Examining the FP receptor-triggered CREB activation in cell-based assays

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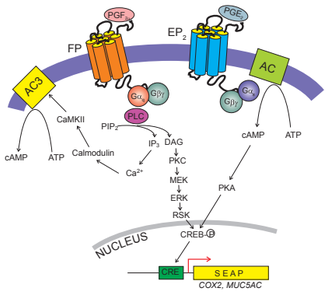
Abstract

Prostaglandins (PGs) play a crucial role in mediating a wide range of cellular processes and progression in inflammation, cancer, cardiovascular, and other diseases. Therefore, enzymes involved in the production of prostanoids, such as cyclooxygenases and prostaglandin synthases, as well as the receptors that mediate their biological responses, are important therapeutic targets. Several families of G protein-coupled receptors have been identified for prostanoids with different agonist selectivity and subsequent signaling pathways. The $PGF_{2\alpha}$ receptor (FP) was initially characterized as a G_q-coupled receptor, which leads to an increase in inositol triphosphate/diacylglycerol, intracellular calcium mobilization, and protein kinase C (PKC) activation. Interestingly, $PGF_{2\alpha}$ has also been suggested to induce CREB-regulated genes in human amniotic fibroblasts and tracheobronchial epithelial cells through PKC activity. The activation of CREB by the FP receptor has been reproduced in this study by co-transfecting the FP receptor with a CRE-SEAP reporter into HEK293T cells using reverse transfection. Replicate transfection plates were used to examine receptor activations by agonists in a calcium mobilization assay and an ELISA for cAMP. In the calcium assay, cloprostenol, as well as the free acids of bimatoprost and latanoprost, exhibited similar potencies and efficacies of $PGF_{2\alpha}$ with EC_{50} values around 50 nM. In contrast, agonists induced only subtle increases of cAMP unless a G_s/G_q chimeric G protein was co-expressed to redirect the receptor activation signal to the adenylate cyclase pathway. This verified that CREB activation by the FP receptor is not mediated by the adenylate cyclase pathway. In the presence of the G_s/G_q chimeric G protein, all four agonists that were tested led to the same maximal increase in cAMP levels after a 45-minute incubation. Latanoprost free acid and $PGF_{2\alpha}$ exhibited comparable EC_{50} values in the cAMP assay performed in parallel with the calcium assay on replicate plates, whereas bimatoprost and cloprostenol were one order more potent in the G_s/G_q-facilitated cAMP assay than in the calcium mobilization assay. This suggests the FP receptor may preferentially interact with the chimeric G protein upon certain agonist binding. On the other hand, the expression of G_s/G_q is not required in the CRE-SEAP reporter assay for FP receptor activity, although the addition of G_s/G_q produced a left shift in the dose-response curves. This direct stimulation of CRE-SEAP reporter using the same assay platform was not observed with other G_q-coupled receptors, such as orexin 1 and 2 receptors, that can also activate the PKC pathway. In other words, the activation of PKC alone may not be sufficient to mediate the effect of the FP receptor on CREB.

Introduction

The prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) receptor, PTGFR or FP, is a G protein-coupled receptor that has been shown to activate the G_q signaling pathway leading to elevation of intracellular calcium levels.[1] HEK293 cells stably transfected with FP have been tested with various agonists in calcium mobilization assays and exhibited comparable potencies in these cell lines compared to endogenous expression of the FP receptor.[2] Nevertheless, the stimulation of FP receptors has also been linked to the activation of CREB and upregulation of *COX2* and *MUC5AC*, suggesting a cross-talk between signaling pathways through PKC.[3-5] On the other hand, a different model has been proposed that the FP receptor-triggered calcium signal may potentiate the $EP_2/G\alpha_s$ pathway-mediated cAMP increase through CaMKII and isoform 3 of adenylate cyclase.[6] In contrary, a recent study reported the opposite effects of EP_2/cAMP/PKA and FP/calcium/PKC pathways in the expression of OVGP1 in bovine oviductal epithelial cells.[7] In this study, a transfection complex with an optimized ratio of FP expression plasmid and CRE-SEAP reporter construct was coated on 96-well microplates for reverse transfection of HEK293T cells. The FP receptor was stimulated with several known agonists the day after seeding cells, and the receptor activation and downstream gene regulation were monitored by calcium mobilization, ELISA for cAMP, and reporter gene assays. By comparing the relative potencies of these agonists in different assays, with and without the supplement of chimeric G protein, and the use of kinase inhibitors, the signaling mechanism models of the FP receptor were examined on this reverse transfection platform.



Materials and Methods

DNA constructs

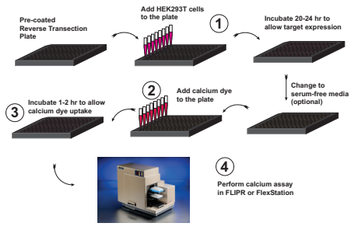
An FP2A cDNA fragment with optimized codon usage and Kozak sequence was designed and assembled into pCMV6-XL5 vector. A G_s/G_q chimera expression construct was produced by replacing the coding sequence for the C-terminal 5 amino acid of G_s cDNA with corresponding sequence from G_q as described by Komatsuzaki, *et al.*[8] CRE-SEAP reporter plasmid contains the cDNA of a secreted form of the human placental alkaline phosphatase (SEAP) under the regulation of five cyclic AMP response elements as previously described.[9,10]

Cell culture and transfection

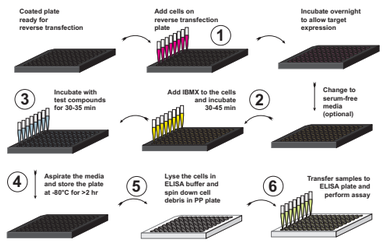
Transfection complex containing lipids for transfection and optimized amount of FP2A expression construct plasmid and CRE-SEAP reporter DNA was immobilized on tissue culture treated 96-well microplates using a proprietary protocol. Expression construct for G_s/G_q was added to the DNA mixture in the experiment that required the chimeric G protein. HEK293T/17 cells were grown in complete medium (Dulbecco's modified essential medium supplemented with 10% fetal calf serum). Actively dividing HEK293T cells were plated on the microplates with transfection complex at 50,000 cells per well 18-24 hours before assay in complete medium, Opti-MEM with 0.5% FBS, or Opti-MEM alone depending on the assay.

Cell-based assays

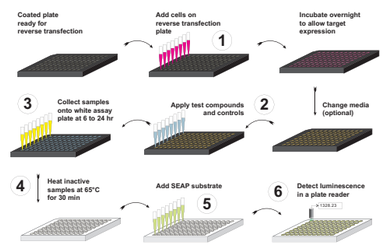
For the calcium mobilization assay, cells were plated in complete medium, which was changed to 100 µl/well serum-free DMEM at 20-24 hours after plating cells. Calcium 6 dye (Molecular Devices) was dissolved in assay buffer with probenecid according to manufacturer's instruction. The dye was dispensed at 100 µl/well to the plate and incubated at 37°C for 90 minutes. Test compounds were dissolved in DMSO at 10 mM stock concentration and diluted in assay buffer to 5x of final concentration on a polypropylene deep well plate. Assay was performed in FlexStation 3 after equilibrating the cell plate to ambient temperature in the machine for 30 minutes. Fluorescence at ex/em 485/525 nm was monitored every 3 seconds. Dilutions of testing compounds were delivered to the well in 50 µl volume at 20 seconds.



For the cAMP ELISA, cells were plated in Opti-MEM with 0.5% FBS, which was replaced with 160 µl/well Opti-MEM containing 0.5 mM IBMX at 20-24 hours after plating cells on the transfection plate. After 45 minutes of incubation, 40 µl of serial dilutions of test compounds in Opti-MEM+IBMX were added to the well. After 30-45 minutes of incubation at 37°C, media were aspirated, and the plates were put on dry ice before transferring to -80°C. After storing the plates at -80°C for at least two hours, the plates were removed from the freezer and thawed on bench. Cells were lysed in ELISA buffer containing 0.1% Tween-20, and the cell lysates were centrifuged to pellet the cell debris. The supernatants were examined with a cAMP ELISA kit (Cayman Item No. 501040).



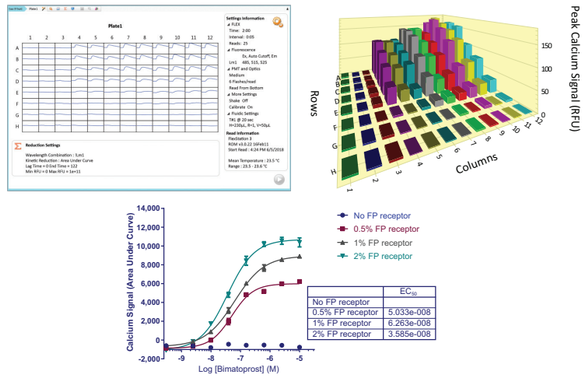
For the reporter assay, cells were plated in serum-free Opti-MEM and serial dilutions of test compounds were applied to the cells after they were seeded on the transfection plate for 20-24 hours before stimulation. Media samples were collected at 6-24 hours after stimulation and assayed for SEAP reporter activity using a luminescence substrate.



Results

Titration of FP cDNA in a Reverse Transfection Complex by Calcium Assay

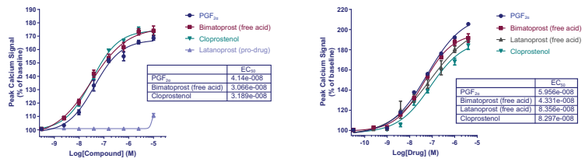
The expression level of FP receptors was titrated by diluting the expression plasmid in empty vector DNA for transfection. Transfection complexes with a fixed amount of total DNA, but different percentages of FP2A, were coated on TC-treated 96-well plates in triplicated columns using a proprietary protocol. Cells were added to the plates and stimulated with serial dilutions of a control agonist, bimatoprost (free acid), according to the calcium assay protocol. An optimal transfection complex was selected based on the robustness of assay and EC_{50} values.



	EC_{50}
No FP receptor	
0.5% FP receptor	5.033e-008
1% FP receptor	6.263e-008
2% FP receptor	3.585e-008

Validation of the Assay with Selected Agonists

Using the same transfection complex recipe, two batches of plates were produced separately. One plate from each batch was plated with different passages of HEK293T/17 cells on different days and was tested in the calcium mobilization assay with several known agonists serially diluted in slightly different schemes. The EC_{50} values obtained for $PGF_{2\alpha}$, bimatoprost (free acid), and cloprostenol are comparable between the two experiments.

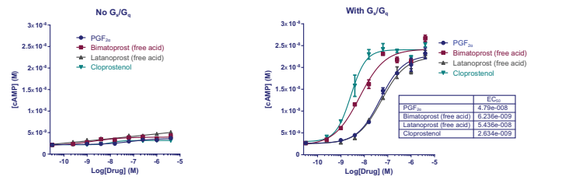


	EC_{50}
$PGF_{2\alpha}$	4.14e-008
Bimatoprost (free acid)	3.066e-008
Cloprostenol	3.189e-008

	EC_{50}
$PGF_{2\alpha}$	5.956e-008
Bimatoprost (free acid)	4.331e-008
Latanoprost (free acid)	8.356e-008
Cloprostenol	8.297e-008

Examining the Role of cAMP in FP Receptor Signaling Pathway

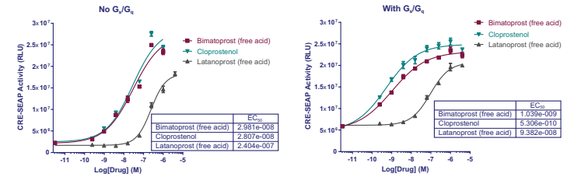
The same serial dilutions of FP receptor agonists were tested on an identical reverse transfection plate from the calcium mobilization assay above in a cAMP assay. With the endogenous $G\alpha_q$ signaling pathway, there are only marginal increases in cAMP levels upon activation of the FP receptor by these agonists. This indicates the activation of a CREB-regulated gene by the FP receptor cannot be mediated by the cAMP pathway directly. With the supplement of an expression plasmid for the G_s/G_q chimeric G protein α-subunit in the transfection complex, the originally $G\alpha_q$-coupled receptor activation was redirected to a $G\alpha_s$ signaling pathway, leading to drastic increases in cAMP. $PGF_{2\alpha}$ and latanoprost (free acid) exhibited EC_{50} values comparable to the calcium mobilization assays above, whereas cloprostenol and bimatoprost (free acid) shifted the dose-response curves to the left by around 1 to 1.5 orders of magnitude. This suggests the chimeric G protein may mimic the effect of EP_2/G_s pathway coactivation when the FP receptor is activated by certain agonists. In other words, the activation of the FP receptor may trigger both the $G\alpha_q$ and $G\alpha_s$ (through G_s/G_q) pathways and exhibits a synergistic effect in cAMP increase depending on the agonist.



	EC_{50}
$PGF_{2\alpha}$	4.79e-008
Bimatoprost (free acid)	6.226e-009
Latanoprost (free acid)	5.436e-008
Cloprostenol	2.634e-009

Downstream Signaling Activity Revealed by Reporter Assay

The same batch of plates examined in the calcium mobilization and cAMP assays above was used to show the downstream effect of FP receptor activity by SEAP reporter assay after overnight incubation with test compounds. In the reverse transfection plate without G_s/G_q chimeric G protein, both cloprostenol and bimatoprost (free acid) exhibited comparable EC_{50} values as in the calcium mobilization assay while latanoprost (free acid) showed a few-fold higher EC_{50}. This confirmed the activation of CREB even without a significant increase in cAMP level by FP receptor activation. With the supplement of G_s/G_q chimeric G protein, the FP receptor activation can be coupled to the cAMP/PKA/CREB pathway directly. Under this condition, the basal reporter activity was elevated presumably due to a constitutive activity by the expression of G_s/G_q chimeric G protein. In addition, the dose-response curves of bimatoprost (free acid) and cloprostenol shifted to the left by 1.5 orders of magnitude, while the latanoprost (free acid) exhibited a much smaller shift. These findings are consistent with the kinetic changes by G_s/G_q observed in the assays for secondary messengers in the upstream signaling pathways upon FP receptor activation above.

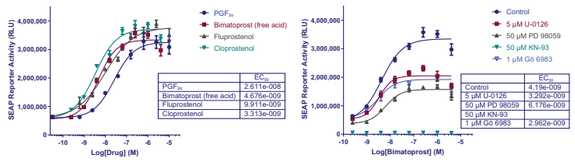


	EC_{50}
Bimatoprost (free acid)	2.981e-008
Cloprostenol	2.807e-008
Latanoprost (free acid)	2.404e-007

	EC_{50}
Bimatoprost (free acid)	1.039e-009
Cloprostenol	5.306e-010
Latanoprost (free acid)	9.382e-008

Dissecting the Signaling Pathway Leading to CREB Activation by the FP Receptor

To investigate the signaling pathway linking the FP/$G\alpha_q$ activation to CREB phosphorylation, inhibitors for MEK1/2, PKC, and CaMKII were examined in the reporter assay. To avoid the toxic effect of prolonged incubation with kinase inhibitors, a 6-hour (instead of 16-hour) stimulation protocol was used after longer post-transfection incubation. The new protocol was found to have lower EC_{50} values with several control agonists examined, while the relative potencies were not changed. Serial dilutions of bimatoprost (free acid) were added to the cells after pre-incubation with kinase inhibitors for 20 minutes. The CaMKII inhibitor KN-93 at 50 µM revealed severe cytotoxic effects and abolished the activity of the FP receptor. Both MEK1/2 inhibitors U-0126 (5 µM) and PD 98059 (50 µM) showed significant suppression of the FP receptor trigger reporter activity, while the latter also led to a decrease in basal reporter activity. The PKC inhibitor Gö 6983 (1 µM) produced a suppressive effect similar to that of the MEK1/2 inhibitors. With these MEK1/2 and PKC inhibitors, the EC_{50} of bimatoprost (free acid) was not affected. These findings confirmed the involvement of PKC and MEK1/2 in the signaling pathway connecting the FP receptor to the phosphorylation of CREB.



	EC_{50}
$PGF_{2\alpha}$	2.611e-008
Bimatoprost (free acid)	4.676e-009
Fluprostenol	9.911e-009
Cloprostenol	3.313e-009

	EC_{50}
Control	4.19e-009
5 µM U-0126	3.292e-009
50 µM PD 98059	6.176e-009
50 µM KN-93	
1 µM Gö 6983	2.962e-009

Conclusion and Discussion

1. A robust assay platform has been developed to study FP receptor activity.
2. The same reverse transfection plate can be used in calcium mobilization, cAMP, and reporter assay.
3. G_s/G_q chimeric G protein can efficiently couple the FP receptor to the $G\alpha_s$ signaling pathway and may mimic the effect of EP_2 co-stimulation.
4. FP receptor activation leads to phosphorylation of CREB through the PKC/MEK pathway without increasing intracellular cAMP levels.

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