Inhibition of Rac activation as a mechanism for negative regulation of actin cytoskeletal reorganization and cell motility by cAMP

Shin-ya NAGASAWA*†, Noriko TAKUWA*, Naotoshi SUGIMOTO*, Hiroshi MABUCHI† and Yoh TAKUWA*¹

*Department of Physiology, Kanazawa University Graduate School of Medicine, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8640, Japan, and †Molecular Genetics of Cardiovascular Medicine, Kanazawa University Graduate School of Medicine, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8640, Japan

cAMP has been found to play a role in mediating the negative regulation of cell motility, although its underlying molecular mechanism remains poorly understood. By using CHO (Chinese-hamster ovary) cells that express the EP2 subtype of PGE₂ (prostaglandin E₂) receptors, we provide evidence that an increase in cellular cAMP content leads to inhibition of cellular Rac activity, which serves as a mechanism for this negative regulation. In CHO cells expressing EP2, but not in vector control cells, PGE₂ dose-dependently inhibited chemotaxis towards IGF-I (insulin-like growth factor-I), which is a Rac-dependent process, with the maximal 75 % inhibition observed at 10⁻⁸ M PGE₂. EP2 stimulation failed to inhibit tyrosine phosphorylation either of IGF-I receptor or IRS-1 (insulin receptor substrate-1), or activation of phosphoinositide 3-kinase or Akt in response to IGF-I, but potently and dose-dependently inhibited IGF-I-induced activation of cellular Rac activity and membrane ruffling. However, PGE₂ failed to inhibit Val¹²-Rac-induced membrane ruffling. Similar to

the case of CHO cells, PGE₂ inhibited PDGF (platelet-derived growth factor)-induced Rac activation and chemotaxis in vascular smooth muscle cells endogenously expressing EP2. The inhibitory effects of PGE₂ on IGF-I-induced chemotaxis, membrane ruffling and Rac activation were faithfully reproduced by a low concentration of forskolin, which induced a comparable extent of cAMP elevation as with 10⁻⁸ M PGE₂, and were potentiated by isobutylmethylxanthine. The protein kinase A inhibitor Rp isomer of adenosine 3',5'-cyclic monophosphorothioate reduced PGE₂ inhibition of Rac activation and chemotaxis. These results indicate that EP2 mediates Rac inhibition through a mechanism involving cAMP and protein kinase A, thereby inhibiting membrane ruffling and chemotaxis.

Key words: actin cytoskeleton, cAMP, cell motility, prostaglandin E₂, protein kinase A, Rac.

INTRODUCTION

Cell migration plays a critical role in a wide variety of physiological and pathological phenomena, including morphogenic processes during embryogenesis, inflammatory responses, wound healing, atherogenesis and tumour cell dissemination [1]. Cell migration is positively and negatively regulated by chemoattractants and inhibitory mediators respectively [2]. The former instructs cells to advance towards a site where higher concentrations of chemoattractants are present. These chemoattractants include chemokines, other inflammatory mediators and growth factors. Chemoattractant receptors, on ligand binding, activate complex signalling cascades involving protein tyrosine kinases, PI3K (phosphoinositide 3-kinase) and the low-molecular-mass (GEF; guanine-nucleotide-exchange factor) GTP-binding proteins, and particularly the Rho family GTPases [3]. The Rho family GTPases, primarily Rac, Cdc42 and Rho, are well-known regulators of actin organization and myosin motor function and thereby of cell motility [4]. These Rho GTPases show distinct activities on actin cytoskeletons: Rho mediates stress fibre formation and focal adhesion, whereas Rac and Cdc42 direct peripheral actin assembly that results in the formation of lamellipodia and filopodia respectively. Many chemoattractants stimulate cellular Rac activity [5]. Furthermore, expression of dominant-negative forms of Rac and Cdc42 inhibits stimulation of migration in response to chemoattractants, implying their essential roles in cell migration [4]. In addition, PI3K was demonstrated to act upstream

of Rac to be involved in lamellipodium formation as well as cell migration [3]. These observations indicate that the signalling pathway comprising PI3K, Rac and other Rho family G-protein members plays a critical role in the regulation of cell migration.

On the other hand, available information about the inhibitory extracellular mediators is by far limited compared with chemoattractants. Previous investigations demonstrated that several cAMP-elevating agents, including PGE₂ (prostaglandin E₂) [6], ISO (isoprenaline) [7], dopamine [8] and adrenomedullin [9], which all act on canonical G_s-coupled cell-surface receptors, inhibited cell migration towards chemoattractants. However, the molecular mechanisms behind the anti-migratory effects of these cAMP-elevating agents remain poorly understood.

PGE₂ is a bioactive eicosanoid that is implicated in biological processes including reproduction, immune responses, regulation of renal electrolyte and water transport, and circulatory homeostasis [10]. PGE₂ receptors, designated as EP receptors, are heptahelical G-protein-coupled receptors, and are divided into four distinct subclasses, EP1–EP4 [10]. The EP1 receptor couples through G_q with phospholipase C stimulation, whereas both EP2 and EP4 couple through G_s with stimulation of adenylate cyclase. The EP3 receptor couples with diverse signalling pathways including G_i, G_s and G_q in isoform-specific manners. PGE₂ inhibits migration of several different cell types, including neutrophils [6,7], lymphocytes [11] and fibroblasts [12], with an increase in cellular cAMP content. In neutrophils, selective EP2 agonists mimicked PGE₂ inhibition of cell migration [6], suggesting that

Abbreviations used: CHO cells, Chinese-hamster ovary cells; CHO-EP2 cells, CHO cells that stably express EP2 receptor; dbcAMP, dibutyryl cAMP; FSK, forskolin; Gα_q-CT, C-terminal peptide of G_q; GEF, guanine-nucleotide-exchange factor; GFP, green fluorescent protein; IBMX, isobutylmethylxanthine; IGF-I, insulin-like growth factor-I; IGF-IR, IGF-I receptor; IRS-1, insulin receptor substrate-1; ISO, isoprenaline; PDGF, platelet-derived growth factor; PGE₂, prostaglandin E₂; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PTHrP, parathyroid hormone-related peptide; Rp-cAMPS, Rp-isomer of adenosine 3',5'-cyclic monophosphorothioate; TRITC, tetramethylrhodamine isothiocyanate.

¹ To whom correspondence should be addressed (email ytakuwa@med.kanazawa-u.ac.jp).

the EP2 receptor mediated PGE₂ inhibition of neutrophil migration. The adenylate cyclase activator, FSK (forskolin), and the cell-permeant cAMP, dbcAMP (dibutyl cAMP), mimicked the PGE₂ action. However, an adenylate cyclase inhibitor and PKA (protein kinase A) inhibitors failed to prevent migration by PGE₂ inhibition. Thus the correlation of the ability to inhibit migration with that to increase cAMP and PKA activity was poor [6,7]. The exact molecular mechanisms for PGE₂ inhibition of cell migration is still not fully defined.

To understand better the role of cAMP in the regulation of cell migration, we established CHO (Chinese-hamster ovary) cells that overexpress EP2, in which PGE₂ inhibited cell migration directed towards a chemoattractant through EP2. We provide evidence that the cAMP–PKA pathway negatively regulates Rac and that this inhibition of Rac is a mechanism for EP2-mediated inhibition of cell migration.

EXPERIMENTAL

Materials

PGE₂ was purchased from Wako Pure Chemicals (Osaka, Japan). FSK and IBMX (isobutylmethylxanthine) were purchased from Sigma. They were dissolved in ethanol and stored at –20 °C. ISO and dbcAMP were obtained from Sigma. Recombinant human IGF-I (insulin-like growth factor-I) and Rp-cAMPS (Rp-isomer of adenosine 3',5'-cyclic monophosphorothioate) were purchased from R & D Systems (Minneapolis, MN, U.S.A.) and Calbiochem (San Diego, CA, U.S.A.) respectively. Mouse monoclonal anti-Rac and anti-Cdc42 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.) and Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) respectively. A rabbit polyclonal anti-Akt antibody and a mouse monoclonal anti-phospho-Akt (Ser-473) antibody were obtained from Cell Signaling (Beverly, MA, U.S.A.). Rabbit anti-IGF-IR (IGF-I receptor) antibody (C-20) and anti-IRS (insulin receptor substrate)-1 antibody (C-20) were obtained from Santa Cruz Biotechnology. Mouse monoclonal anti-phosphotyrosine antibodies 4G10 and PY20 were purchased from Upstate Biotechnology and BD Biosciences (San Jose, CA, U.S.A.) respectively. TRITC (tetramethylrhodamine isothiocyanate)-labelled phalloidin was obtained from Sigma. GST (glutathione S-transferase)-human PAK1 (p21-activated kinase amino acids 75–131) fusion protein was prepared as described in [5].

Cells, plasmids, adenoviruses and transfection

CHO-K1 cells were grown in Ham's F12 medium supplemented with 10 % (v/v) fetal bovine serum (Equitech-Bio, Ingram, TX, U.S.A.), 100 units/ml penicillin and 100 µg/ml streptomycin (Wako Pure Chemicals). Rat aortic smooth-muscle cells were cultured as described previously [13].

Mouse EP2 and human adrenergic β1 receptor cDNA, which were donated by Dr S. Narumiya (Kyoto University Graduate School of Medicine) and Dr R. J. Lefkowitz (Duke University Medical Center) respectively, was ligated on to the *EcoRI* site of pCAGGS to generate pCAGGS-EP2. pME18S-myc-V¹⁴RhoA, pME18S-myc-V¹²Rac1, pME18S-myc-V¹²Cdc42 and adenoviruses encoding myc-N¹⁹RhoA, N¹⁷Rac1 and N¹⁷Cdc42 were described previously [5,13,14]. pCAGGS-Gα_q-CT (where Gα_q-CT stands for the C-terminal peptide of G_q) and pCAGGS-Gα_s-CT, which encode myc-tagged C-terminal sequences of Gα_q (residues 306–359) and Gα_s (residues 319–377) respectively were described previously [13]. pCAGGS-LacZ and adenovirus encoding LacZ were donated by Dr I. Saito (Institute of Medical Sciences, University of Tokyo).

The cells were infected with adenoviruses at a multiplicity of infection of 200 by incubating cells with adenovirus-containing medium for 1 h, which conferred successful gene transduction in nearly 100 % of cells [5,13]. Transient transfection with an expression plasmid with or without GFP (green fluorescent protein) expression vector pEGFP-C1 (Clontech), which was employed as a transfection marker, was performed by using LIPOFECT-AMINE™ (Invitrogen) 48 h before each experiment. After recovery in growth medium for 24 h, the cells were serum-deprived for 24 h.

To establish CHO-EP2 cells (CHO cells that stably express EP2 receptor), cells were co-transfected with pCAGGS-EP2 and the neomycin resistance gene expression vector pKM3, and selected in the presence of 0.7 mg/ml G418 (Nacalai, Kyoto, Japan) as described previously [5]. To establish CHO-EP2 cells that stably express myc-tagged Gα_q-CT and Gα_s-CT, CHO-EP2 cells were co-transfected with either pCAGGS-Gα_q-CT or pCAGGS-Gα_s-CT and the Zeocin resistance gene expression vector pCMV/Zeo (Invitrogen) and selected in the presence of 50 µg/ml Zeocin (Invitrogen) and 0.7 mg/ml G418.

Transwell migration assay

Chemotactic migration of cells was measured in a modified Boyden chamber (Neuroprobe, Cabin John, MA, U.S.A.) using polycarbonate filters with 8 µm pores as described previously [5,15].

Determination of the activities of Rac and Cdc42, and cellular cAMP content

Pull-down assay methods to determine GTP-bound active forms of Rac and Cdc42 were described previously [5,13]. Briefly, cell extracts were incubated with GST-PAK CRIB (Cdc42/Rac inter-active-binding region) domain immobilized to glutathione-Sepharose 4B beads (Amersham Biosciences) at 4 °C for 45 min, followed by washing three times. Bound Rac and Cdc42 proteins were quantitatively detected by Western blotting using specific monoclonal antibodies against Rac and Cdc42. Cellular cAMP content was determined as described previously [17].

Analysis of tyrosine phosphorylation of IGF-IR and IRS-1

Cells were washed with ice-cold Ca²⁺- and Mg²⁺-free Dulbecco's PBS and lysed in either lysis buffer A (50 mM Tris/HCl, pH 8.0, 120 mM NaCl, 0.5 % Nonidet P40, 100 mM NaF, 1 mM Na₃VO₄, 0.1 % SDS, 2 mM EGTA, 0.19 mM leupeptin, 370 units/l aprotinin and 0.6 mM PMSF) for the determination of IGF-IR phosphorylation, or lysis buffer B (50 mM Tris/HCl, pH 7.4, 1 % Nonidet P40, 0.25 % sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM NaF, 0.19 M leupeptin, 370 units/l aprotinin and 0.6 M PMSF) for the determination of IRS-1 phosphorylation. After centrifugation at 8000 g for 5 min, the supernatants were subjected to immunoprecipitation with anti-IGF-IR antibody or anti-IRS-1 antibody for 2 h at 4 °C. The immunoprecipitates were recovered by incubation with Protein A-Sepharose, and bound IGF-IR and IRS-1 proteins were quantitatively detected by Western blotting using anti-phosphotyrosine antibody (4G10), anti-IGF-IR antibody or anti-IRS-1 antibody and by densitometry of the corresponding bands using the Quantity One image analysing system (PDI, San Diego, CA, U.S.A.).

PI3K assay

The PI3K activity assay was performed as described previously [5]. PI3K activity was measured in immunoprecipitates with an anti-phosphotyrosine antibody (PY20), using phosphoinositide and [γ-³²P]ATP as the substrates.

Figure 1 The EP2 PGE₂ receptor and β_1 adrenergic receptor mediate inhibition of IGF-I-directed chemotaxis by PGE₂ and ISO respectively

(A, C) Migration towards IGF-I (100 ng/ml) across the porous filter of CHO cells that stably express EP2 (CHO-EP2) or β_1 adrenergic receptor (CHO- β_1) or vector control CHO cells (CHO-vector) was determined in the presence of indicated concentrations of PGE₂ or ISO in the lower wells of the Boyden chamber. (B, D) The effects of PGE₂ (10⁻⁸ M) (C) or ISO (10⁻⁵ M) (D) were determined when they were added exclusively to the upper or lower chamber or both the upper and lower chambers as indicated. Results are representative of three independent experiments with similar results.

Western blotting and fluorescence microscopy

Western blotting was performed as described previously [13,16]. To evaluate the actin cytoskeleton, the cells were transfected 48 h before experiments and serum-starved as described above. After treatment with receptor agonists for the indicated time periods, the cells were fixed in 3.7% (v/v) formaldehyde in Dulbecco's PBS and processed as described previously [13,14]. F-actin was visualized with TRITC-labelled phalloidin under an inverted fluorescence microscope IX70 (Olympus, Tokyo, Japan).

Statistics

Results are presented as means $\pm$ S.E.M. for at least three determinations, and are representative of at least three independent experiments with similar results. The statistical significance of differences among multiple data was analysed by Scheffe's test.

RESULTS

EP2 subtype of PGE₂ receptor and β_1 adrenergic receptor mediate negative regulation of chemotaxis

As shown in Figure 1(A), PGE₂ dose-dependently inhibited IGF-I-stimulated chemotaxis in CHO-EP2 cells in the Boyden chamber assay, with the maximal 75% inhibition at 10⁻⁸ M PGE₂. In vector control cells, in contrast, PGE₂ did not inhibit IGF-I-stimulated chemotaxis. Both cell types showed comparable extents of chemotaxis towards IGF-I in the absence of PGE₂. These results indicate that EP2 is responsible for mediating negative regulation of chemotaxis. The PGE₂ inhibition of migration did not depend on a PGE₂ concentration gradient: similar extents of migration inhibition were observed whether PGE₂ was added exclusively to the lower or upper chamber or both the lower and upper chambers

(Figure 1B). We tested whether overexpression of β_1 adrenergic receptor, which is also coupled through G_s with the cAMP signalling pathway, mediated inhibition of chemotaxis. Indeed, in CHO cells that overexpress β_1 adrenergic receptor, ISO dose-dependently inhibited IGF-I-stimulated chemotaxis, with the maximal effect obtained for 10⁻⁶ M ISO. ISO was without any effect in vector control cells (Figure 1C). The inhibitory regulation by β_1 receptor was not dependent on a concentration gradient of ISO (Figure 1D), such as EP2-mediated inhibition.

Cellular Rac activity, which is required for cell migration, is negatively regulated by PGE₂-EP2 signalling

As shown in Figure 2(A), adenovirus-mediated expression of dominant-negative forms of Rac (N¹⁷Rac) and Cdc42 (N¹⁷Cdc42) potently inhibited IGF-I-directed chemotaxis in CHO-EP2 cells. In contrast, the expression of a dominant-negative form of RhoA (N¹⁹Rho) did not inhibit the chemotactic response compared with the LacZ control under our experimental conditions. These findings are consistent with our previous observations [5] and indicate that cellular activities of Rac and Cdc42 are required for IGF-I-directed chemotaxis in CHO-EP2 cells. We next studied the effect of EP2 stimulation on cellular Rac activity. We found that pretreatment of CHO-EP2 cells with PGE₂ (10⁻⁸ M) for 10 min potently suppressed the IGF-I-induced increase in the cellular amount of a GTP-bound active form of Rac (GTP-Rac) (Figure 2B). Thus IGF-I induced a rapid 11-fold increase in GTP-Rac at 1 min, which then gradually decreased but remained high at least for 10 min. In PGE₂-pretreated cells, the peak activation of Rac at 1 min was inhibited to a 3.5-fold increase above the basal level, which decreased to a near-basal level at 10 min (Figure 2B). The inhibitory effect was PGE₂ dose-dependent, with the maximal inhibition obtained at 10⁻⁸ M (Figure 2C). In

Figure 2 The expression of dominant-negative Rho family GTPase mutants inhibits chemotaxis, and PGE₂ inhibits cellular Rac activity

(A) Adenovirus-mediated expression of dominant-negative mutants of Rac (N¹⁷Rac) and Cdc42 (N¹⁷Cdc42), but not RhoA (N¹⁹RhoA), inhibits chemotaxis towards IGF-I. CHO-EP2 cells were transduced with adenoviral vectors encoding LacZ, N¹⁷Rac, N¹⁷Cdc42 or myc-N¹⁹Rho and, 48 h later, assayed for chemotaxis towards IGF-I (100 ng/ml). (B) PGE₂ inhibits IGF-I-induced Rac activation. CHO-EP2 cells were pretreated with or without PGE₂ (10⁻⁸ M) for 10 min and then stimulated with IGF-I (100 ng/ml) for the indicated time periods. Cellular amount of GTP-bound active form of Rac (GTP-Rac) was quantified by the pull-down technique (upper). A fraction (1/100) of the total Rac present in the cell lysates analysed is also shown (lower). (C, D) PGE₂ dose-dependently inhibited cellular Rac activity (C) but not Cdc42 activity (D). CHO-EP2 cells were pretreated with the indicated concentrations of PGE₂ and then stimulated with IGF-I (100 ng/ml) for 1 min and analysed as described in (B). (E, F) PGE₂ inhibits PDGF-B chain-induced chemotaxis (E) and Rac activation (F) in vascular smooth-muscle cells. The experiments for the determination of migration and GTP-Rac were conducted as in (C, D). Results are representative of three independent experiments with similar results.

contrast, PGE₂ failed to inhibit cellular Cdc42 activity at up to 10⁻⁷ M (Figure 2D). IGF-I itself also failed to affect cellular Cdc42 activity for at least 10 min. Taken together, these observations strongly suggest that the inhibition of cellular Rac activity is a mechanism responsible for EP2-mediated inhibition of chemotaxis towards IGF-I.

Vascular smooth-muscle cells express the endogenous EP2 receptor [18]. PGE₂ (10⁻⁶ M) inhibited PDGF (10 ng/ml)-induced chemotaxis and Rac activation (Figures 2E and 2F).

EP2 signalling inhibits IGF-I-induced Rac activation at the site distal to PI3K activation

To delineate the molecular mechanism by which EP2 mediates suppression of IGF-I-induced Rac activation, we examined whether PGE₂ inhibited IGF-I signalling events at the sites upstream of Rac activation in CHO-EP2 cells. We failed to detect any inhibition by PGE₂ pretreatment of IGF-IR activation, which included IGF-I-stimulated tyrosine phosphorylation of IGF-IR and

Figure 3 PGE₂ does not inhibit IGF-I-induced tyrosine phosphorylation of IGF-IR or its substrate, IRS-1, or stimulation of PI3K

(A, B) CHO-EP2 cells were pretreated with PGE₂ (10⁻⁸ M) for 10 min or left without pretreatment, and then stimulated with IGF-I (100 ng/ml) for 1 min. Total cell extracts were subjected to immunoprecipitation (IP) using anti-IGF-IR (A) or IRS-1 (B) antibody and the immunocomplexes were analysed by Western immunoblotting (IB) using 4G10 anti-phosphotyrosine (P-Tyr) antibody. The levels of tyrosine-phosphorylated IGF-R or IRS-1 were quantified by densitometry. (C) CHO-EP2 cells were treated as in (A, B) and assayed for PI3K activity, as described in the Experimental section. Autoradiographs of TLC plates and quantified results are shown. (D) CHO-EP2 cells were treated either with IGF-I (100 ng/ml) alone or with IGF-I and PGE₂ (10⁻⁸ M). PGE₂ was added 10 min before the addition of IGF-I. Akt stimulation was evaluated by Western analysis using anti-phospho-Akt antibody as described in the Experimental section. Results shown are representative of three or four experiments with similar results.

its substrate IRS-1 (Figures 3A and 3B). We previously demonstrated that IGF-I-induced Rac activation was dependent on PI3K [5]. As shown in Figure 3(C), IGF-I induced a 12-fold increase in the PI3K activity at 1 min, which was slightly inhibited by pretreatment with PGE₂. In addition, IGF-I induced comparable extents of activation of Akt (Figure 3D), which is one of the direct downstream effectors of PI3K, in the presence or absence of PGE₂ pretreatment. These results indicate that the major site of the inhibitory action of PGE₂ is located distal to IGF-I-stimulated PI3K activation.

cAMP mediates PGE₂ inhibition of IGF-I-stimulated chemotaxis, Rac activation and membrane ruffling

As shown in Figure 4(A), PGE₂ induced dose-dependent increases in the cellular cAMP content, with the half-maximal and maximal effects obtained at 5 × 10⁻¹⁰ and 10⁻⁸ M respectively. The addition of IBMX, a phosphodiesterase inhibitor, sensitized this effect in such a way that the PGE₂ dose-response curve was shifted to the left. Consequently, the effect of the combination of 10⁻⁹ M PGE₂ and IBMX was similar to the effect of 10⁻⁸ M PGE₂ (Figure 4A). Stimulation of CHO-EP2 cells with 2.5 × 10⁻⁷ M FSK resulted in an increase in the cellular cAMP content, which was comparable with that induced by 10⁻⁸ M PGE₂ (Figure 4B). We found that either the combination of 10⁻⁹ M PGE₂ and IBMX or 2.5 × 10⁻⁷ M FSK induced levels of inhibition of chemotaxis towards IGF-I comparable with that by 10⁻⁸ M PGE₂ (Figure 4C). In addition, dbcAMP inhibited IGF-I-directed chemotaxis (Figure 4C).

We tested the possibility that an increase in the cellular cAMP content might mediate inhibition of Rac activity. Indeed, as shown in Figure 4(D), 2.5 × 10⁻⁷ M FSK induced a similar extent of

inhibition of cellular Rac activity to that induced by either 10⁻⁸ M PGE₂ or 10⁻⁹ M PGE₂ plus IBMX. We also found that dbcAMP inhibited cellular Rac activity (Figure 4D). The results provide evidence that cAMP is a negative regulator of cellular Rac activity and suggest that cAMP mediates PGE₂ inhibition of Rac activation. We further examined whether FSK and dbcAMP inhibited IGF-I signalling at the sites upstream of Rac activation and found that, similar to PGE₂, these agents did not inhibit IGF-IR activation, IRS-1 tyrosine phosphorylation, PI3K activation or Akt activation (results not shown). These results provide further support to the notion that cAMP acts as the intracellular messenger that negatively regulates Rac in the EP2 signalling pathway.

We also examined the effects of these cAMP-elevating agents on IGF-I-induced membrane ruffling, which is a Rac-dependent process [5]. PGE₂, FSK or dbcAMP strongly inhibited IGF-I-induced membrane ruffling (Figure 5A). In contrast, neither FSK, PGE₂ (Figure 5B) nor dbcAMP (results not shown) inhibited membrane ruffling that was induced by a constitutively active mutant of Rac1, V¹²Rac. The observations suggest that cAMP does not directly act on the actin cytoskeletal machinery itself to modulate its organization at physiologically relevant concentrations.

EP2 inhibition of Rac and migration is specifically reversed by the expression of a C-terminal peptide of G_s, but not of G_q

We have previously demonstrated that the expression of the C-terminal peptides of G_α subunits inhibited the G_α-effector coupling in a G_α species-specific manner [13]. In the present study, we found that the expression of the C-terminal peptide of G_s, G_α-CT, almost completely reversed EP2-mediated inhibition of cell migration (Figure 6A) and Rac activity (Figure 6B). In

Figure 4 The phosphodiesterase inhibitor IBMX potentiates, and with FSK and dbcAMP mimics, PGE₂ inhibition of IGF-I-stimulated migration and Rac activation

(A) IBMX potentiated the cellular cAMP response to PGE₂. CHO-EP2 cells were stimulated with various concentrations of PGE₂ for 6 min without or with IBMX (2×10^{-5} M) pretreatment for 6 min. (B) CHO-EP2 cells were stimulated with either PGE₂ (10^{-8} M) or FSK (2.5×10^{-7} M) for 6 min and assayed for cAMP. (C) Migration towards IGF-I (100 ng/ml) across the porous membrane of CHO-EP2 cells was determined in the presence or absence of indicated concentrations of PGE₂ (10^{-8} M), IBMX (2×10^{-5} M), FSK (2.5×10^{-7} M) and dbcAMP (10^{-3} M) in the lower wells of the Boyden chamber. (D) CHO-EP2 cells were pretreated with indicated concentrations of PGE₂ (10^{-8} M), IBMX (2×10^{-5} M), FSK (2.5×10^{-7} M) and dbcAMP (10^{-3} M), stimulated with IGF-I (100 ng/ml) for 1 min and analysed for GTP-Rac as described for Figure 2. Results are representative of three independent experiments with similar results. ns, statistically not significant.

contrast, the expression of the $G\alpha_q$ C-terminal peptide, $G\alpha_q$ -CT, was without effect. In contrast, the inhibition by dbcAMP of both cell migration (Figure 6A) and Rac (results not shown) was resistant to $G\alpha_s$ -CT expression. The expression of $G\alpha_s$ -CT inhibited PGE₂-induced cAMP increase by 90%, but that of $G\alpha_q$ -CT did not significantly affect the cAMP response (results not shown). These results indicate that the inhibitory EP2 signalling is mediated by G_s , which is consistent with the notion that cAMP mediates PGE₂ inhibition of Rac and migration.

EP2 inhibition of Rac and migration is reversed by a specific inhibitor of PKA

We tested the effect of Rp-cAMPS, which is a specific inhibitor of PKA, on EP2-mediated cAMP-dependent inhibition of cell migration and Rac activity. As shown in Figure 7, the PKA inhibitor partially reversed the inhibition of both Rac and cell migration by PGE₂ in CHO-EP2 cells. It is therefore suggested that PKA is involved in cAMP-dependent down-regulation of Rac and cell migration.

DISCUSSION

The role of cAMP in the regulation of cell migration is not fully understood. The present study demonstrates, by using the heterologous expression in CHO cells, that PGE₂ induces inhibition of chemotaxis towards IGF-I through the EP2 receptor in the

signalling pathway comprising G_s , cAMP and PKA. We explored the molecular mechanisms of chemotaxis inhibition by the cAMP signalling pathway, and found that the EP2 receptor mediated the inhibition of the small GTPase Rac, a molecular switch of cell motility regulation, through cAMP and PKA.

It was demonstrated previously that PGE₂ inhibited chemotaxis of neutrophils towards chemoattractants [6,7]. However, it was not conclusive whether cAMP mediated the anti-migratory effect of PGE₂. In the present study, we found that IBMX, a phosphodiesterase inhibitor, potentiated both the PGE₂-induced cAMP accumulation and migration inhibition, and that a sub-maximal concentration of FSK reasonably reproduced the PGE₂ effects on cAMP and chemotaxis (Figures 4C and 4D). Also, FSK and the cell-permeant dbcAMP mimicked the PGE₂ inhibition of IGF-I-induced membrane ruffling (Figure 5A) and Rac activation (Figures 2B and 4D), and IBMX potentiated the effect of PGE₂ inhibition of Rac (Figure 4D). All these observations together suggest that cAMP mediates PGE₂ inhibition of chemotaxis. Consistent with this notion, the PGE₂ inhibition of chemotaxis was abolished by the blockade of EP2 receptor- G_s coupling (Figure 6). At present, the molecular basis for the discrepancies concerning the ability of cAMP in mediating negative regulation of cell migration in different cell types is unknown. In addition to possible cell-type-specific differences in subcellular compartmentalization of cAMP, cAMP metabolism and PKA, an intriguing possibility is that distinct regulators of Rac activity, including GEFs and GAPs (GTPase-activating proteins), or their distinct regulation may be operating in neutrophils and CHO cells.

Figure 5 PGE₂ inhibits IGF-I-induced membrane ruffling, but not V¹²-Rac-induced membrane ruffling

(A) CHO-EP2 cells were stimulated with IGF-I (100 ng/ml) with or without PGE₂ (10⁻⁸ M), FSK (2.5 × 10⁻⁷ M) or dbcAMP (1 mM) for 30 min and then fixed and stained for visualizing F-actin with TRITC-phalloidin. (B) CHO-EP2 cells were co-transfected with the GFP expression vector pEGFP-C1 and the V¹²-Rac expression vector at a weight ratio of 1:10, 48 h before stimulation with FSK (2.5 × 10⁻⁷ M) or PGE₂ (10⁻⁸ M) for 30 min. The cells were then fixed, and stained for visualizing F-actin with TRITC-phalloidin or visualized with fluorescence for GFP. Results shown are representative of three experiments with similar results.

Figure 6 Blockade of the CHO-EP2 receptor coupling with G_s by the expression of Gα_s-CT abolishes PGE₂ inhibition of IGF-I-stimulated migration and Rac activation

(A) The migration towards IGF-I (100 ng/ml) of the CHO-EP2 cells stably transfected with Gα_s-CT, Gα_q-CT or empty vector was determined in the presence or absence of indicated concentrations of PGE₂ (10⁻⁸ M) and dbcAMP (10⁻³ M) in the lower wells of the Boyden chamber. (B) CHO-EP2 cells stably overexpressing Gα_s-CT and Gα_q-CT and vector control cells were pretreated with PGE₂ (10⁻⁸ M) for 10 min, stimulated with IGF-I (100 ng/ml) for 1 min and analysed for GTP-Rac as described for Figure 2. Results are representative of three independent experiments with similar results.

A large body of evidence indicates that cAMP regulates many physiological processes through the activation of PKA. However, recent investigations also indicate that cAMP regulates specific cellular functions through PKA-independent pathways, which include direct regulation by cAMP of the Rap-GEF, Epac [19], and membrane ion channels [20]. In the present study, we observed that the PKA inhibitor Rp-cAMPS partially blocked PGE₂-induced inhibition of chemotaxis (Figure 7), suggesting that PKA, at least in part, mediates the PGE₂ inhibition. The inability of

Figure 7 The PKA inhibitor Rp-cAMPS inhibits PGE₂ inhibition of IGF-I-stimulated migration and Rac activation

(A) The PKA inhibitor Rp-cAMPS reversed PGE₂ inhibition of migration towards IGF-I. The migration of CHO-EP2 cells towards IGF-I (100 ng/ml) was determined in the presence or absence of PGE₂ (10⁻⁹ M) in the lower chambers and Rp-cAMPS (10⁻⁵ M) in the upper chambers. (B) Rp-cAMPS reversed PGE₂ inhibition of IGF-I-induced Rac activation. CHO-EP2 cells were pretreated with Rp-cAMPS (10⁻⁵ M) for 3 h and PGE₂ (10⁻⁹ M) for 10 min or left without pretreatment, and then stimulated with IGF-I (100 ng/ml) for 1 min and analysed for GTP-Rac as described for Figure 2. Results are representative of three independent experiments with similar results.

Rp-cAMPS to achieve a full extent of inhibition might result from the relatively insufficient intracellular Rp-cAMPS concentration because of its limited diffusion across the plasma membrane, compared with the intracellular endogenous cAMP concentration.

The present study showed that PGE₂ inhibited IGF-I-induced Rac stimulation with the dose–response relationship similar to that

for PGE₂ inhibition of chemotaxis (Figures 1A and 2C). Since Rac is essentially required for IGF-I-directed chemotaxis (Figure 2A) and lamellipodia formation [5], our results strongly suggest that PGE₂-induced Rac inhibition at least in part underlies the anti-migratory effect of PGE₂. FSK and dbcAMP mimicked, and IBMX potentiated, PGE₂ inhibition of IGF-I-induced Rac activation, like chemotaxis (Figure 4D). Furthermore, the PKA inhibitor Rp-cAMPS reversed PGE₂ inhibition of migration and Rac activation (Figure 7). These observations together suggest that the PGE₂ inhibition of Rac is mediated through the cAMP–PKA signalling pathway.

Recently, it was reported that PTHrP (parathyroid-hormone-related peptide) induced inhibition of Rac and cell migration in vascular endothelial cells through PKA [21], which is consistent with the present results. We have observed that PGE₂ inhibited Rac and chemotaxis through endogenous EP2 receptor in vascular smooth-muscle cells (Figures 2E and 2F), suggesting a potential anti-atherogenic role for PGE₂. Thus exogenous administration of cAMP-elevating agents, including PGE₂ and PTHrP, may have beneficial effects on certain vascular diseases that involve smooth-muscle migration and angiogenesis. In addition, the fact that PGE₂ and PTHrP are produced in the vascular bed suggests that the cAMP-mediated inhibitory actions on motility of vascular smooth-muscle and endothelial cells may play some regulatory roles in vascular development. In contrast with the present results and the reported effects of PTHrP on endothelial cells [21], it was demonstrated in several cell types, including breast carcinoma cells [22], melanoma cells [23] and neuronal cells [24], that cAMP mediated Rac stimulation, rather than its inhibition. In these cell types, however, the effects of cAMP on cell migration were not determined. Understanding the precise mechanisms for the bimodal actions of cAMP on cellular Rac activity await elucidation of a diversity of Rac regulatory molecules and their regulation by cAMP and PKA in various cell types.

We observed that PGE₂ did not affect IGF-I stimulation of PI3K activity, which is required for Rac activation [5]. This observation suggests that PGE₂ inhibits Rac by acting on a site downstream or independent of PI3K. It is possible that PGE₂ could either inhibit a Rac-GEF activity or stimulate a Rac-GAP activity, resulting in inhibition of IGF-I-induced Rac activation. The exact mechanism of PGE₂ inhibition of Rac remains to be clarified.

In conclusion, we have demonstrated that PGE₂ inhibits cellular Rac and migration via the EP2 receptor through the cAMP–PKA signalling pathway. The cAMP-mediated inhibitory regulation of Rac probably contributes to the regulation of many biological and pathological phenomena in which cell motility plays important roles.

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