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Prostaglandin Receptors in NIH 3T3 Cells: Coupling of One Receptor to Adenylate Cyclase and of a Second Receptor to Phospholipase C

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SUMMARY

In intact NIH 3T3 murine fibroblasts, prostaglandins (PGs) $F_{2\alpha}$ and E_2 induce dose-dependent stimulation of inositol monophosphate generation. $PGF_{2\alpha}$ is >50-fold more potent than PGE_2 in eliciting this response. In streptolysin O-permeabilized NIH 3T3 cells, $PGF_{2\alpha}$ and PGE_2 induced dose-dependent accumulations of inositol bis- and trisphosphates, which were dependent on the presence of the guanine nucleotide guanosine-5′-*O*-(3-thio)triphosphate (GTPγS) (10 μM). Pretreatment of cells for 16 hr with 100 nM $PGF_{2\alpha}$ resulted in a significant reduction of not only subsequent $PGF_{2\alpha}$- and PGE_2-induced but also GTPγS-induced stimulation of inositol phosphate formation in permeabilized cells. $PGF_{2\alpha}$-induced accumulation of inositol phosphates was partially inhibited by pretreatment with pertussis toxin (1 μg/ml, 4 hr). The inhibition by pertussis toxin was small but was not related to cyclic AMP formation, because forskolin, which activates adenylate cyclase, did not mimic pertussis toxin-induced inhibition. In the same cell line, $PGF_{2\alpha}$ and PGE_2 induced a dose-dependent accumulation of cAMP and a dose-dependent potentiation of 0.5 μM forskolin-stimulated cAMP formation. $PGF_{2\alpha}$ and PGE_2 were almost equipotent in eliciting both responses. However, $PGF_{2\alpha}$ was less efficacious than PGE_2 and, in the presence of forskolin, $PGF_{2\alpha}$ at 10 μM induced an inhibitory effect on cAMP accumulation. Such inhibition may be related to $PGF_{2\alpha}$-mediated phospholipase C activation and subsequent stimulation of protein kinase C, because the phorbol ester phorbol 12-myristate-13-acetate, which directly activates protein kinase C, also inhibited forskolin- and PGE_2-induced cAMP accumulation. Pretreatment with $PGF_{2\alpha}$ for 16 hr did not reduce subsequent stimulation of cAMP accumulation by $PGF_{2\alpha}$ or PGE_2. The results indicate that in NIH 3T3 cells two receptors for PGs are present, one that couples to adenylate cyclase, probably through G_s, and does not exhibit selectivity between $PGF_{2\alpha}$ and PGE_2 and a second receptor that couples to phospholipase C through a guanine nucleotide-binding protein that is not sensitive to pertussis toxin pretreatment. The latter shows at least 40-fold selectivity towards $PGF_{2\alpha}$ over PGE_2. Because long treatment with $PGF_{2\alpha}$ resulted in desensitization of the GTPγS-induced response, it is possible that long exposure to $PGF_{2\alpha}$ may down-regulate the guanine nucleotide-binding protein involved in phospholipase C signal transduction.

PGs mediate various physiological phenomena, including inflammation, smooth muscle contraction and relaxation, and platelet aggregation. Such actions are attained through the interaction of PGs at specific receptors (1). PGEs stimulate the generation of the second messenger cAMP in a variety of cells. Such stimulation is mediated by a receptor coupled to the enzyme adenylate cyclase through a G protein, G_s. PG receptors can also stimulate another second messenger-generating system, namely the hormone- and neurotransmitter-sensitive phospholipase C. In bovine luteal cells, $PGF_{2\alpha}$ induces phosphoinositide breakdown (2), which results in the formation of the second messengers IP_3 and diacylglycerol. In bovine chromaffin cells, PGEs and $PGF_{2\alpha}$ elicit both phosphoinositide breakdown and cAMP formation (3). However, neither of these actions seems to account for PG-mediated catecholamine release (3). Another study suggests that only the PGEs activate phosphoinositide breakdown in bovine chromaffin cells and that activation of phosphoinositide breakdown does mediate catecholamine release (4).

In NIH 3T3 cells, we report evidence for the presence of two different receptors for PGs. They can be differentiated by their selectivity towards PGE_2 and $PGF_{2\alpha}$ and by the second messenger system to which they couple.

Experimental Procedures

Materials. $PGF_{2\alpha}$ and GTPγS were from Fluka. PGE_2 was from Sigma (St. Louis, MO). [^{3}H]Inositol (specific activity, 14 Ci/mmol) was

ABBREVIATIONS: PG, prostaglandin; PMA, phorbol 12-myristate-13-acetate; IP_1, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate; G protein, guanine nucleotide-binding protein; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

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from DuPont (Boston, MA). The cAMP kit was from Amersham (Arlington Heights, IL).

Cell culture. NIH 3T3 murine fibroblasts were cultured in Dulbecco's modified Eagle's medium (GIBCO, Gaithersburg, MD) containing 10% calf serum (ABI, Rockville, MD) and antibiotics.

Phosphoinositide breakdown in NIH 3T3 cells. NIH 3T3 cells were subcultured in 12-well plates, at a density of 5×10^5 cells/well, in the presence of 10 μCi/ml [^{3}H]inositol. The following day, medium was aspirated, cells were washed twice with 1 ml of incubation buffer (118 mM NaCl, 4.7 mM KCl, 10 mM LiCl, 1.2 mM $MgSO_4$, 0.5 mM EDTA, 10 mM glucose, 20 mM HEPES, pH 7.4), and cells were preincubated for 10 min at 37° in incubation buffer containing 3 mM $CaCl_2$. Then agents were added at the indicated concentrations, and incubations were carried out for 15 min at 37°, in a final volume of 500 μl. Incubations were stopped by addition of 250 μl of 12% trichloroacetic acid. Cells were scraped from the bottom of the wells with a rubber policeman, transferred to microfuge tubes, and centrifuged for 2 min at 12,000 × *g*. [^{3}H]IP_1 was analyzed in the supernatant by anion exchange chromatography (5).

Phosphoinositide breakdown in permeabilized NIH 3T3 cells. NIH 3T3 cells were subcultured in 12-well plates and labeled with [^{3}H] inositol as indicated above. The following day, medium was aspirated and cells were washed twice with 1 ml of incubation buffer containing no calcium. Then 500 μl of permeabilization buffer were added (incubation buffer containing 200 μM ATP, 7 μM $CaCl_2$, and 0.4 unit/ml streptolysin O) (calculated concentration of free calcium, 100 nM). After 10 min at 37°, agents were added at the indicated concentrations, and incubations were carried out for 15 min or for the indicated times. Incubations were terminated by the addition of 250 μl of 12% trichloroacetic acid, as indicated for whole-cell experiments (see above).

ADP-ribosylation of G proteins in NIH 3T3 cells. NIH 3T3 cells were treated for 4 hr with pertussis toxin (1 μg/ml) at 37° in Dulbecco's modified Eagle's medium. Cells were then used for phosphoinositide breakdown (previously labeled with [^{3}H]inositol) or for *in vitro* ADP-ribosylation, as follows. Cells were washed twice with phosphate-buffered saline and harvested. After a low speed (500 × *g*) centrifugation (5 min), cells were homogenized in 1 ml of lysing buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin) by forcing of the cell suspension through a 22-gauge syringe needle. The suspension was centrifuged at 500 × *g* for 5 min, and the resultant supernatant was centrifuged at 100,000 × *g*, in a Beckman Airfuge, for 1 hr at 4°. Membrane pellets were suspended in incubation buffer (50 mM Tris, pH 7.5, 4 mM EDTA, 10 mM $MgSO_4$, 40 mM KH_2PO_4). Incubations contained 1.3 mM ATP, 6.7 mM thymidine, 6.7 μM GTP, 3.3 μM NAD containing 10^6 cpm/10 μl [^{32}P]NAD, 1 μg/ml pertussis toxin where indicated, and 100 μg of membrane protein, in a final volume of 150 μl. They were carried out at 30° for 1 hr. At the end of the incubation, membranes were centrifuged at 12,000 × *g* for 10 min, the supernatants were discarded, and the pellets were dissolved in loading buffer, heated at 95° for 5 min, and run on sodium dodecyl sulfate gels containing 12% acrylamide. After completion of electrophoresis, gels were dried and exposed to X-ray film for 18 hr.

cAMP determinations in NIH 3T3 cells. NIH 3T3 cells were subcultured in 24-well plates at a density of 5×10^5 cells/well. The following day, medium was aspirated and cells were washed with incubation buffer and preincubated in incubation buffer containing 3 mM calcium and 30 μM rolipram, a phosphodiesterase inhibitor, for 10 min at 37°. Agents were then added at the indicated concentrations. Incubations were carried out for 15 min at 37° and stopped by aspiration of the incubation buffer and addition of 500 μl of 0.1 N HCl. After 30 min at room temperature, the HCl was removed, the reaction was neutralized, and cAMP concentration was determined in a 50-μl aliquot, using a commercial kit.

Results

$PGF_{2\alpha}$ and PGE_2 induced the formation of [^{3}H]IP_1 in NIH 3T3 cells in a dose-dependent manner. EC_{50} values were 200 nM for $PGF_{2\alpha}$ and >10 μM for PGE_2 (Fig. 1). Because these experiments were performed in whole cells in the presence of LiCl, stimulation of phospholipase C under these conditions results in accumulation of IP_1 (5).

NIH 3T3 cells can be permeabilized with streptolysin O, as reported for other cell types (6, 7). Such a permeabilization procedure allows for the introduction of agents in the cytosolic compartment of cells. Phospholipase C activation in permeabilized cells results in accumulation of IP_2 and IP_3, because the enzyme that dephosphorylates IP_2 is cytosolic (8) and the activity is highly diluted during the permeabilization procedure. GTPγS, a nonhydrolyzable analog of GTP, induced a significant stimulation of [^{3}H]IP_2 plus [^{3}H]IP_3 in permeabilized NIH 3T3 cells (Fig. 2). GTPγS-mediated stimulation of [^{3}H]IP_2 plus [^{3}H]IP_3 formation was potentiated in a dose-dependent manner by both $PGF_{2\alpha}$ and PGE_1 (Fig. 2). The EC_{50} values for $PGF_{2\alpha}$ and PGE_2 in increasing GTPγS-mediated stimulation were 20 and 800 nM, respectively (Fig. 2). In the absence of GTPγS, $PGF_{2\alpha}$ and PGE_2 had no effect on stimulation of [^{3}H]IP_2 plus [^{3}H]IP_3 formation (Fig. 3).

Overnight treatment (16 hr) of NIH 3T3 cells with 100 nM $PGF_{2\alpha}$ resulted in a significant inhibition of subsequent stimulation with GTPγS (10 μM), $PGF_{2\alpha}$ (1 μM) plus GTPγS, or PGE_2 (1 μM) plus GTPγS, in permeabilized cells (Table 1).

Pretreatment of NIH 3T3 cells with pertussis toxin (1 μg/ml, 4 hr), which results in almost complete inhibition of subsequent *in vitro* ADP-ribosylation of G proteins, induced a small inhibition of $PGF_{2\alpha}$-mediated effects on [^{3}H]IP_2 plus [^{3}H] IP_3 formation (Fig. 4). Pretreatment of cells with forskolin plus rolipram, a phosphodiesterase inhibitor that, similar to pertussis toxin, would result in an increase in cAMP, did not affect GTPγS or $PGF_{2\alpha}$ plus GTPγS effects on [^{3}H]IP_2 plus [^{3}H]IP_3

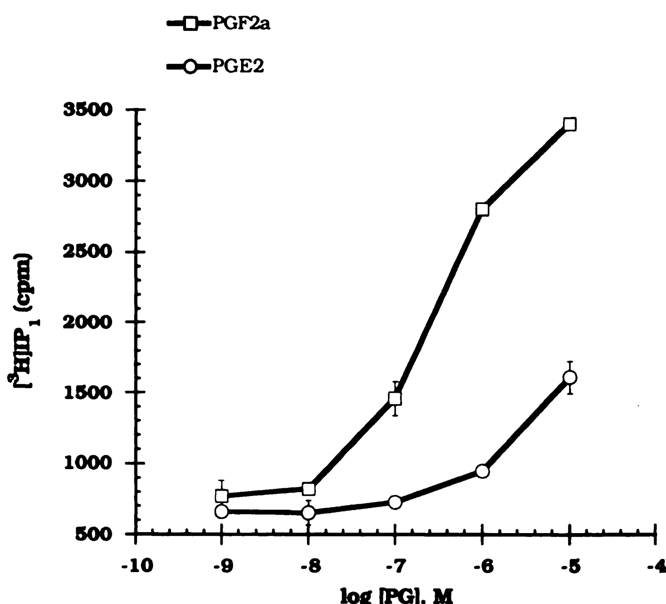

Fig. 1. Dose-dependent stimulation of [^{3}H]IP_1 formation in NIH 3T3 cells by $PGF_{2\alpha}$ and PGE_2. Cells were incubated overnight in medium containing [^{3}H]inositol. After washing and preincubation with buffer, cells were incubated with the indicated concentrations of $PGF_{2\alpha}$ (□) or PGE_2 (○), for 15 min. [^{3}H]IP_1 was extracted and analyzed as described in Experimental Procedures. Data correspond to a representative experiment performed in duplicate (mean ± range), which was repeated three times with similar results. Control value for unstimulated cells was 808 ± 44 cpm.

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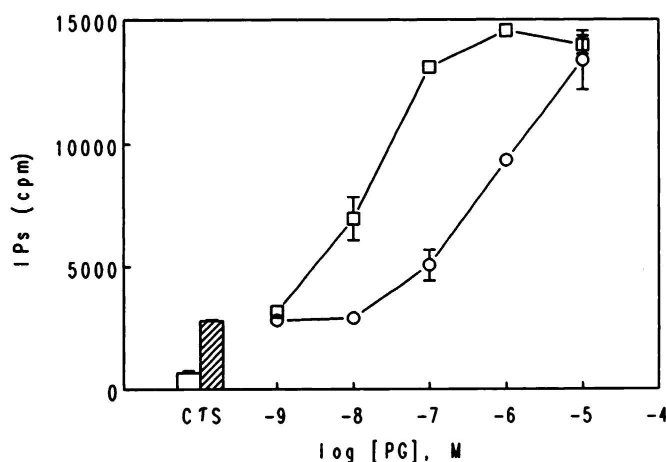

Fig. 2. Dose-dependent potentiation of GTPγS-stimulated [^{3}H]IP$_2$ plus [^{3}H]IP$_3$ formation in permeabilized NIH 3T3 cells. [^{3}H]Inositol-labeled cells were permeabilized with streptolysin O-containing buffer. After 10 min at 37°, GTPγS (10 μM) or GTPγS plus the indicated concentrations of PGF$_{2\alpha}$ (□) or PGE$_2$ (○) were added, and incubations were carried out for 15 min. [^{3}H]IP$_2$ plus [^{3}H]IP$_3$ were extracted and analyzed as described in Experimental Procedures. □, Control (C); ▨, 10 μM GTPγS (γS). Data correspond to a representative experiment performed in duplicate (mean ± range), which was repeated three times with similar results.

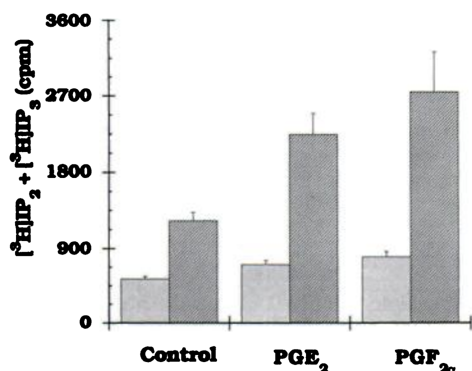

Fig. 3. PG induced [^{3}H]IP$_2$ plus [^{3}H]IP$_3$ formation in permeabilized NIH 3T3 cells only in the presence of GTPγS. [^{3}H]Inositol-labeled cells were permeabilized with streptolysin O-containing buffer. After 10 min at 37°, PGE$_2$ or PGF$_{2\alpha}$, at 10 μM, alone (□) or in combination with 10 μM GTPγS (▨), was added, and incubations were carried out for 15 min. [^{3}H]IP$_2$ plus [^{3}H]IP$_3$ were extracted and analyzed as described in Experimental Procedures. Data correspond to the means ± SEM of three independent experiments performed in triplicate.

formation (Fig. 5). Rolipram at the concentration used (30 μM) did not alter either basal, GTPγS-stimulated, or GTPγS- plus PGF$_{2\alpha}$-stimulated [^{3}H]IP$_2$ plus [^{3}H]IP$_3$ formation (data not shown).

PGF$_{2\alpha}$ and PGE$_2$ induced an accumulation of cAMP in NIH 3T3 cells (Fig. 6). EC$_{50}$ values for this response were 100 nM for PGF$_{2\alpha}$ and 300 nM for PGE$_2$. Incubation times and buffer were the same as for phosphoinositide breakdown experiments, except for the addition of 30 μM rolipram, a phosphodiesterase inhibitor. In the presence of 0.5 μM levels of the diterpene forskolin, which directly activates adenylate cyclase (9), similar dose dependencies for PGF$_{2\alpha}$ and PGE$_2$ were obtained (Fig. 7); however, in the case of PGE$_2$ the potency could not be accurately determined, due to the fact that a maximum response was not attained even at the highest concentration tested (Fig. 7). In both series of experiments (in the presence and absence of forskolin), PGF$_{2\alpha}$-induced accumulation of cAMP reached

TABLE 1

Effects of overnight treatment with PGF$_{2\alpha}$ on GTPγS- and PGF$_{2\alpha}$-induced phosphoinositide breakdown in permeabilized NIH 3T3 cells

Cells were labeled overnight with [^{3}H]inositol in the presence (PGF$_{2\alpha}$) or absence (No pretreatment) of 100 nM PGF$_{2\alpha}$. Cells were washed and permeabilized with streptolysin O-containing buffer. After 10 min, agents (GTPγS and PGF$_{2\alpha}$) were added and incubations were carried out for 15 min at 37°. [^{3}H]IP$_2$ plus [^{3}H]IP$_3$ were extracted and analyzed as described in Experimental Procedures. Control values were 1423 ± 20 cpm for cells with no pretreatment and 1858 ± 64 cpm for PGF$_{2\alpha}$-treated cells. Values are shown as means ± SEM of percentage of control values. Data correspond to the means ± SEM of three independent experiments performed in duplicate.

Treatment	[^{3}H]IP$_2$ + [^{3}H]IP$_3$	
	No pretreatment	PGF$_{2\alpha}$
	% of control	
GTPγS (10 μM)	352 ± 17	255 ± 23^a
PGF$_{2\alpha}$ (1 μM) + GTPγS	983 ± 48	561 ± 65^b
PGE$_2$ (1 μM) + GTPγS	747 ± 38	419 ± 35^b

^{a} $p < 0.05$.
^{b} $p < 0.01$.

lower maximal values than PGE$_2$-induced accumulation. Moreover, in the presence of forskolin an inhibitory effect was observed on cAMP accumulation with 10 μM PGF$_{2\alpha}$ (Fig. 7). Overnight treatment (16 hr) of NIH 3T3 cells with 100 nM PGF$_{2\alpha}$ resulted in no significant effect on subsequent stimulation of cAMP formation with PGE$_2$ (1 μM) or PGF$_{2\alpha}$ (1 μM) (Table 2). The protein kinase C activator PMA induced a significant inhibition of forskolin- and forskolin- plus PGE$_2$-induced but not PGF$_{2\alpha}$-induced stimulation of cAMP formation (Fig. 8).

Discussion

PGEs stimulate adenylate cyclase in a variety of cells. PGEs and PGFs can also activate the phospholipase C system in some cells (2–4, 10). These actions are known, for other neurotransmitters and hormones, to be mediated by receptors coupled to G proteins. G proteins are heterotrimeric proteins that serve as mediators for signal transduction, in a variety of systems, between receptors and effectors (11, 12). Mechanistically, it has been proposed that, upon binding of an agonist, the activated receptor induces the exchange of GDP for GTP on the G protein α subunit, causing dissociation of the α subunit from a βγ complex; only then does interaction of the α subunit with the effector occur (11). Although the G protein involved in phospholipase C signal transduction has not been fully characterized, numerous examples have been reported of a guanine nucleotide requirement in receptor activation of phospholipase C in cell-free preparations (13, 14). Recently, G proteins from liver and brain membranes have been purified and determined to be responsible for signal transduction with phospholipase C (15, 16).

PGF$_{2\alpha}$ induced phosphoinositide breakdown in NIH 3T3 cells (15) (Fig. 1). PGE$_2$ was less potent than PGF$_{2\alpha}$ (Fig. 1). The stimulation of phosphoinositide breakdown by PGs was examined in permeabilized NIH 3T3 cells. GTPγS induced a significant stimulation of [^{3}H]IP$_2$ plus [^{3}H]IP$_3$ formation (Fig. 2). Such stimulation was enhanced, in a dose-dependent manner, by PGF$_{2\alpha}$ and PGE$_2$ (Fig. 2), suggesting that upon activation a PG receptor induces the exchange of GDP for GTP, or GTPγS, on a G protein α subunit, leading to activation of phospholipase C. The potencies for stimulation were higher than those ob-

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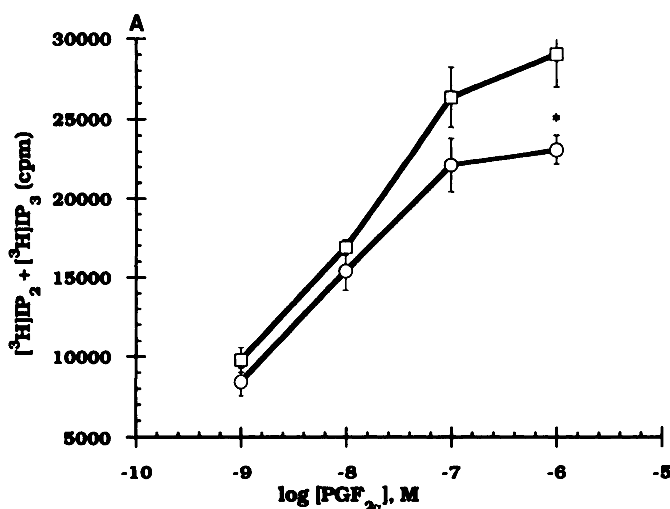

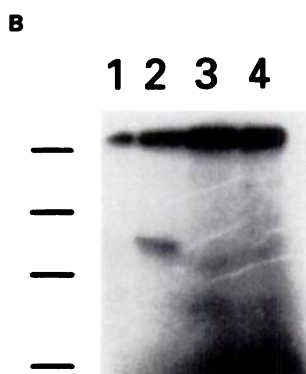

Fig. 4. A, Pertussis toxin effects on $PGF_{2\alpha}$-mediated stimulation of $[^3H]IP_2$ plus $[^3H]IP_3$ formation in permeabilized NIH 3T3 cells. $[^3H]$Inositol-labeled cells were treated with pertussis toxin (1 μg/ml) (○) or regular medium (□) for 4 hr at 37°. After permeabilization, cells were treated with the indicated concentrations of $PGF_{2\alpha}$ in the presence of 10 μM GTPγS. $[^3H]IP_2$ plus $[^3H]IP_3$ were extracted and analyzed as described in Experimental Procedures. Data correspond to the means ± SEM of four independent experiments performed in triplicate. Values for untreated cells were 1643 ± 226 and 1589 ± 110 cpm for control and pertussis toxin, respectively. Values for GTPγS-treated cells were 7237 ± 378 and 6241 ± 129 cpm for control and pertussis toxin, respectively. *, $p < 0.05$ in a paired *t* test. B, Inhibition of *in vitro* ADP-ribosylation by pretreatment of cells with pertussis toxin. NIH 3T3 cells were treated with regular medium (*lanes 1* and *2*) or medium containing pertussis toxin (1 μg/ml) (*lanes 3* and *4*), for 4 hr. Cells were washed and membranes were prepared. ADP-ribosylation of membranes was performed in the absence (*lanes 1* and *3*) or in the presence (*lanes 2* and *4*) of pertussis toxin, as described in Experimental Procedures. *Marks on left*, migration of molecular mass standards of (in descending order) 69, 46, 30, and 21.5 kDa.

served in whole cells. The reason for the disparity is not clear; however, the much higher potency of $PGF_{2\alpha}$, compared with PGE_2, in both cases suggests that the same receptor population is affected in both whole-cell and permeabilized cell experiments.

The G protein involved in transducing stimulatory signals between the PG receptor in NIH 3T3 cells and phospholipase C does not seem to be a good substrate for pertussis toxin-induced ADP ribosylation. Pretreatment with pertussis toxin (1 μg/ml) for 4 hr induced a small inhibition of $PGF_{2\alpha}$-mediated responses (0.1 and 1 μM $PGF_{2\alpha}$) (Fig. 4). Such treatment seems to induce complete ADP-ribosylation of G proteins in NIH 3T3 cells, because it eliminated subsequent pertussis toxin-induced

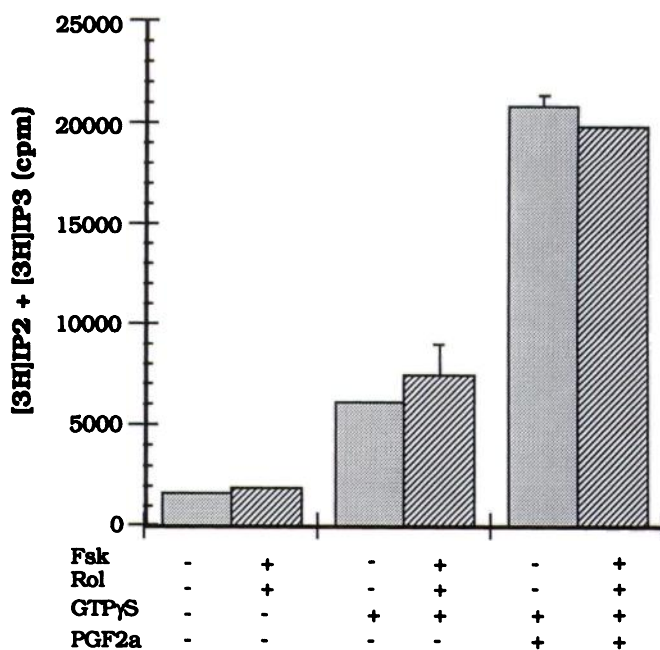

Fig. 5. Lack of effect of forskolin (*Fsk*) [plus rolipram (*Rol*)] on stimulation of $[^3H]IP_2$ plus $[^3H]IP_3$ formation in permeabilized NIH 3T3 cells. $[^3H]$Inositol-labeled cells were washed with incubation buffer and incubated for 10 min with 30 μM rolipram at 37°, where indicated. Forskolin (1 μM) (▨) or buffer (□) was then added, and cells were incubated for 15 min at 37°. After permeabilization, cells were treated with buffer, GTPγS (10 μM), or $PGF_{2\alpha}$ (1 μM) plus GTPγS, as indicated. $[^3H]IP_2$ plus $[^3H]IP_3$ were extracted and analyzed as described in Experimental Procedures. Data correspond to a representative experiment performed in duplicate (mean ± range), which was repeated four times with similar results.

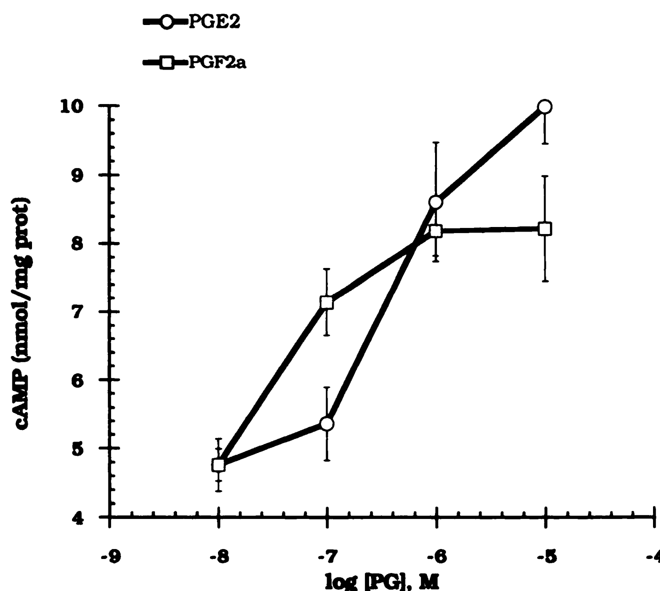

Fig. 6. Dose-dependent stimulation of cAMP formation in NIH 3T3 cells by $PGF_{2\alpha}$ and PGE_2. After washing with medium, cells were preincubated with buffer containing rolipram (30 μM), for 10 min, and then the indicated concentrations of PGE_2 (○) or $PGF_{2\alpha}$ (□) were added. Incubations were carried out for 15 min at 37°. Extraction and determination of cAMP levels were performed as described in Experimental Procedures. Data correspond to means ± SEM of at least three independent experiments. Control value for unstimulated cells was 3.8 ± 0.2 nmol/mg of protein.

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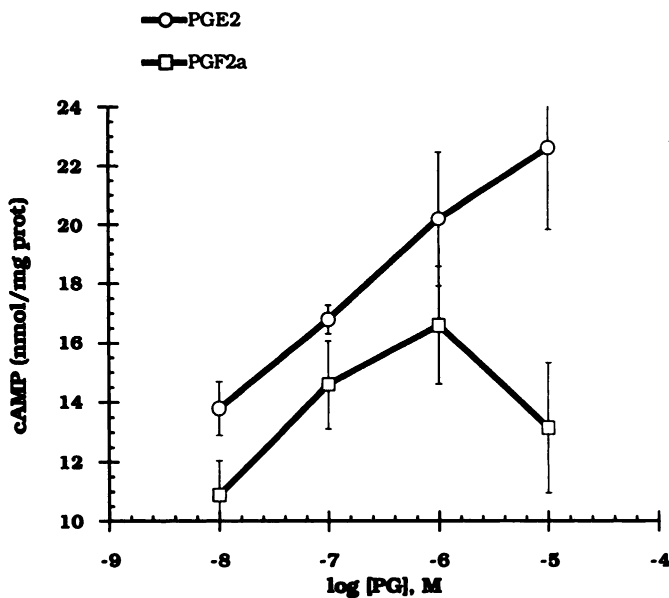

Fig. 7. Dose-dependent potentiation of forskolin-induced cAMP formation in NIH 3T3 cells by $PGF_{2\alpha}$ and PGE_2. After washing with medium, cells were preincubated with buffer containing rolipram (30 μM), for 10 min, and then forskolin (0.5 μM) plus the indicated concentrations of PGE_2 (○) or $PGF_{2\alpha}$ (□) were added. Incubations were carried out for 15 min at 37°. Extraction and determination of cAMP levels were performed as described in Experimental Procedures. Data correspond to means ± SEM of at least three independent experiments. Control values for unstimulated cells and forskolin-treated cells were 3.6 ± 0.2 and 13.3 ± 0.38 nmol/mg of protein, respectively.

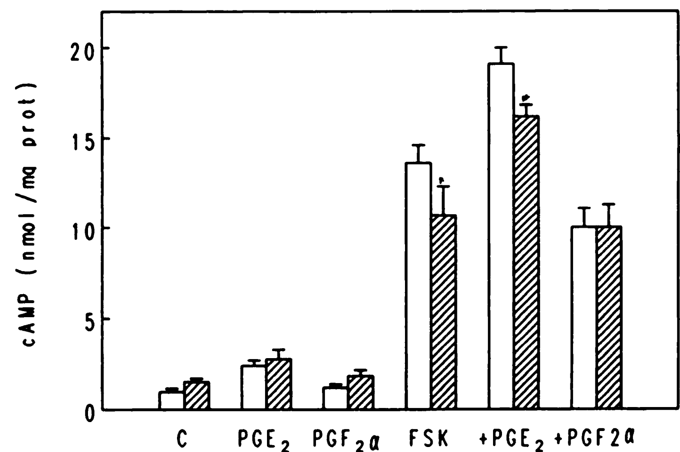

Fig. 8. Effects of PMA on PGE_2- and $PGF_{2\alpha}$-induced cAMP accumulation in NIH 3T3 cells. After washing with medium, cells were preincubated with buffer containing rolipram (30 μM), for 10 min, and then PGE_2 (10 μM), $PGF_{2\alpha}$ (10 μM), or forskolin (0.5 μM) plus PGE_2 (10 μM) or $PGF_{2\alpha}$ (10 μM) were added without (□) or with (■) 1 μM PMA. Incubations were carried out for 15 min at 37°. Extraction and determination of cAMP levels were performed as described in Experimental Procedures. Data correspond to means ± SEM of three independent experiments performed in duplicate. *C*, control. *, $p < 0.05$.

TABLE 2

Effects of overnight treatment with $PGF_{2\alpha}$ on PGE_2- and $PGF_{2\alpha}$-induced cAMP accumulation in NIH 3T3 cells

Cells were treated overnight with regular medium (No pretreatment) or medium containing 100 nM $PGF_{2\alpha}$ ($PGF_{2\alpha}$). Cells were washed twice and preincubated with incubation buffer containing 30 μM rolipram, for 10 min at 37°. Agents were added at the indicated concentrations and incubations were carried out for 15 min at 37°. Cyclic AMP was extracted and analyzed as described in Experimental Procedures. Data correspond to means ± SEM of three experiments performed in duplicate. Values for no treatment versus $PGF_{2\alpha}$ are not statistically different.

Treatment	cAMP	
	No pretreatment	$PGF_{2\alpha}$
	nmol/mg of protein	
Control	4.63 ± 0.03	4.23 ± 0.12
PGE_2 (1 μM)	7.73 ± 0.58	7.03 ± 0.50
$PGF_{2\alpha}$ (1 μM)	5.56 ± 0.24	5.07 ± 0.20

[^{32}P]ADP-ribose incorporation *in vitro* in treated cells (Fig. 4). Moreover, incubation for longer times (14 hr) with pertussis toxin did not result in greater inhibition (data not shown). Because pertussis toxin treatment would result in accumulation of cAMP as a consequence of inactivation of G_i, the possibility of cAMP itself being responsible for inhibition of phospholipase C activation was tested. Pretreatment with forskolin (1 μM) (15 min in the presence of 30 μM rolipram), which results in a 4-5-fold increase in cAMP levels (data not shown), did not induce any significant inhibition of a subsequent stimulation of phospholipase C activity by either GTPγS or $PGF_{2\alpha}$ plus GTPγS (Fig. 5). It is, thus, unlikely that the actions of pertussis toxin are mediated through accumulation of intracellular cAMP. Two interpretations of the effects observed after pertussis toxin are possible. (i) The PG receptor can interact with more than one G protein in order to induce phospholipase C stimulation, and not all of these G proteins are substrates for pertussis toxin-induced ADP ribosylation. (ii) The PG receptor interacts with one G protein involved in inducing phospholipase C activity, and this G protein is only poorly ribosylated by pertussis toxin. Similar partial inhibition of a phospholipase C system has been demonstrated in HL-60 cells, where P_2 purinergic receptor-mediated activation of phospholipase C was partially inhibited by pertussis toxin (17). It was proposed that P_2 purinergic receptors can activate phospholipase C through pertussis toxin-"sensitive" and -"insensitive" pathways (17).

Long term treatment with $PGF_{2\alpha}$ resulted in a reduction of $PGF_{2\alpha}$ plus GTPγS, PGE_2 plus GTPγS, and GTPγS responses (Table 1). In NG108 × 15 cells, long exposure to PGE_2 resulted in down-regulation of G_s, the G protein involved in stimulation of adenylate cyclase (18). Such down-regulation was due to increased disappearance of $G_{s\alpha}$, probably by proteolytic degradation (18). A similar phenomenon, namely the down-regulation of the G protein α subunit involved in phospholipase C signal transduction, may pertain in NIH 3T3 cells.

PG receptors are coupled in many cell types to another second messenger system, the adenylate cyclase system. In NIH 3T3 cells, PGs induced accumulation of cAMP (Fig. 6). The potencies for $PGF_{2\alpha}$ and PGE_2 were similar for such action. PGE_2-mediated cAMP formation appeared to be more efficacious (Fig. 6). The higher efficacy of PGE_2 for inducing cAMP accumulation was even more pronounced in experiments where a submaximal concentration of forskolin was used in combination with the PGs (Fig. 7). Low concentrations of forskolin seem to enhance or "prime" receptor coupling to adenylate cyclase (19). Under such conditions, $PGF_{2\alpha}$ induced, at the same concentrations, a lower potentiation of forskolin-induced cAMP formation than did PGE_2 (Fig. 7). Moreover, at 10 μM $PGF_{2\alpha}$ an inhibitory effect was observed, which may be the result of diacylglycerol generation and subsequent stimulation of protein kinase C (see below). Thus, in NIH 3T3 cells a receptor for PGs is coupled to adenylate cyclase, probably through G_s, by analogy with other cells. This receptor does not

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show a great selectivity for $PGF_{2\alpha}$ versus PGE_2 (Figs. 6 and 7). It seems likely that PG-mediated actions on phosphoinositide breakdown and on cAMP accumulation are attained by activation of two different receptors, based on the differences in potency of $PGF_{2\alpha}$ and PGE_2 observed for phosphoinositide breakdown. This is further supported by the observation that overnight treatment with 100 nM $PGF_{2\alpha}$, which induced a desensitization of PG-induced phosphoinositide breakdown (Table 1), did not affect PG-induced cAMP formation (Table 2).

The generation of cAMP is significantly affected by protein kinase C (20). Protein kinase C, in fact, comprises a family of isozymes (21), and the activation of different isozymes has distinct actions on the generation of cAMP (20). In NIH 3T3 cells protein kinase C α subtype (but not γ subtype) is present, and activation of this isozyme results in inhibition of cAMP generation (20). Because $PGF_{2\alpha}$ and PGE_2 can stimulate phospholipase C activity, with concomitant generation of diacylglycerol, which activates protein kinase C, it is likely that the generation of cAMP by PGs in NIH 3T3 cells may be reduced to some extent, due to the activation of the α isozyme of protein kinase C. Such reduction in cAMP generation was observed with $PGF_{2\alpha}$ at high concentrations (Fig. 5). Thus, at 10 μM $PGF_{2\alpha}$ the potentiation of forskolin-induced cAMP formation was reduced, compared with 1 μM $PGF_{2\alpha}$. It seems that $PGF_{2\alpha}$, but not PGE_2, can induce the release of sufficient amounts of diacylglycerol to result in a sustained protein kinase C activation and subsequent inhibitory action on cAMP formation. PMA, which directly activates protein kinase C, inhibited significantly forskolin- and forskolin-plus PGE_2-induced accumulation of cAMP (Fig. 8). However, the combination of forskolin plus $PGF_{2\alpha}$ was not affected by pretreatment with PMA (Fig. 6), suggesting that maximal inhibition through a protein kinase C pathway is attained with $PGF_{2\alpha}$ alone through diacylglycerol generation.

Another aspect of interaction between second messenger systems may be of relevance in NIH 3T3 cells. Stimulation of proliferation of fibroblasts in culture can be achieved by several agents that induce phosphoinositide breakdown (22). In NIH 3T3 cells, however, $PGF_{2\alpha}$ failed to induce DNA synthesis (10), in spite of the fact that phosphoinositide breakdown is observed (10, this work). On the other hand, it has been proposed that cAMP analogs inhibit proliferation in fibroblasts (23). Because $PGF_{2\alpha}$ stimulation would also generate cAMP (Figs. 6 and 7), the lack of action on proliferation may be determined by the resultant of both signals, the generation of cAMP and the generation of inositol lipid metabolites.

In summary, two receptors for PGs are present in NIH 3T3 cells, (i) a receptor coupled to adenylate cyclase, probably via G_s, by analogy with other systems (PGE2 is more efficacious than $PGF_{2\alpha}$ in activating this receptor), and (ii) a receptor coupled to phospholipase C via one or more G protein(s) ($PGF_{2\alpha}$ is 30-fold more potent than PGE_2 in activating this receptor).

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