MOLECULAR PHARMACOLOGY, 21:664–670

Stimulation of Neuroblastoma Adenylate Cyclase by Arachidonic Acid Metabolites

ALLYN C. HOWLETT

Department of Pharmacology, St. Louis University School of Medicine, St. Louis, Missouri 63104

Received August 31, 1981; Accepted December 7, 1981

SUMMARY

This report is a comprehensive examination of the regulation of N18TG2 neuroblastoma adenylate cyclase by physiological prostaglandins. Cyclic AMP accumulation *in situ* was provoked by prostaglandin (PG) E_2, PGI_2, and 6-keto-PGE_1. These agonists exhibited the same intrinsic activity as PGE_1 toward stimulation of adenylate cyclase in membranes from these cells. The potency order was $PGI_2 > PGE_1 >$ 6-keto-$PGE_1 > PGE_2$. The stimulation was consistent with a hormone-mediated mechanism in that each agonist increased the capacity of GTP to activate adenylate cyclase and increased the rate of activation by a nonhydrolyzable GTP analogue. In addition, the Mg^{2+} requirement was reduced by these prostanoids. The prostaglandin receptor(s) was down-regulated by incubating the cells with either PGE_1 or 6-keto-PGE_1. The refractory state produced extended equally to each of the prostanoid agonists. PGE_2 and the stable endoperoxide analogues were poor agonists, and neither $PGF_{2\alpha}$ nor 6-keto-$PGF_{1\alpha}$ stimulated adenylate cyclase in this system.

INTRODUCTION

PG[1] E_1 was originally shown by Gilman and Nirenberg (1) to stimulate adenylate cyclase in neuroblastoma cells. Brunton *et al.* (2) extensively examined PGE_1 binding to a membrane site and correlated this receptor with stimulation of adenylate cyclase in neuroblastoma and other cultured cells. Since those studies were performed, PGI_2 (prostacyclin) and its metabolites have been shown to alter the cyclic nucleotide content in platelet (3–5), myometrium (6), gut (7), and liver (8), systems in which the effects of arachidonic acid metabolites are believed to be mediated at least in part by cyclic AMP. Demonstrations that PGD_2 and PGI_2 activate adenylate cyclase in certain neuroblastoma cell lines have appeared in the recent literature (9–11).

It is the goal of the present investigation to examine more comprehensively the regulation of neuroblastoma adenylate cyclase by prostaglandins metabolically derived from arachidonic acid, the predominate C_{20} unsaturated fatty acid in mammalian cell membranes. Evidence is presented that certain of these physiologically relevant prostaglandins stimulate adenylate cyclase in a manner consistent with current models of hormone-receptor regulation of a guanine nucleotide-binding component of the enzyme. The possibility of subtypes of prostaglandin receptors in a single cell is addressed.

MATERIALS AND METHODS

Cell culture. Neuroblastoma cell stock (C-1300 clone N18TG2, Passage 25) was obtained from A. G. Gilman. Cells were maintained in Dulbecco's modified Eagle's medium containing 10% heat-inactivated calf serum under an atmosphere of 5% CO_2. Cells intended for *in situ* studies and membrane preparations were plated at 10^6 cells per 150-cm^2 flask. Confluent cells were harvested on day 4 following a change of media on day 3. Fresh stock was thawed from storage at intervals of 50–60 days.

Cellular cyclic AMP determinations. Cells were harvested by gentle pipetting with a dissociation medium (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.0 mM glucose, and 0.625 mM EDTA). Cells were sedimented, rinsed once, and resuspended at 2×10^6 cells/ml in GBSS (12) in which carbonate and phosphate buffers were replaced with 10 mM sodium glycylglycine (pH 7.4 at 37°). Following a 30-min incubation at 37° with a cyclic nucleotide phosphodiesterase inhibitor (either IBMX or RO20-1724), cells (450 μl) were added to 50 μl of GBSS containing the designated agonist. When PGI_2 was the agonist, dilutions were made in GBSS adjusted to pH 9.0 and were kept on ice until the addition of cells. After 4 min at 37°, the incubation was terminated by addition of 50 μl of 500 mM sodium acetate (pH 4.5) and boiling for

This work was supported in part by a Pharmaceutical Manufacturers Association Foundation Research Starter Grant and by United States Public Health Service Grant NS 16513.

[1] The abbreviations used are: PG, prostaglandin; GBSS, Gey's balanced salt solution (12); IBMX, 3-isobutyl-1-methylxanthine; RO20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; Gpp(NH)p, guanosine 5′-(β,γ-imino)-triphosphate; K_{act}, activation constant, n_H, Hill coefficient; U-44069, (15*S*)-hydroxy-9α,11α-(epoxymethano)prosta-5Z,13E-dienoic acid; U-46619, (15*S*)-hydroxy-11α,9α-(epoxymethano)-prosta-5Z,13E-dienoic acid.

0026-895X/82/030664-07$02.00/0

4 min. The particulate matter was sedimented, and protein was determined according to the method of Lowry *et al.* (13). The cyclic AMP content of the supernatant was assayed by the modification of Brostrom and Kon (14) of the protein kinase-binding method of Gilman (15).

Adenylate cyclase in membranes. Cells were harvested as above and a plasma membrane fraction was prepared according to the method of Ross *et al.* (16). Unless otherwise described, adenylate cyclase activity was determined in a reaction mixture containing 50 mM sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 8.0), 1 mM EDTA, 10 mM $MgCl_2$, 1 μM GTP, 0.1 mM RO20-1724, bovine serum albumin (0.1 mg/ml), pyruvate kinase (10 μg/ml), 1.5 mM potassium phosphoenolpyruvate, 0.5 mM ATP, and [^{32}P]ATP (1–5 μCi). Maximal adenylate cyclase activity occurred between pH 8 and 8.5 for stimulation by NaF, Gpp(NH)p, PGE_1, or PGI_2. The reaction was initiated by the addition of 10 μg of membrane protein to the reaction mixture such that the final volume was 100 μl. The reaction progressed for 15 min at 30°, and ^{32}P-labeled cyclic AMP was isolated by the procedure of Salomon *et al.* (17). Ethanol concentrations in the reaction mixture, as the prostaglandin vehicle (maximally 0.7%), did not alter the rates of basal, Gpp(NH)p-, or NaF-activated enzyme.

Chromatography of prostaglandins. Thin-layer chromatographic analysis of PGE_1 and metabolites was performed in solvent systems A IV and A III of Änggård and Samuelsson (18).

Materials. Tissue culture media and serum were obtained from either KC Biologicals (Kansas City, Mo.) or Flow Laboratories (Rockville, Md.). Reagent-grade chemicals were obtained from standard sources. ATP was Sigma A2383 and GTP was Sigma G5881. [^{32}P]ATP and [^{3}H]PGE_1 were purchased from Amersham Corporation (Arlington Heights, Ill.). The prostaglandins were generous donations from Upjohn (Kalamazoo, Mich.), and RO20-1724 from Hoffmann-La Roche (Nutley, N. J.). PGI_2 and 6-keto-$PGF_{1\alpha}$ were stored in 10 mM potassium phosphate (pH 9.2), and other prostaglandins were stored in 95% ethanol at 1 mg/ml at −15°. The PGE_2 was dissolved at 5 mg/ml for the experiments shown in Fig. 2.

RESULTS

Intact N18TG2 cells were examined for their ability to synthesize cyclic AMP in response to PGI_2 and prostaglandins of the E type (Table 1). The maximal response to 2-Cl-adenosine is shown for comparison. PGI_2 stimulated cyclic AMP accumulation to the same maximal value as PGE_1; however, the ED_{50} of PGI_2 was 10-fold lower than PGE_1. 6-Keto-$PGF_{1\alpha}$, the chemical breakdown product of PGI_2, did not increase cellular cyclic AMP significantly above basal values. However, 6-keto-PGE_1, an enzymatic derivative of PGI_2 (19), stimulated cyclic AMP accumulation to the same maximal value as PGE_1. At 10 μM, PGE_2 did not produce a maximal response. Log dose-response curves (not shown) demonstrate that this is so because PGE_2 is less potent than PGE_1. The 15-methyl analogue of PGE_1 appeared to be more efficacious than PGE_1, possibly because the latter is not subject to the same metabolic pathways as other prostaglandins, although there is no evidence that N18TG2 cells have 15-OH-dehydrogenase activity (see below).

Control experiments (not shown) demonstrated that cellular cyclic AMP accumulation in response to PGE_1, PGI_2, or 2-Cl-adenosine increased linearly for 5 min in the presence of either IBMX or RO20-1724 as a phosphodiesterase inhibitor. Thereafter, cyclic AMP levels were constant for at least 15 min. Cellular metabolism of PGE_1 is negligible under these assay conditions. This was determined by incubating 5×10^6 cells/ml in GBSS containing 1 μM [^{3}H]PGE_1 for 30 min at 37°. The medium was extracted with ethyl acetate containing 1% acetic acid and analyzed by thin-layer chromatography as described under Materials and Methods. Over 98% of the ^{3}H-labeled counts per minute traveled with a PGE_1 peak visualized by phosphomolybdic acid reagent spray. The two solvent systems chosen adequately separate PGE_1 from its less polar metabolites that result from transformation by lung tissue. Therefore, any significant transformation of PGE_1 by N18TG2 cells would have been detected. Maintenance of the PGI_2 concentration during the assay is impossible owing to its chemical instability in an aqueous medium. Interpolating from data of Cho and Allen (20), the $t_{1/2}$ for nonenzymatic hydrolysis of PGI_2 in 30 mM sodium phosphate buffer (pH 7.4) taken

TABLE 1

Cyclic AMP accumulation (means ± standard deviation) in N18TG2 cells

Agonist	Cyclic AMP				ED_{50}	
	Expt. 1[a, b]	Expt. 2[a, b]	Expt. 3[c, d]	Expt. 4[b, c]	Expt. 3[c]	Expt. 5[a]
	pmoles/mg protein				*nM*	
None	23 ± 2	19 ± 3	44 ± 17	56 ± 12		
PGE_1	570 ± 112	511 ± 40	531 ± 67	441 ± 59	410	160
15-CH_3-PGE_1	900 ± 87	789 ± 87	—	—		
PGI_2	679 ± 76	473 ± 25	617 ± 76	—	30	16
6-Keto-$PGF_{1\alpha}$	—	—	—	47 ± 10		
6-Keto-PGE_1	709 ± 76	536 ± 43	—	—		
PGE_2	262 ± 30	216 ± 33	—	—		
2-Cl-Adenosine	—	—	141 ± 11	—		

[a] The phosphodiesterase inhibitor was 0.2 mM IBMX.

[b] All agonists were at a final concentration of 10 μM. Three determinations were made and each was assayed in duplicate.

[c] The phosphodiesterase inhibitor was 0.1 mM RO20-1724.

[d] Concentrations of agonists were 10 μM 2-Cl-adenosine and 1 μM PGE_1 and PGI_2. Two determinations were made, and each was assayed in duplicate.

to ionic strength 0.5 M with NaCl at 37°, is 3.1 min. During the assay period ($1.3 \times t_{1/2}$), the PGI_2 concentration can be expected to decline logarithmically to 40%. Thus, the ED_{50} values for PGI_2 given in Table 1 overestimate the true value. If an average concentration during the assay of 65% of the initial value is assumed, the ED_{50} values become 20 and 10 nM, respectively, for Experiments 3 and 4. Although a more acid medium significantly increases the rate of PGI_2 hydrolysis, the time course of cyclic nucleotide accumulation at pH 7.2 is similar to that at pH 7.6. This suggests that the cell's ability to respond is a more significant determinant of the time course than is the degradation of the agonist.

Adenylate cyclase in membranes. A similar pattern of response to prostaglandins is observed when adenylate cyclase activity is examined in membranes prepared from N18TG2 cells (Fig. 1). At 10 μM, maximal activity was achieved by PGE_1, 15-methyl-PGE_1, PGI_2, and 6-keto-PGE_1. This represents a stimulation of >6-fold over that with GTP alone. The rank order of activation was $PGE_1 > PGA_1 > PGB_1 > PGF_{1\alpha}$ for the eicosatrienoic acid series. Of the arachidonic acid metabolites, PGE_2 and PGD_2 stimulated 4.5- and 1.75-fold over basal at 10 μM, and $PGF_{2\alpha}$ did not stimulate at all. In contrast to the maximal activity observed with PGI_2 and 6-keto-PGE_1, 6-keto-$PGF_{1\alpha}$ had no ability to activate adenylate cyclase. Two stable endoperoxide analogues, U46619 and U44069, exhibited a small stimulation at 10 μM.

A pharmacological characterization of the arachidonic acid derivatives compared with PGE_1 is shown in Fig. 2. These data were linearized according to the Hill equation to obtain the K_{act} and Hill coefficients shown in the *inset*. Hill coefficients approximated 1.0 for each agonist, consistent with an absence of cooperative interactions. As was true *in situ*, PGI_2 was one order of magnitude more potent than PGE_1 in stimulating adenylate cyclase activity. The apparent K_{act} values for PGI_2 and PGE_1 were similar to the ED_{50} values shown in Table 1. The true K_{act} for PGI_2 must be estimated because of hydrolysis during the assay. According to the data of Cho and Allen (20), inactivation in an organically buffered (50 mM) saline solution (ionic strength 0.5 M), pH 8.1 at 30°, occurs with a $t_{1/2}$ = 22 min. Under the conditions of adenylate cyclase assay, PGI_2 decays with a $t_{1/2} \geq 35$ min. Evidence suggests that the bovine serum albumin present in the reaction may stabilize the conformation of PGI_2 as has been shown for other prostanoids (21). Using the latter value, the PGI_2 would decline to 75% of the initial concentration during the 15-min assay period, giving an average concentration of 86% during the assay. Thus, the K_{act} value is overestimated by less than 0.2 log unit.

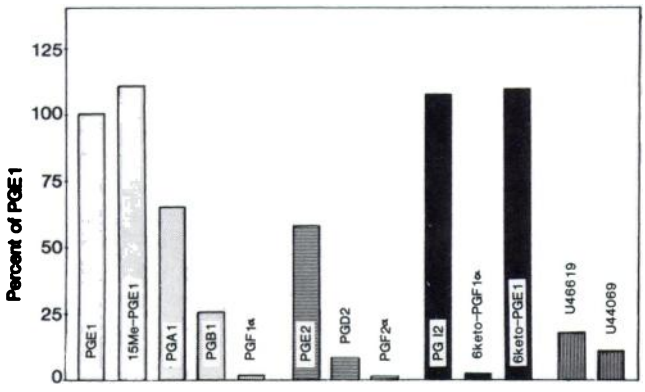

FIG. 1. *Prostaglandin-stimulated adenylate cyclase activity in N18TG2 membranes*

The adenylate cyclase assay was described in the text. All agents were tested at 10μM. The concentration of ethanol was 0.3–0.4% in all determinations except PGI_2 and 6-keto-$PGF_{1\alpha}$. Data are represented as a percentage of PGE_1-stimulated activity (minus basal). Specific activities (picomoles of cyclic AMP per minute per milligram of protein) were 42 for basal (+GTP) and 260 for PGE_1-stimulated. The coefficient of variation was less than 10% for each agent. This experiment is representative of three separate experiments using different membrane preparations.

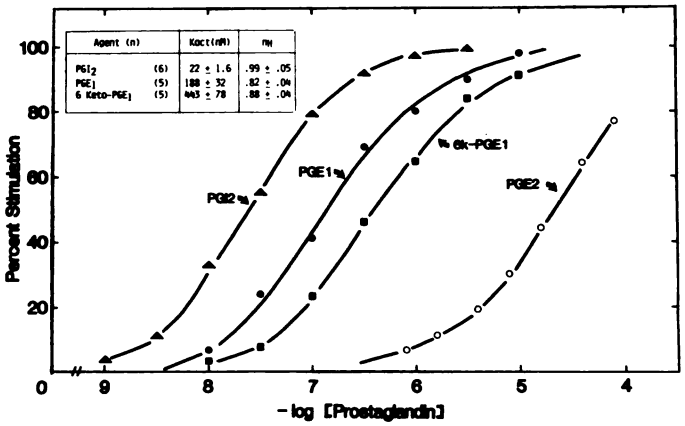

FIG. 2. *Log dose-response curves for prostaglandin-stimulated adenylate cyclase*

The points on each curve represent the mean percentage stimulation for the number of experiments (n) shown in the *inset*. For PGE_2, n = 2. Coefficients of variation were less than 10% for each point. The maximal stimulation for each experiment was defined as the activity obtained with 30 μM PGE_1. The *inset* gives activation constants (K_{act}) and Hill coefficients (n_H) (mean ± standard error of the mean) for the data shown.

6-Keto-PGE_1 was less potent than PGE_1 but exhibited maximal efficacy. PGE_2 was tested at concentrations less than 0.1 mM in order to avoid any membrane perturbations by a higher concentration of the lipophilic agonist and to keep the ethanol concentration in the assay less than 0.7%. If one assumes that the maximal PGE_2-stimulated value is the same as the maximal PGE_1 response (as the data have been plotted), then the apparent K_{act} for PGE_2 is 15.3 ± 0.5 μM and $n_H = 0.76 \pm 0.02$.

The alternative that PGE_2 is a partial agonist in this system was ruled out by examining the additivity of PGE_2 with either PGE_1 or PGI_2. This is shown in Fig. 3. Log dose-response curves were determined for PGE_1 in the absence or presence of 3 μM PGE_2 (Fig. 3A). Similar plots were obtained for PGI_2 in the absence or presence of 3 μM (Fig. 3B) or 10 μM (Fig. 3c) PGE_2. The data were plotted over computer-generated curves that describe the behavior expected of PGE_2 as either a full agonist at the same or different receptors or as a partial agonist at the same or different receptors (22, 23). The parameters of K_{act} and intrinsic activity used were taken from within the same experiment to eliminate variability between experiments. In each case, the data points for PGE_1 or

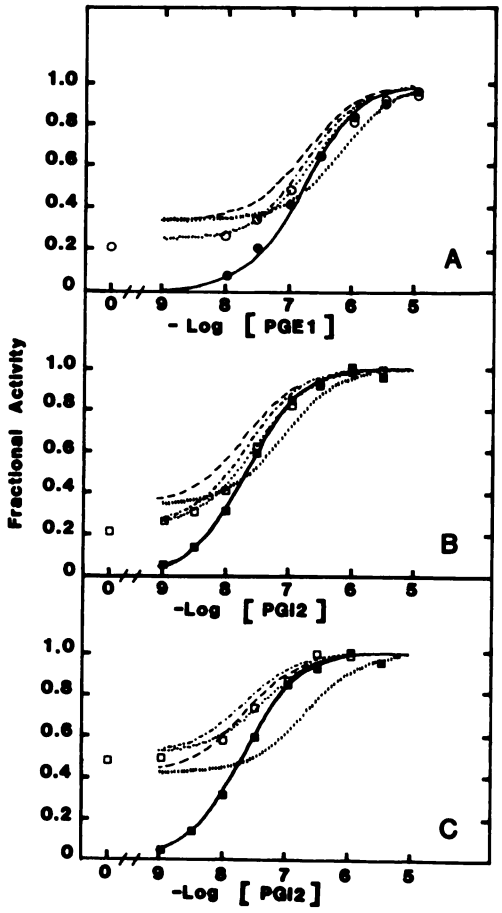

FIG. 3. *Additive interactions of PGE_2 with PGE_1 or PGI_2*

Theoretical curves were plotted by an Apple II-plus desk-top computer to describe the following relationships of PGE_2 with a full agonist: in the absence of PGE_2 (——); PGE_2 as a full agonist competing at the same receptor (·····); PGE_2 as a full agonist acting at a distinct receptor (·-·-·); PGE_2 as a partial agonist at the same receptor (''''); or PGE_2 as a partial agonist at a distinct receptor (– – –). Equations are those of Ariëns and Simonis (22, 23). The values of K_{act} were determined from the same experiment by least-squares fit to a linear Hill plot and were as follows: PGE_2 as a full agonist: intrinsic activity = 1.0, K_{act} = 9.5 μM; PGE_2 as a partial agonist: intrinsic activity = 0.47, K_{act} = 1.1 μM; PGE_1 (A): intrinsic activity = 1.0, K_{act} = 174 nM; PGI_2 (B and C): intrinsic activity = 1.0, K_{act} = 21.9 nM. PGE_2 was absent (*closed symbols*) or present (*open symbols*) at 3 μM in A and B and 10 μM in C. The *symbols* represent actual data points from the experiment.

PGI_2 fall accurately on curves generated for that agonist using the K_{act} obtained by least-squares fit of the data. Similarly, in each case the data points in the presence of PGE_2 most closely resemble the behavior of a full agonist. In the experiment shown, theoretical plots were obtained for PGE_2 as a partial agonist having an intrinsic activity of 0.47, the percentage of maximal activity of 10 μM PGE_2 in this experiment. However, similar plots obtained for PGE_2 having an intrinsic activity of 0.77 (see Fig. 2) evoke similar conclusions. These were not shown in the diagram for the sake of clarity.

Characterization of a receptor-mediated response. Because of the numerous examples of lipid influences on adenylate cyclase (see ref. 24 and references contained therein), it is important to establish that stimulation of the enzyme results from an interaction of prostaglandins with a receptor moeity rather than from membrane lipid bilayer perturbations due to the hormone or the solvent. The following experiments were performed to determine whether the stimulation by these arachidonic acid metabolites is consistent with a receptor-mediated mechanism. Guanine nucleotides are essential regulators of receptor-mediated adenylate cyclase activity in mammalian cells. According to current concepts (for review, see ref. 25), GTP binds to a regulatory component that subsequently activates the catalytic protein. Concurrent with hydrolysis of GTP, the activity of the catalytic protein returns to a basal rate. The hormone-receptor complex interacts with the guanine nucleotide regulatory component, possibly by altering the affinity for bound GDP, thereby permitting rebinding of GTP. Thus, the hormone would provide the stimulus for guanine nucleotide-promoted activation. Figure 4A demonstrates that PGE_1 and the arachidonic acid metabolites increase the capacity of GTP to activate adenylate cyclase. GTP half-maximally activated at 0.1 μM for each agonist, including PGE_2. The maximal stimulation was 8- to 10-fold over hormone alone for PGE_1, 6-keto-PGE_1, and PGI_2 when tested at fully activating concentrations. The GTP dependence of PGD_2 is difficult to assess because PGD_2 is a relatively poor stimulator of adenylate cyclase at any GTP concentration. In this experiment, stimulation by 20 μM PGD_2 was 51% above basal in the absence of exogenous nucleotide; however, at 1 μM GTP the hormonal effect increased to 84% above GTP alone.

The ability of the agonist to facilitate a receptor-mediated alteration in guanine nucleotide-dependent activity can also be assessed using Gpp(NH)p, a nonhydrolyzable GTP analogue. In Fig. 4B, the agonists present at their maximally stimulating concentrations facilitated activation of adenylate cyclase by Gpp(NH)p to the same extent (2- to 3-fold). Enhancement of activation by PGE_2 (at its estimated half-maximal concentration) was nearly as great. These results, using Gpp(NH)p as the activating nucleotide, suggest that PGD_2 also facilitates guanine nucleotide exchange. In contrast, 6-keto-$PGF_{1\alpha}$, a prostaglandin having essentially no agonist potential in the presence of GTP (Fig. 1), was unable significantly to enhance Gpp(NH)p activation.

The initial rate of Gpp(NH)p activation is particularly influenced by the presence of hormones, possibly reflecting a hormone receptor-mediated release of bound nucleotide or a hysteretic modification of the protein. As shown in Fig. 4C, the rate of adenylate cyclase activity increased with time when Gpp(NH)p activated in the absence of hormone. However, the addition of maximally stimulating concentrations or PGE_1 and PGI_2 permitted nearly linear activity from the earliest time point (30 sec at 30°). PGE_2 at its half-maximally stimulating concentration partially precluded the lag.

A modification in the requirement for the divalent cation Mg^{2+} is also associated with hormonal stimulation of adenylate cyclase (for review see ref. 26). The $MgCl_2$ concentration required for half-maximal activation in the presence of 1 μM GTP was 2.5 mM. Upon addition of maximally stimulating concentrations of either PGE_1 or PGI_2, the ED_{50} was reduced to 1 mM $MgCl_2$ (data not shown). For both hormones, 90% of maximal activity was attained at 5 mM $MgCl_2$ and the plateau in activity

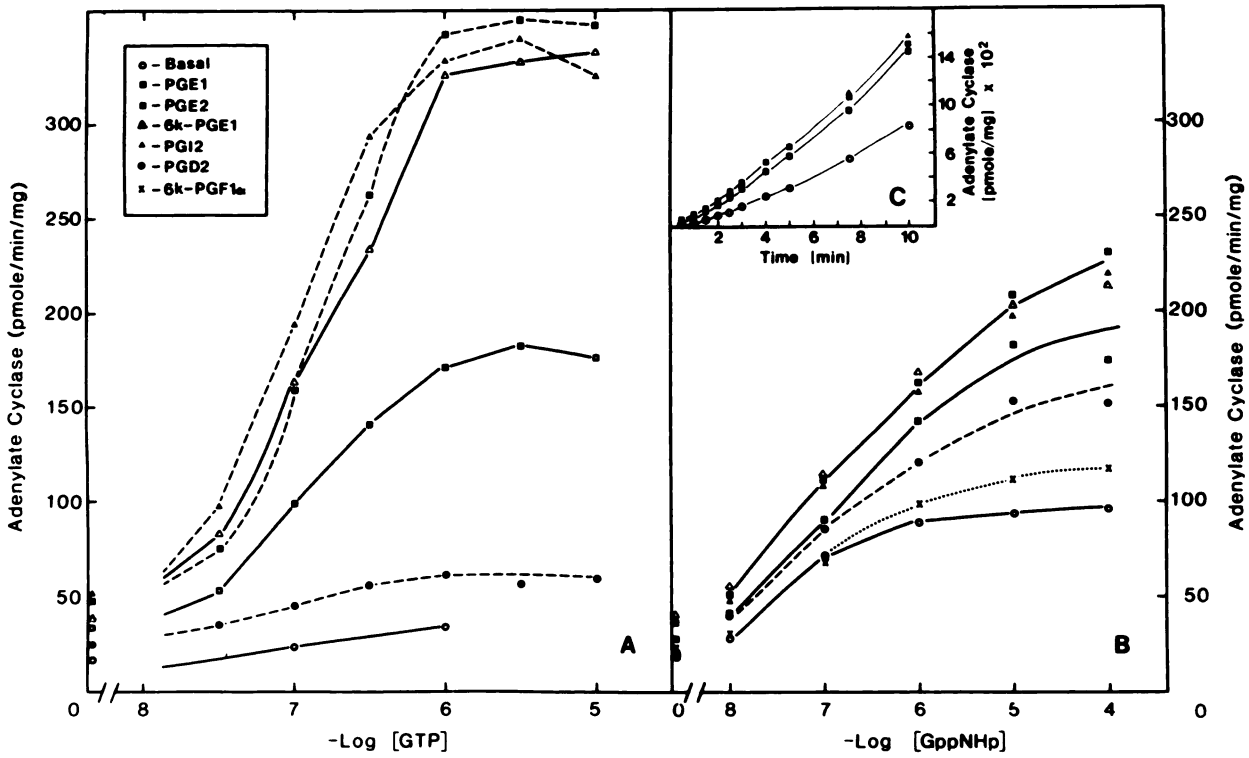

FIG. 4. *Guanine nucleotide requirement for prostanoid agonists*

A. Adenylate cyclase activity was measured as described in the text at the GTP concentrations indicated. The agonists were present at 10 μM with the exception of PGD_2 (20 μM) and PGI_2 (1 μM).

B. Adenylate cyclase activity was measured as above except that GTP was omitted and the Gpp(NH)p concentration was varied. The assay proceeded for 10 min at 30°. All agonists were present at 10 μM.

C. Time-course of Gpp(NH)p activation. Gpp(NH)p (1 μM) and agonists were present in a reaction mixture warmed to 30°. At 0 min, 0.1 volume of protein, also warmed to 30°, was added. Aliquots were removed and placed in stopping solution at the times indicated. Agonists were present at 10 μM PGE_2, 1 μM PGE_1, and 0.2 μM PGI_2.

extended beyond 15 mM. In contrast, activity peaked sharply at 1.5 mM $MnCl_2$ for either PGE_1 or PGI_2, and the ED_{50} decreased from 0.95 mM to 0.69 mM Mn^{2+} in the presence of prostanoid agonist. Thus, for both agonists, divalent cations are required at a concentration somewhat greater than equimolar to ATP.

Desensitization of response. Some insight into the question of identity of receptors was provided by examining the refractory state that develops in response to exposure *in situ* to an agonist. Cells were incubated with PGE_1 until a subsequent exposure to PGE_1 would not promote the accumulation of cyclic AMP. Membranes were fractionated so that hormone stimulation of cyclic AMP synthesis could be determined in the absence of

TABLE 2

Adenylate cyclase activity in membranes from refractory cells

Confluent cells on 150-cm^2 plates were rinsed twice and subsequently incubated with 10 ml of medium (minus serum) with or without 1 μM PGE_1 or 1 μM 6-keto-PGE_1. After 150 min at 37°, the cells were rinsed and then removed from the plate with the dissociation medium as described under Materials and Methods. Cells were rinsed twice by sedimentation, and a membrane fraction was prepared as described except that the sucrose gradient step was omitted and adenylate cyclase activity was determined immediately. Concentrations of activators were as follows: 1 μM GTP, 100 μM Gpp(NH)p, 10 μM 2-Cl-adenosine, 10 μM prostaglandins, and 10 mM NaF. Values are means of triplicate determinations. The coefficient of variation was less than 10% for each value.

Activator	Adenylate cyclase desensitized by					
	PGE_1			6-Keto-PGE_1		
	Control	Treated	% Decrease	Control	Treated	% Decrease
	pmoles/min/mg protein			*pmoles/min/mg protein*		
GTP	19.4	12.9	34	14.0	12.3	12
Gpp(NH)p	70.2	33.8	52	62.0	52.0	16
GTP + 2-Cl-Adenosine	32.7	24.3	26	23.9	26.1	8
GTP + PGE_1	136.4	18.7	86	148.8	52.3	65
GTP + PGI_2	125.8	16.3	87	102.2	33.3	67
GTP + 6-keto-PGE_1	116.0	12.6	89	145.1	46.8	68
NaF	79.8	78.5	2	86.2	87.1	0

the contribution of soluble phosphodiesterase induced by cyclic AMP. A parallel experiment was performed in which cells were made refractory with 6-keto-PGE_1.[2] The results are shown in Table 2. When compared with adenylate cyclase from cells incubated in the absence of hormone, the NaF response in membranes from refractory cells remained unaltered. In contrast, the responses to all hormonal stimuli and guanine nucleotides were reduced in the PGE_1-desensitized cell membranes. This is consistent with the occurrence of a "heterologous" desensitization such as reported for other cell types following exposure to PGE_1 (6, 27, 28). Nevertheless, the responses to the prostanoid agonists shown here were reduced more than the responses to guanine nucleotides or to 2-Cl-adenosine. Similarly, the adenylate cyclase responses to prostaglandins but not to NaF were reduced in cells made refractory to 6-keto-PGE_1. In this case, the 2-Cl-adenosine activity was not significantly reduced. These results suggest a strong functional association, if not identity, of the receptors for PGE_1, 6-keto-PGE_1, PGE_2, and PGI_2.

DISCUSSION

This study was undertaken to develop a better understanding of the prostaglandin-stimulated adenylate cyclase of N18TG2 neuroblastoma cells. This clone was chosen because the adenylate cyclase response and a [^{3}H]PGE_1 binding activity associated with it had been previously described by Brunton *et al.* (2). Numerous studies have demonstrated that several arachidonic acid metabolites stimulate cyclic AMP production in specific organ systems (3, 4, 6–10). The experiments communicated here confirm earlier reports that PGI_2 stimulates adenylate cyclase activity in neuroblastoma cells (10, 11). In contrast, cyclic AMP accumulation in response to PGD_2, reported for two neuroblastoma clones (9), could not be demonstrated in N18TG2. The present studies are the first to report neuroblastoma cell adenylate cyclase stimulation by 6-keto-PGE_1.

This investigation has pharmacologically compared the arachidonic acid metabolites to PGE_1 and determined that PGE_2, PGI_2, and 6-keto-PGE_1 stimulate adenylate cyclase with the same intrinsic activity. Although each is a full agonist, the potencies differ over several orders of magnitude. A very similar pattern was observed in a second C1300 clone, N4TG1.[3]

The receptor(s) characterized in this investigation exhibits properties typical of receptors for other hormones that stimulate adenylate cyclase. Maximally activating prostanoid agonists augment the response to GTP and Gpp(NH)p. This is consistent with a mechanism of more rapid occupancy of the GTP site by a nonhydrolyzable analogue. In addition, the prostanoid agonists reduce the requirement for free divalent cation.

The notion of prostaglandin receptor subtypes is suggested by studies in a variety of systems characterized by a differential functional response to prostaglandins (5, 7, 8). The singularity of a prostaglandin receptor associated with adenylate cyclase is neither intuitively obvious nor previously established by pharmacological investigations. Certainly the steric structure and charge distribution of prostacyclin should differ from that of PGE_2 or PGD_2, suggesting different binding requirements in a receptor molecule (29, 30). In this study, the subject of multiplicity of receptors has been approached indirectly by the examination of down-regulation of the response. Others have also observed that desensitization to PGE_1 or PGE_2 induced a concomitant desensitization to PGI_2 (5, 31). A single receptor for these arachidonic acid metabolites could have been made refractory by exposure either to PGE_1 or to 6-keto-PGE_1 in the N18TG2 cells. Alternatively, distinct prostanoid receptor types, each associated with adenylate cyclase, may be present. However, these receptors as a class could be more sensitive to the effects of a prostanoid agonist than are receptors for structurally unrelated agonists. Because we do not understand the mechanism by which the desensitization occurs, this latter explanation is highly speculative. Receptor binding analyses are necessary before a definitive statement can be made.

[2] PGI_2 was ineffective in producing a refractory state, probably because a sufficiently high concentration could not be maintained for a prolonged period of time under the protocols used.

[3] A. C. Howlett, unpublished observations.

ACKNOWLEDGMENTS

The technical assistance of R. M. Fleming and M. M. McDonnell and the clerical help of J. Herbert are greatly appreciated.

REFERENCES

1. Gilman, A. G., and M. Nirenberg. Regulation of adenosine 3′,5′-cyclic monophosphate metabolism in cultured neuroblastoma cells. *Nature* (*Lond.*) **234**:356–358 (1971).
2. Brunton, L. L., R. A. Wiklund, P. M. Van Arsdale, and A. G. Gilman. Binding of [^{3}H]prostaglandin E_1 to putative receptors linked to adenylate cyclase of cultured cell clones. *J. Biol. Chem.* **251**:3037–3044 (1976).
3. Tateson, J., S. Moncada, and J. R. Vane. Effects of prostacyclin (PGX) on cyclic AMP concentrations in human platelets. *Prostaglandins* **13**:389–399 (1977).
4. Gorman, R. R., S. Bunting, and O. V. Miller. Modulation of human platelet adenylate cyclase by prostacyclin (PGX). *Prostaglandins* **13**:377–388 (1977).
5. Eggerman, T. L., L. A. Harker, N. H. Andersen, and C. H. Wilson. Evaluation of prostaglandin and prostacyclin antagonism at platelet receptors by aggregometry. *Adv. Prostaglandin Thromboxane Res.* **6**:389–393 (1980).
6. Tougui, Z., L. Do Khac, and S. Harbon. Modulation of cyclic AMP content of the rat myometrium: desensitization to isoproterenol, PGE_2 and prostacyclin. *Mol. Cell. Endocrinol.* **20**:17–34 (1980).
7. Simon, B., and H. Kather. Human colonic adenylate cyclase: stimulation of enzyme activity by vasoactive intestinal peptide and various prostaglandins via distinct receptor sites. *Digestion* **20**:62–67 (1980).
8. Robertson, R. P., D. R. Storm, N. H. Andersen, and K. R. Westcott. Prostaglandin E and prostacyclin stimulate a common adenylate cyclase but bind to separate receptors in liver plasma membrane. *Clin. Res.* **27**:446A (1979).
9. Shimizu, T., N. Mizuno, T. Amano, and O. Hayaishi. Prostaglandin D_2, a neuromodulator. *Proc. Natl. Acad. Sci. U. S. A.* **76**:6231–6234 (1979).
10. Ortmann, R. Effect of PGI_2 and stable endoperoxide analogues on cyclic nucleotide levels in clonal cell lines of CNS origin. *F. E. B. S. Lett.* **90**:348–352 (1978).
11. Blair, I. A., C. M. Hensby, and J. MacDermot. Prostacyclin-dependent activation of adenylate cyclase in a neuronal somatic cell hybrid: prostanoid structure-activity relationships. *Br. J. Pharmacol.* **69**:519–525 (1980).
12. Paul, J. *Cell and Tissue Culture.* Churchill Livingstone, New York, 95 (1975).
13. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
14. Brostrom, C. O., and C. Kon. An improved protein binding assay for cyclic AMP. *Anal. Biochem.* **58**:459–468 (1974).
15. Gilman, A. G. A protein binding assay for adenosine 3′:5′-cyclic monophosphate. *Proc. Natl. Acad. Sci. U. S. A.* **67**:305–312 (1970).
16. Ross, E. M., M. E. Maguire, T. W. Sturgill, R. L. Biltonen, and A. G. Gilman. Relationship between the β-adrenergic receptor and adenylate cyclase: studies of ligand binding and enzyme activity in purified membranes of S49 lymphoma cells. *J. Biol. Chem.* **252**:5761–5775.
17. Salomon, Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548 (1974).
18. Änggård, E., and B. Samuelsson. Prostaglandins and related factors. Metab-

olism of prostaglandin E_1 in guinea pig lung: the structures of two metabolites. *J. Biol. Chem.* **239**:4097–4100 (1964).
19. Quilley, C. P., J. C. McGiff, W. H. Lee, F. F. Sun, and P. Y.-K. Wong. 6-Keto-PGE_1: a possible metabolite of prostacyclin having platelet antiaggregatory effects. *Hypertension* **2**:524–528 (1980).
20. Cho, M. J., and M. A. Allen. Chemical stability of prostacyclin (PGI_2) in aqueous solutions. *Prostaglandins* **15**:943–954 (1978).
21. Wynalda, M. A., and F. A. Fitzpatrick. Albumins stabilize prostaglandin I_2. *Prostaglandins* **20**:853–861 (1980).
22. Ariëns, E. J., and A. M. Simonis. A molecular basis for drug action. *J. Pharm. Pharmacol.* **16**:137–157 (1964).
23. Ariëns, E. J., and A. M. Simonis. A molecular basis for drug action: the interaction of one or more drugs with different receptors. *J. Pharm. Pharmacol.* **16**:289–312 (1964).
24. Sinensky, M., K. P. Minnemin, and P. B. Molinoff. Increased membrane acyl chain ordering activates adenylate cyclase. *J. Biol. Chem.* **254**:9135–9141 (1979).
25. Abramowitz, J., R. Iyengar, and L. Birnbaumer. Guanyl nucleotide regulation of hormonally-responsive adenyl cyclases. *Mol. Cell. Endocrinol.* **16**:129–146 (1979).
26. Cech, S. Y., W. C. Broaddus, and M. E. Maguire. Adenylate cyclase: the role of magnesium and other divalent cations. *Mol. Cell. Biochem.* **33**:67–92 (1980).
27. Su, Y.-F., L. X. Cubeddu, and J. P. Perkins. Regulation of adenosine 3′:5′-monophosphate content of human astrocytoma cells: desensitization to catecholamines and prostaglandins. *J. Cyclic Nucleotide Res.* **2**:257–270 (1976).
28. Clark, R. B., and R. W. Butcher. Desensitization of adenylate cyclase in cultured fibroblasts with prostaglandin E_1 and epinephrine. *J. Biol. Chem.* **254**:9373–9378 (1979).
29. Kothekar, V. Specificity and molecular mechanism of abortificient action of prostaglandins. *Int. J. Quant. Chem.* **20**:167–178 (1981).
30. DeTitta, G. T., D. A. Langs, and M. G. Erman. Structure-activity relationships: a look at the structures of PG-related molecules. *Adv. Prostaglandin Thromboxane Res.* **6**:381 (1980).
31. Gorman, R. R., and N. K. Hopkins. Agonist-specific desensitization of PGI_2-stimulated cyclic AMP accumulation by PGE_1 in human foreskin fibroblasts. *Prostaglandins* **19**:1–15 (1980).

Send reprint requests to: Dr. Allyn C. Howlett, Department of Pharmacology, St. Louis University School of Medicine, St. Louis, Mo. 63104.