\alpha}$	$EP_{3\beta}$	$EP_{3\gamma}$
None	100.0 ± 6.1	100.0 ± 3.2	100.0 ± 3.5
PGE_2	102.7 ± 7.8	108.4 ± 2.7	96.4 ± 2.7

Table IV. Effects of U-73122 on PGE_2-induced increase in $[Ca^{2+}]i$ in cells expressing each EP_3 isoform. CHO cells expressing each EP_3 isoform were loaded with fura-2/AM. After preincubation with 10 μM U-73122 for 5 min at 37°C, the cells were stimulated with 1 μM PGE_2 in HEPES-buffered saline ($+Ca^{2+}$) or in Ca^{2+}-free buffer containing 1 mM EGTA ($-Ca^{2+}$). The Peak $[Ca^{2+}]i$ levels induced by PGE_2 were determined as described in "Materials and Methods". Values are the means ± S.E.M. for three independent experiments.

	$\Delta[Ca^{2+}]i$ (nM)		
Addition	$EP_{3\alpha}$	$EP_{3\beta}$	$EP_{3\gamma}$
$+Ca^{2+}$	66 ± 23	33 ± 10	72 ± 16
$-Ca^{2+}$	20 ± 4	26 ± 7	8 ± 5

of extracellular Ca^{2+}, indicating that the EP_3-operated two pathways, Ca^{2+} entry and Ca^{2+} mobilization, are mediated by inositol phosphates. IP_4 as well as IP_3 can evoke Ca^{2+} entry [18, 19]. Since no detectable IP_4 accumulation was observed in PGE_2-stimulated cells expressing EP_3 (data not shown), and an electrophysiological study showed that CHO cells have no IP_4-dependent Ca^{2+}-permeable channels [17], the PGE_2-induced Ca^{2+} entry in EP_3-expressing CHO cells may be mediated by IP_3 rather than by IP_4. Thus, EP_3 causes the stimulation of phospholipase C through Gi, leading to Ca^{2+} mobilization and Ca^{2+} entry in CHO cells. Recently, substance P receptor, a well known Gq-coupled receptor, has been shown to exert remarkable IP_3 accumulation (230% above the control), and cause $[Ca^{2+}]i$ increase ($\Delta[Ca^{2+}]i$; 330 nM) in the receptor cDNA transfected CHO cells [17]. In contrast to the substance P receptor, Gi-coupled EP_3 induces only small amount of IP_3 accumulation (13%- 65% above the control) (Table II), while it induced remarkable $[Ca^{2+}]i$ increase ($\Delta[Ca^{2+}]i$; 306- 476 nM) (Table I), comparable with that induced by the substance P receptor. A small amount of IP_3 appears to be sufficient for the Gi-coupled receptor-mediated $[Ca^{2+}]i$ increase in CHO cells.

PGE receptors can be pharmacologically divided into three subtypes, EP_1, EP_2 and EP_3, and these are presumed to be coupled to Ca^{2+} mobilization and stimulation and inhibition of adenylate cyclase, respectively [23, 24]. Thus, it is believed that the PGE_2-induced $[Ca^{2+}]i$ increase is mediated by EP_1. We recently cloned the cDNA for EP_1, and showed that EP_1 causes extracellular Ca^{2+} entry via a PT-insensitive G protein [25]. However, pharmacological studies showed that PGE_2 induced contraction of the uterus, where EP_3 is abundantly expressed [4], due to $[Ca^{2+}]i$ increase in a PT-sensitive manner [7]. In addition, EP_3-selective agonists also induced uterus contraction [26]. Furthermore, it was also reported that PGE_2 produced a prompt increase in $[Ca^{2+}]i$ by a mechanism involving Ca^{2+} mobilization and Ca^{2+} entry in rabbit collecting ducts resulting in inhibition of osmotic water flow

[27], suggesting that some PGE_2-induced $[Ca^{2+}]i$ increase is mediated by EP_3. Our present study strongly supported that in addition to adenylate cyclase inhibition, EP_3 causes $[Ca^{2+}]i$ increase via Gi and mediates some biological actions of PGE_2 such as induction of uterus contraction and inhibition of osmotic water flow in the kidney through this signal transduction pathway. It has been reported that dopaminergic D_2 receptor expressed in the fibroblast cell line Ltk^- [28], angiotensin II receptor in the adrenocortical cell line Y1 [29] and luteinizing hormone-releasing hormone receptor in the pituitary cell line GH_3 [30], caused the influx of extracellular Ca^{2+} via a PT-sensitive G protein. The present results clearly demonstrate that EP_3 causes $[Ca^{2+}]i$ increase due to Gi-mediated phospholipase C activation. Our present study showed that Gi-phospholipase C coupling is the other important Ca^{2+}-signaling pathway in addition to Gq-phospholipase C coupling.

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