Classification of prostaglandin receptors based on coupling to signal transduction systems

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A wide spectrum of prostaglandins (PG) stimulate both the production of cyclic AMP and an increase in free cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in the osteogenic osteosarcoma cell line, UMR-106-01, which has characteristics compatible with osteoblasts. Using PG-stimulated determinations of the second messengers cyclic AMP and $[\text{Ca}^{2+}]_i$, a method for classification of PG receptors is presented. UMR-106-01 cells demonstrate three subclasses of PG receptors. One receptor interacts with $\text{PGF}_{2\alpha}$, PGD_2 , and thromboxane B_2 (TxB_2) to increase $[\text{Ca}^{2+}]_i$. A second receptor binds PGE_2 , PGE_1 , PGI_2 , PGA_2 and 6-oxo- $\text{PGF}_{1\alpha}$ to increase $[\text{Ca}^{2+}]_i$ by stimulation of a second separate phospholipase C pool. A third receptor accepts PGE_2 , PGE_1 , PGA_2 , PGI_2 and to a lesser extent $\text{PGF}_{2\alpha}$, PGD_2 and TxB_2 to increase cyclic AMP. Such a classification system may be applicable to other cells responding to multiple PGs by inducing changes in cellular second messengers.

INTRODUCTION

Prostaglandins (PGs) are considered to be autocrine or paracrine hormones. They appear to bind to specific receptors [1–4], and their actions on a given cell depend on cell type, location, metabolic activity of the cell and the simultaneous actions on the cell of other humoral factors. It is clear that the same PGs can trigger opposite metabolic or physiological effects in different types of cells. Previous methods to classify types of PG receptors have relied on the assessment of a physiological function or action of a PG on specific tissues with unique profiles of responsiveness to the PGs [5]. Since PG antagonists for each of the naturally occurring PGs have not been available, and binding studies with PGs produce small signal-to-noise ratios that are exceedingly difficult to interpret, previous PG classification has been done by observing a physiological or metabolic response to varying doses of a number of different PGs. However, the observed responses of a given cell, tissue or organ may be the net result of a cascade of biochemical reactions distal to the actual binding of PGs to their receptors. Thus, the earliest membrane or intracellular change following the binding of the PG to its receptor may not be represented by the final cellular response. Furthermore, such a method of classification is not suitable for cells with more than one type of PG receptor, particularly when different PG receptors mediate the same biological functions. The rapidly propagating signal transduction systems that are activated immediately upon PG binding are intimately coupled to PG receptors, so that assessment of second messenger generation should reflect PG receptor binding. In the phenotypically osteoblastic osteosarcoma cell line UMR-106-01, PGs have previously been shown to stimu-

late the production of cyclic AMP and an increase in free cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) [6]. Based on measurements of the effect of PGs on second messenger levels, we now present a method for classification of receptors for those PGs stimulating an increase in $[\text{Ca}^{2+}]_i$ and/or cyclic AMP.

EXPERIMENTAL

UMR-106-01 cells were generously donated by Dr. T. J. Hahn, VAMC West Los Angeles, Los Angeles, CA, U.S.A. $\text{PGF}_{2\alpha}$, PGE_2 and 6-oxo- $\text{PGF}_{1\alpha}$ were obtained from Upjohn Pharmaceuticals (Kalamazoo, MI, U.S.A.). PGI_2 , PGE_1 , PGA_2 , PGD_2 and thromboxane B_2 (TxB_2) were obtained from Calbiochem–Behring (La Jolla, CA, U.S.A.). Fura 2/AM was obtained from Molecular Probes (Eugene, OR, U.S.A.). ^{125}I -labelled cyclic AMP (succinyltyrosine ester) and rabbit anti-(cyclic AMP) antibody were from ICN Radiochemicals (Irvine, CA, U.S.A.) and ICN Immune Biologicals (Lisle, IL, U.S.A.) respectively. UMR-106-01 cells were maintained in culture as previously described [6,7]. To measure $[\text{Ca}^{2+}]_i$, cell suspensions were loaded with Fura 2 and suspended in incubation solutions containing (in mM): NaCl, 140; KCl, 5; MgCl_2 , 1; CaCl_2 , 1.5; Hepes, 10 (pH 7.4 with Tris); sodium pyruvate, 10; glucose, 10; and 0.1% bovine serum albumin. Release of cells from tissue culture flasks, Fura 2 loading, fluorescence measurements and calibration of fluorescence signals to calculate $[\text{Ca}^{2+}]_i$ were as previously described [7].

Measurements of cellular cyclic AMP were done on UMR-106-01 cells grown on 48-well plates as previously described [6]. Incubation with or without hormones occurred in the above medium containing 0.2 mM-3-

Abbreviations used: PG, prostaglandin; $[\text{Ca}^{2+}]_i$, free cytosolic calcium concentration; Tx, thromboxane; PTH, parathyroid hormone; PLC, phospholipase C.

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isobutyl-1-methylxanthine. Incubation were for 5 min at 37 °C. Reactions were stopped by aspirating the incubation medium, washing the cells with phosphate-buffered saline (Sigma) and extracting cyclic AMP with 90 % n-propanol. Propanol was driven off by a stream of N₂. Residues were reconstituted with sodium acetate buffer (pH 6.2), and cyclic AMP was assayed by radioimmunoassay as previously described [6,7].

RESULTS AND DISCUSSION

The stimulation of a rise in $[Ca^{2+}]_i$ in UMR-106-01 cells by PGs has previously been shown ([6,7a]. It was determined that the potency rank for a PG-stimulated increase in $[Ca^{2+}]_i$ was $F_{2\alpha} > D_2 > E_2 > TxB_2 > E_1 > I_2 > A_2 > 6\text{-oxo-F}_{1\alpha}$. The increase in $[Ca^{2+}]_i$ caused by the PGs occurs via release of Ca^{2+} from intracellular stores. We have shown that the phosphoinositide cycle is stimulated by PGs and that the increase in $Ins(1,4,5)P_3$ levels is related to a release of Ca^{2+} from intracellular stores [6]. For a given receptor coupled to the phospholipase C (PLC) signal transduction system, maximum doses of an agonist should maximally stimulate PLC coupled to this receptor to generate $Ins(1,4,5)P_3$, which triggers the release of Ca^{2+} from intracellular stores. If a second agonist is also able to bind to the same receptor as the first agonist, occupancy of the receptor by the first agonist would prevent further stimulation of the signal transduction system and prevent any further increase in $[Ca^{2+}]_i$. If the second agonist binds to a separate receptor coupled to its own cellular pool of

PLC, then one would expect a further release of Ca^{2+} from intracellular stores. To explore the possibility of PG binding to more than one type of receptor, we stimulated cells sequentially or simultaneously with two different PGs and measured their response as an increase in $[Ca^{2+}]_i$.

Fig. 1 shows that following maximal stimulation with $PGF_{2\alpha}$, PGD_2 or TxB_2 did not further increase $[Ca^{2+}]_i$. Stimulation with PGI_2 , PGE_2 , PGE_1 , PGA_2 or 6-oxo- $PGF_{1\alpha}$ did result in a second peak increase in $[Ca^{2+}]_i$ following stimulation by a maximal dose of $PGF_{2\alpha}$. Similarly, maximal doses of $PGF_{2\alpha}$ or TxB_2 following maximal increases in PGD_2 -stimulated $[Ca^{2+}]_i$ had little or no further effect on $[Ca^{2+}]_i$ (Fig. 2). Prestimulation with either PGD_2 (Fig. 2) or TxB_2 (results not shown), followed by stimulation with PGE_2 , PGE_1 , PGI_2 or PGA_2 , resulted in a second peak in $[Ca^{2+}]_i$. The results depicted in Figs. 1 and 2 strongly imply that PGE_2 , PGE_1 , PGI_2 , PGA_2 and 6-oxo- $PGF_{1\alpha}$ do not bind to the same receptor as do $PGF_{2\alpha}$, PGD_2 and TxB_2 , while $PGF_{2\alpha}$, PGD_2 and TxB_2 each bind to the same receptor in UMR-106-01 cells.

Cells initially stimulated with a concentration of PGE_2 that resulted in a maximal increase in $[Ca^{2+}]_i$ gave no further rise in $[Ca^{2+}]_i$ when subsequently stimulated with maximal doses of PGE_2 , PGE_1 , PGI_2 , PGA_2 or 6-oxo- $PGF_{1\alpha}$ (Fig. 3). Similarly, pretreatment of the cells with PGE_1 followed by the addition of PGE_2 , PGE_1 , PGI_2 or PGA_2 resulted in minimal or no subsequent increase in $[Ca^{2+}]_i$ (Fig. 2). Addition of $PGF_{2\alpha}$, PGD_2 or TxB_2 to cells pretreated with a maximal dose of PGE_2 (Fig. 3),

Fig. 1. Effect of prostaglandins on $[Ca^{2+}]_i$ of $PGF_{2\alpha}$ -stimulated cells

Fura 2-loaded cells were maintained at 37 °C under continuous stirring. Where indicated, the cells were initially stimulated with 1 μM - $PGF_{2\alpha}$. When $[Ca^{2+}]_i$ was decreased to near basal levels, the cells were exposed to the indicated concentration of the different prostaglandins. Each tracing showing serial stimulation by $PGF_{2\alpha}$, followed by another PG, is representative of at least three other experiments.

Fig. 2. Effect of prostaglandins on $[Ca^{2+}]_i$ of PGD_2 - or PGE_1 -stimulated cells

Cells were stimulated with $10 \mu M$ - PGD_2 or $25 \mu M$ - PGE_1 prior to a second stimulation with the indicated concentrations of the prostaglandins. Each tracing is representative of two or three similar experiments.

Fig. 3. Effects of prostaglandins on $[Ca^{2+}]_i$ of PGE_2 -stimulated cells

Fura 2-loaded cells, stirred and maintained at $37^\circ C$, were prestimulated with $10 \mu M$ - PGE_2 prior to a second stimulation with the indicated concentrations of the prostaglandins. Each tracing showing serial stimulation by PGE_2 followed by another PG is representative of at least three other experiments.

Fig. 4. Effect of ionomycin on $[Ca^{2+}]_i$.

Fura 2-loaded cells were suspended in albumin- and Ca^{2+} -free solution A. Then the cells were exposed to ionomycin (IM) prior to or after stimulation with the indicated concentration of the different prostaglandins.

PGE_1 (Fig. 2) or PGI_2 (results not shown) resulted in a further increase in $[Ca^{2+}]_i$.

Fig. 4 shows that the absence of a further rise in $[Ca^{2+}]_i$ was not due to depletion of a common intracellular Ca^{2+} pool. This was tested by the addition of ionomycin to cells following stimulation by PG in Ca^{2+} -free medium. Ionomycin was able to release further Ca^{2+} from intracellular stores even after initial PG stimulation. Furthermore, the effect of ionomycin on $[Ca^{2+}]_i$ was reduced by prestimulation with PGs as expected from PG mediated Ca^{2+} release from intracellular stores [6].

An additional protocol for testing the relationship between different PGs acting on the same cell is shown in Fig. 5. In these experiments, the effect of individual PGs on $[Ca^{2+}]_i$ was compared with the effect of different PG combinations added simultaneously. The combination of maximal concentrations of $PGF_{2\alpha}$ with PGD_2 or TxB_2 , and PGD_2 with TxB_2 , increased $[Ca^{2+}]_i$ to the same levels as those evoked by the individual PGs. The combination of $PGF_{2\alpha}$ with PGE_2 , PGE_1 or PGI_2 and of PGD_2 with PGE_2 , PGE_1 or PGI_2 increased $[Ca^{2+}]_i$ to higher levels than those evoked by the individual PGs. Finally, no increased response was observed with the combinations of PGE_2 with PGI_2 or PGE_1 , or of PGE_1 with PGI_2 (Fig. 5). These data are consistent with a common receptor for PGE_2 , PGI_2 , PGE_1 and PGA_2

coupled to a PLC pool which is separate from that coupled to a common receptor for $PGF_{2\alpha}$, PGD_2 and TxB_2 .

The potency rank for PG-evoked $[Ca^{2+}]_i$ increases in osteoblast-like cells is similar to that reported for PG-mediated contraction of dog and cat iris smooth muscle cells, and was suggested to reflect binding of PGs to a single type of receptor termed FP [5,8]. The PG-induced contraction of smooth muscle derived from guinea pig ileum or fundus, chick ileum [9], dog fundus [5] or cat trachea [10] had a similar PG potency rank. On the basis of such findings, it was suggested that in these latter tissues, all the PGs bind to a single type of receptor, termed EP [5]. A similar approach was used to identify other types of PG receptors. Thus, based on rank potency of PGs on cellular activities, the presence of specific receptors for each naturally occurring PG has been proposed [5]. Furthermore, it was concluded that all of the PGs can interact with varying specificities with any of these classes of receptors.

Our results show that a similar potency rank to that obtained in previous studies [5,8] reflects the binding of PGs to two separate receptors, both of which result in an increase in $[Ca^{2+}]_i$ in osteoblast-like cells. Thus determination of cellular activity tightly coupled to the PG receptors, such as the PLC signal transduction system, is a preferable method in classification of PG receptors. This latter approach clearly differentiates between different PG receptors coupled to a similar signal transduction system. The resolution of these PG receptors, particularly when expressed in the same cells, would be impossible on the basis of potency rank alone.

In addition to stimulating an increase in $[Ca^{2+}]_i$, some of the PGs stimulate an increase in cellular cyclic AMP [1,6,11,12]. Cyclic AMP is another second messenger whose signal transduction system is intimately coupled to PG receptors. We have found that the potency rank for PG-stimulated cyclic AMP generation in osteoblast-like cells is $E_2 > E_1 > A_2 > I_2 \gg F_{2\alpha} > D_2 > TxB_2$ [7a]. This potency rank for cyclic AMP generation is completely different from the potency rank for the increase in $[Ca^{2+}]_i$ stimulated by these same PGs. This observation suggests that the PLC and adenylate cyclase transduction systems are coupled to different PG receptors.

The same approach as that used for the classification of PG receptors coupled to PLC was used to classify PG receptors coupled to adenylate cyclase. Table 1 summarizes the ability of individual PGs or PGs in combination with other PGs or with parathyroid hormone (PTH) to stimulate cyclic AMP production. PGE_2 , PGE_1 , PGA_2 and PGI_2 alone significantly stimulated cyclic AMP production in UMR-106-01 cells. However, when either PGE_2 or PGE_1 was used in combination with PGE_1 , PGA_2 or PGI_2 to stimulate the cells, no additivity of cyclic AMP generation was observed. The data are consistent with a single type of adenylate cyclase-coupled PG receptor.

To demonstrate that individual PGs do not activate the entire cellular adenylate cyclase pool and to show that the adenylate cyclase-coupled PG receptor is distinct from other receptors coupled to different pools of adenylate cyclase, studies combining the stimulation of cyclic AMP production by PTH and the PGs were done. PTH binds to specific receptors in bone cells and can also stimulate an increase in $[Ca^{2+}]_i$ and cyclic AMP [7]. Doses of PGs and PTH that maximally stimulate cyclic

Fig. 5. Effect of prostaglandins alone and in combination on $[Ca^{2+}]_i$

Cells were stimulated with the indicated concentrations of the individual or combination of prostaglandins and $[Ca^{2+}]_i$ was estimated from the change in the fluorescence signal.

Table 1. Effects of prostaglandins and PTH alone and in combination on cyclic AMP levels in osteoblasts

UMR-106-01 cells in 48-multiwell plates were incubated with 0.2 mM-3-isobutyl-1-methylxanthine, the indicated concentrations of the different prostaglandins, PTH, or a combination of prostaglandins and PTH for 5 min at 37 °C. At the end of the incubations, the reaction was terminated and cyclic AMP was extracted by removal of the medium and addition of 0.5 ml of *n*-propanol. The propanol extract was removed to clean tubes and evaporated under a stream of N_2 . The dry extract was reconstituted in 0.1 ml of sodium acetate buffer, pH 6.2. Assay of cyclic AMP was done by radioimmunoassay. Means $\pm$ S.D. of six replicates are given.

Prostaglandins (μ M)	Cyclic AMP formed (p mol/well)	
	– PTH	+ PTH (10^{-8} M)
Control	1.03 $\pm$ 0.25	31.0 $\pm$ 0.9
E_2 (1.0)	29.0 $\pm$ 2	67.3 $\pm$ 2
E_1 (2.5)	23.3 $\pm$ 1.5	55.5 $\pm$ 3.2
A_2 (10)	8.56 $\pm$ 0.15	40.6 $\pm$ 1.8
I_2 (25)	4.06 $\pm$ 0.41	37.1 $\pm$ 1.2
E_2 (1.0) + E_1 (2.5)	27.5 $\pm$ 1.75	
E_2 (1.0) + A_2 (10)	28.6 $\pm$ 1.6	
E_2 (1.0) + I_2 (25)	28.8 $\pm$ 1.4	
E_1 (2.5) + A_2 (10)	23.6 $\pm$ 0.57	
E_1 (2.5) + I_2 (25)	23.5 $\pm$ 0.88	

AMP production individually are additive on cyclic AMP stimulation when added simultaneously to cells. Thus the results suggest that the PGs bind to a single type of receptor coupled to a specific adenylate cyclase pool in UMR-106-01 cells.

Since the same type of PGs increase both $[Ca^{2+}]_i$ and cyclic AMP and with a similar potency rank, it was necessary to obtain evidence that PG-mediated cyclic AMP and $[Ca^{2+}]_i$ increases result from binding of the PGs to separate types of receptors. This was achieved by studying the properties of desensitization of the cyclic AMP and Ca^{2+} signals. Preincubating cells with 0.25 μ M- PGE_2 desensitized the cells to cyclic AMP production after restimulation with PGE_2 , PGE_1 , PGA_2 or PGI_2 (Fig. 6a). Similarly, preincubation with low doses of PGE_1 , PGA_2 or PGI_2 prior to stimulation with PGE_2 , PGE_1 , PGA_2 or PGI_2 also resulted in desensitization of the cyclic AMP response (results now shown). However, cells pretreated with 0.25 μ M- PGE_2 for 24 h responded to 10 μ M- PGE_2 by an increase in $[Ca^{2+}]_i$ (Fig. 6b). Pretreatment with 0.25 μ M- PGE_2 also did not inhibit the increase in $[Ca^{2+}]_i$ stimulated by maximal doses of PGE_1 , PGA_2 or PGI_2 (results not shown). Thus these results provide additional evidence that all of the PGs tested which stimulate cyclic AMP generation in UMR-106-01 cells appear to bind to a common receptor linked to one adenylate cyclase transduction system, since heterologous desensitization among PGs stimulating cyclic AMP is apparent. Furthermore, these results provide evidence

Fig. 6. Desensitization of the prostaglandin-mediated increase in cyclic AMP but not in $[Ca^{2+}]_i$

UMR-106-01 cells were preincubated in serum-free medium containing $0.25 \mu M$ -PGE₂ (closed symbols) or vehicle (controls, open symbols) for 24 h. (a) The ability of vehicle, PGE₂ ($1 \mu M$) (○, ●), PGE₁ ($2.5 \mu M$) (△, ▲), PGA₂ ($10 \mu M$) (□, ■) and PGI₂ ($25 \mu M$) (◇, ◆) to increase cyclic AMP was then measured. (b) UMR-106-01 cells preincubated with vehicle (1) or $0.25 \mu M$ -PGE₂ (2) as above were used to measure the effect of $10 \mu M$ -PGE₂ on $[Ca^{2+}]_i$. Each point represents the mean $\pm$ S.D. of two experiments performed in triplicate.

that the adenylate cyclase-linked PG receptors are distinct from the PLC-linked PG receptors.

In summary, we have utilized the PLC/ Ca^{2+} and adenylate cyclase/cyclic AMP signal transduction systems to classify PG receptors coupled to these two transduction systems. We conclude that this method of receptor classification is more refined and conclusive

than the rank order potency technique for the differentiation of PG receptors. Such an approach could aid the classification of PG receptors in other cells to facilitate the study of signal transduction by PGs and to allow a better understanding of the effect of different PGs on cellular activities. Based on the receptor-transduction method of receptor classification, we define three subclasses of PG receptors: a PLC/ Ca^{2+} -linked receptor accepting PGF_{2 α} , PGD₂ and TxB₂; a PLC/ Ca^{2+} -linked receptor accepting PGE₂, PGE₁, PGA₂, PGI₂ and 6-oxo-PGF_{1 α} ; and an adenylate cyclase-linked receptor accepting PGE₂, PGE₁, PGA₂ and PGI₂.

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