EFFECT OF PGI_2 AND STABLE ENDOPEROXIDE ANALOGUES ON CYCLIC NUCLEOTIDE LEVELS IN CLONAL CELL LINES OF CNS ORIGIN

Rainer ORTMANN

Pharmakologisches Institut der Universität Freiburg, Hermann Herderstrasse 5, D-78 Freiburg, FRG

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1. Introduction

The effects of prostaglandins of the E, A and F series on the adenylate and guanylate cyclase system of various cloned cell lines in culture have been described [1–6]. Since the detection of the endoperoxides PGG_2 and PGH_2 [7], thromboxane A_2 [8] and PGI_2 (prostacyclin) [9–11], many laboratories worked on the effects of these new prostaglandins on platelet aggregation and platelet adenylate cyclase [12–18]. Little is know however about the effects of these prostaglandins on cyclic nucleotide levels in other systems. We therefore compared the effect of PGI_2 and 2 endoperoxide analogues (U 44069 and U 46619) with the effects of prostaglandins of the A, B, E and F series on cAMP and cGMP levels of the murine neuroblastoma cell line N4TG3 and the human astrocytoma cell line 1321N1. We show that PGI_2 is an extremely potent stimulator of cAMP accumulation in N4TG3 cells, while it is relatively inactive in 1321N1 cells. Furthermore cGMP accumulation is stimulated by PGI_2 and PGE_1 in the neuroblastoma cell line.

2. Materials and methods

2.1. *Prostaglandins*

All prostaglandins were a gift from the Upjohn Company, MI. 6βH-5,6α-dihydro-PGI_2 was obtained from the Carlo Erba institute, Milano, which also supplied a sample of PGI_2. Stock solutions (10 mM) of prostaglandins except PGI_2 were prepared in ethanol and kept at −20°C. PGI_2 and 6βH-5,6α dihydro-PGI_2 were dissolved in cold 0.05 M Tris buffer, pH 9.4, immediately before each experiment.

2.2. *Cell culture*

The isolation and growth conditions of human astrocytoma cell line 1321N1 [1,10] and the 6-thioguanine resistant neuroblastoma cell line N4TG3 [20,21] have been described. 1321N1 cells were obtained from R. B. Clark, Worcester, MA. N4TG3 cells were from B. Hamprecht, München. 35 mm 'Lux' plastic dishes were seeded with 1×10^5 (N4TG3) or 2×10^5 (1321N1) cells in 2 ml growth medium and grown for 3 days.

2.3. *Experimental incubations*

For experimental incubations cells were washed with serum free growth medium and challenged with prostaglandins for 2 min at 37°C in serum free growth medium containing 1 mM isobutylmethyl xanthine (IBMX). Incubations were stopped by aspirating the medium and adding 1 ml 5% trichloroacetic acid (TCA). The TCA was extracted with water saturated ether and used for cAMP and cGMP radioimmunoassay without further purification.

2.4. *Cyclic nucleotide determination*

For cAMP and cGMP RIA 10–500 μl of the ether-extracted TCA extract were incubated overnight at 0°C with 20 000 cpm ^{125}I-labeled-2′-O succinyl cyclic cAMP or cGMP tyrosine methyl ester and cAMP or cGMP antiserum (final dilution, 1:200 000) in total vol. 2 ml in isotonic phosphate buffer, pH 7.4, containing 1 g gelatine/liter. For the cGMP RIA the acetylation step in [22] was included.

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Bound and free ^{125}I-labeled-ligand was separated by the addition of 0.2 ml charcoal suspension (Aktivkohle, E. Merck, 20 mg/ml phosphate buffer) and immediate centrifugation.

The inhibitor of the binding of ^{125}I-labeled-ligands in the cAMP and cGMP RIA, respectively, was adsorbed by charcoal treatment, destroyed by beef heart phosphodiesterase. Serial dilutions of the TCA extracts inhibited the binding to the same degree as authentic cAMP or cGMP. Addition of known amounts of cAMP or cGMP standards to TCA extracts after the ether extraction step showed a complete recovery of the added cyclic nucleotides. Cross-reactions of both antisera with other nucleotides and nucleosides are summarized in table 1.

3. Results

3.1. *Effects of PGI_2 on cAMP*

Stimulation of cAMP accumulation by PGE_1 in N4TG3 cells [21,23] and by PGE_1, E_2, A_1 and $F_{2\alpha}$ in 1321N1 cells [1] has been reported. The data of fig.1 compare the effects of PGI_2 and its stable end-product 6-keto $F_{1\alpha}$ with PGE_1 during a 2 min incubation period in both cell lines. While PGI_2 is a more potent stimulator of adenylate cyclase than PGE_1 in N4TG3 cells (ED_{50} 3 nM), its ED_{50} in 1321N1 cells (10 μM) is significantly higher than the

Table 1
Cross-reactions of the cAMP and cGMP antiserum with various nucleotides and nucleosides

Inhibitor	% Cross-reaction	
	cAMP antiserum	cGMP antiserum
3':5' cAMP	100	0.01
3':5' cIMP	1	3
3':5' cGMP	< 0.001	100
Guanosine		0.004
2':3' cAMP		
2':3' cGMP		
ATP, ADP, AMP	< 0.001	< 0.002
GTP, GDP, GMP		
Adenosine		

Assay conditions as described in 2.4; final antiserum dilution 1:200 000

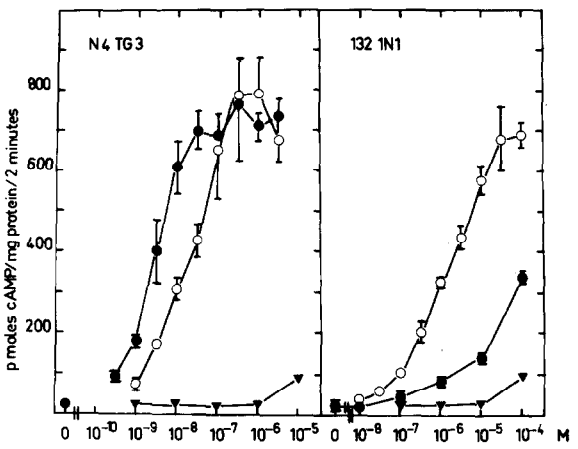

Fig.1. The effect of PGE_1 (o—o—o), PGI_2 (•—•—•) and 6-keto $F_{1\alpha}$ (▲—▲—▲) on cAMP accumulation in N4TG3 cells (left) and 1321N1 cells (right). Results are means ± SD of 3 dishes assayed in duplicate.

ED_{50} measured for PGE_1 (1 μM). 6-Keto $F_{1\alpha}$ has only small effects on cAMP levels in both cell types. At high concentrations the stable PGI_2 derivative 6βH-5, 6α-dihydro-PGI_2 stimulates cAMP accumulation to the same degree as PGI_2 in both cell lines (table 2). Its ED_{50} in N4TG3 cells is however about 100 times higher than the ED_{50} determined for PGI_2 (data not shown).

3.2. *Effects of other prostaglandins on cAMP*

Table 2 compares the effects of maximal concentrations of various prostaglandins on the adenylate cyclase of both cell lines. It confirms the data reported earlier on PGE_1, E_2, A_1 and $PGF_{2\alpha}$ stimulated cAMP accumulation in 1321N1 cells [1] and shows that the pattern of prostaglandin stimulation in N4TG3 cells is the same as reported for the N4TG3 clone [3]. It demonstrates that PGB_1 is a rather poor stimulator in both cell lines and that the 2 stable endoperoxide analogues U 44069 and U 44619 have a different effect on the cAMP level of the astrocytoma line: At high concentration (30 μM) the 9α,11α-epoxymethano derivative U 44069 has a stimulatory effect, while the 11α,9α-epoxymethano compound U 46619 is inactive (table 2). In N4TG3 cells both analogues have the same, small stimulatory effect on cAMP accumulation at 10 μM.

Table 2
Effects of different prostaglandins on cAMP levels in 1321N1 and N4TG3 cells

Prostaglandin	% PGE_1 response				
	1321N1 cells (30 μM)		N4TG3 cells (10 μM)		
	Exp. 1[a]	Exp. 2[a]	Exp. 1[b]	Exp. 2[a]	Exp. 3[b]
PGE_1	100 ± 14	100 ± 13	100 ± 14	100 ± 9	100 ± 4
PGE_2	101 ± 14	89 ± 9	86 ± 6	77 ± 11	60 ± 3
PGA_1	83 ± 10	66 ± 6	83 ± 4	75 ± 7	–
PGB_1	26 ± 3	22 ± 3	44 ± 2	33 ± 2	–
$PGF_{2\alpha}$	9 ± 1	–	36 ± 2	38 ± 4	–
PGI_2	42 ± 5	61 ± 11	129 ± 14	129 ± 9	111 ± 27
6-keto $F_{1\alpha}$	9 ± 1	5 ± 1	10 ± 1	29 ± 2	–
6βH 5,6α-dihydro-PGI_2	46 ± 3	38 ± 5	–	111 ± 8	98 ± 11
U 44069	29 ± 2	–	20 ± 11	11 ± 1	–
U 46619	5 ± 1	–	11 ± 1	10 ± 1	–
None	6 ± 1	6 ± 2	4 ± 1	4 ± 1	3 ± 1

[a] $n = 4, \bar{x} \pm$ SD, each dish assayed in duplicate
[b] $n = 3, \bar{x} \pm$ SD, each dish assayed in duplicate

cAMP accumulation at 30 μM (1321N1) and 10 μM (N4TG3) prostaglandin concentration is compared. In both cell lines cAMP levels in the presence of PGE_1 were set as 100% for each experiment. Maximal stimulation of cAMP accumulation was 398 pmol/mg protein/2 min in 1321N1 cells and varied between 428 and 617 pmol/mg protein/2 min in N4TG3 cells

3.3. *Effects on cGMP levels*

Figure 2 depicts the effects of PGE_1, PGI_2 and 6 keto-$F_{1\alpha}$ on cGMP levels in both cell types. While there is a barely measurable level of cGMP in the astrocytoma cell line (0.3 pmol cGMP/mg protein), the basal cGMP level in N4TG3 cells after a 2 min incubation period in the presence of 1 mM IBMX was 20–30-times higher (6.3–10.9 pmoles cGMP/mg protein). In the absence of IBMX cGMP and cAMP levels were however significantly lower (1.3 pmol cGMP/mg protein), confirming the high phosphodiesterase activity in N4TG3 cells described [21,23]. Furthermore there was no detectable influence of any of the prostaglandins on the cGMP level in 1321N1 cells (table 3, fig.2), while PGE_1 and PGI_2 caused a 5–7-fold increase in cGMP levels in N4TG3 cells at 10 μM. While PGI_2 had a higher affinity to the receptor mediating this effect, the app. V_{max} of PGE_1 was greater in most experiments (table 3, fig.2). In preliminary experiments no stimulation of cGMP levels by 10 μM PGI_2 was found, when Krebs-Ringer solution containing 1 mM IBMX [24]

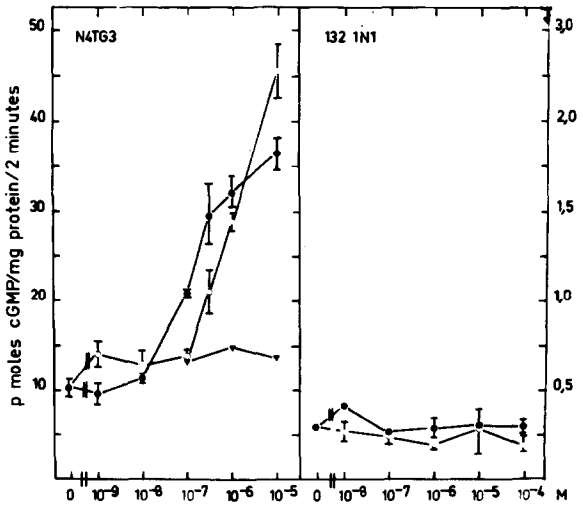

Fig.2. The effect of PGE_1 (○–○–○), PGI_2 (● – ● – ●) and 6-keto-$F_{1\alpha}$ (▲–▲–▲) on cGMP accumulation in N4TG3 (left) and 1321N1 cells (right). Results are the average of 2 dishes, or means of 3 dishes ± SD assayed in duplicate (N4TG3) or single determinations (1321N1). Note the different scale for N4TG3 and 1321N1 results.

Table 3
Effects of various prostaglandins on cGMP levels in 1321N1 and N4TG3 cells

Prostaglandins	(pmol cGMP/mg protein/2 min)				
	1321N1 cells		N4TG3 cells		
	Exp. 1[a]	Exp. 2[a]	Exp. 1[b]	Exp. 2[a]	Exp. 3[c]
PGE_1	0.34 ± 0.02	0.23 ± 0.03	31.1 ± 5.0	38.3 ± 0.9	55.6 ± 4.8
PGE_2	0.37 ± 0.02	0.25 ± 0.02	9.6 ± 1.8	11.0 ± 1.3	13.1 ± 0.7
PGA_1	0.32 ± 0.02	–	7.6 ± 2.0	9.9 ± 1.6	–
PGB_1	0.36 ± 0.04	–	7.0 ± 0.8	7.3 ± 1.9	–
$PGF_{2\alpha}$	0.37 ± 0.03	–	8.8 ± 0.6	9.6 ± 1.0	–
PGI_2	0.34 ± 0.01	0.29 ± 0.03	26.4 ± 2.6	39.1 ± 2.7	36.7 ± 4.1
6-Keto $F_{1\alpha}$	0.36 ± 0.06	–	7.4 ± 0.8	8.6 ± 0.8	–
6βH 5,6α-dihydro-PGI_2	0.34 ± 0.05	0.32 ± 0.09	–	14.8 ± 2.4	14.9 ± 1.8
U 44069	0.37 ± 0.04	–	8.2 ± 0.3	8.9 ± 2.2	–
U 46619	0.36 ± 0.03	–	7.4 ± 0.9	9.3 ± 1.1	–
None[d]	0.34	0.30	6.3	10.9	8.3

[a] $n = 4$, $\bar{x}$ ± SD, single determinations
[b] $n = 3$, $\bar{x}$ ± SD, single determinations
[c] $n = 3$, $\bar{x}$ ± SD, duplicate determinations
[d] $n = 2$

Experiment numbers refer to the same experiments as listed in table 2 for the cAMP system

was used as incubation medium. In the absence of calcium cGMP levels in Krebs-Ringer solution were even lower.

4. Discussion

PGI_2 has been shown to be a more potent stimulator of platelet adenylate cyclase than PGE_1 [13–15]. The main point of this report is to extent this comparison to other adenylate cyclase systems. The data of fig.1 and table 2 show that like in the platelets PGI_2 is also in N4TG3 cells a more potent agonist than PGE_1. The ED_{50} (3 nM) for PGI_2 in N4TG3 cells is 10-times lower than in the human platelet adenylate cyclase system [13]. However in 1321N1 cells PGI_2 is substantially less potent than PGE_1.

Beside the difference in affinity (ED_{50}) of various prostaglandins (compare ED_{50} for PGE_1 in both cell lines) and their effects at maximal concentrations (V_{max}) (table 2, 2–4), the marked difference in potency of PGI_2 found in the 2 cell lines, may represent an interesting characteristic of different types of prostaglandin receptors. Together with the information on antagonists of prostaglandin effects [26], on the stereospecificity for different prostaglandins (compare the effects of U 44069 and U 46619) and binding studies with labeled prostaglandins [3], these data may help to classify multiple types of prostaglandin receptors linked to the adenylate or guanylate cyclase system.

The data of table 2 confirm the low activity of the endoperoxide analogues U 46619 and U 44069 to stimulate cAMP accumulation found in rat osteogenic sarcoma cells [25]. In contrast to the adenylate cyclase inhibiting effect of PGG_2 in platelets [16] and of PGH_2 in adipocyte ghosts [17] 10 μM U 44069 did not alter the stimulation of cAMP or cGMP accumulation in N4TG3 cells by 10 nM or 1 μM PGI_2 (data not shown).

Simultaneous stimulation by PGE_1 of cAMP and cGMP accumulation has been reported [4]. Basal as well as stimulated cGMP levels in N4TG3 cells are in the same concentration range as described for N1E115 neuroblastoma cells [4]. Our data characterize further the receptor mediating the stimulation of cGMP accumulation by PGE_1 and PGE_2:the affinity of PGI_2 (ED_{50} 0.2 μM) and PGE_1 (ED_{50} 2 μM) for this

receptor is 100 times lower than in the cAMP system. The receptor displays a similar specificity for PGE_1 and PGI_2 as the receptor which mediates the stimulation of cAMP accumulation, because at 10 μM only PGE_1, PGI_2 and 6βH-5,6α-dihydro-PGI_2 stimulate consistently cGMP levels. In contrast to the cAMP system 6βH-5,6α-dihydro-PGI_2 stimulates significantly less than PGI_2. Preliminary data show that while the cAMP accumulation is not altered by the use of Krebs-Ringer solution as incubation medium, no stimulation of cGMP levels is observed under these conditions. The reason for this observation is unknown.

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