Proc. Natl. Acad. Sci. USA
Vol. 76, No. 12, pp. 6231–6234, December 1979
Biochemistry

Prostaglandin D_2, a neuromodulator

(prostaglandin D synthetase/enzyme distribution/neuroblastoma cell/cyclic AMP)

TAKAO SHIMIZU, NOBORU MIZUNO*, TAKEHIKO AMANO†, AND OSAMU HAYAISHI

Department of Medical Chemistry, and *Department of Anatomy, Kyoto University Faculty of Medicine, Sakyo-ku, Kyoto 606, Japan; and †Mitsubishi-Kasei Institute of Life Sciences, Machida-shi, Tokyo 194, Japan

Contributed by Osamu Hayaishi, September 17, 1979

ABSTRACT **The distribution of prostaglandin D synthetase activity was determined in various tissues of rat by using the supernatant fraction (10,000 × *g*, 20 min) of the homogenates. The highest activity was found in brain, spinal cord, and alimentary tract. The activity was ubiquitously distributed in all parts of brain, and the highest specific activity was found in hypothalamus and thalamus. Homogenates of two neuroblastoma cell lines were found to produce prostaglandin D_2, whereas a glioma cell line was almost inactive. Prostaglandin D_2 is a potent and specific activator of the adenylate cyclase system of cultured neuroblastoma cells, suggesting the possibility that it may act as a neuromodulator in the central nervous system.**

Prostaglandin (PG) D synthetase (isomerase) was first detected in the cytosol fraction of various rat tissues (1). Recently, the enzyme was purified to homogeneity from rat brain (2) and clearly distinguished from glutathione *S*-transferase. To clarify the physiological role of PGD_2, we carried out a systematic survey of the enzyme's distribution. In this report, we present quantitative data on the occurrence of PGD synthetase in rat tissues, particularly in brain and cultured neuroblastoma cells. Furthermore, PGD_2 at about 10 nM was demonstrated to increase the cyclic AMP level rapidly in the neuroblastoma cells. The possible significance of these findings is discussed.

MATERIALS AND METHODS

Materials. [1-^{14}C]Arachidonic acid (60.2 mCi/mmol;/Ci = 3.7 × 10^{10} becquerels) was purchased from the Radiochemical Centre (Amersham). Arachidonic acid was a product of P-L Biochemicals. PGD_2, $-E_2$, $-E_1$, $-F_{2\alpha}$, $-F_{2\beta}$, and $-B_2$, 6-keto-$PGF_{1\alpha}$, and thromboxane B_2 were kindly donated by M. Hayashi (Ono Central Research Institute). L-Norepinephrine bitartrate, 3-hydroxytyramine (dopamine) HCl, DL-propranolol·HCl, 9,10-dihydroxyergotamine tartrate, and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma. Naloxone·HCl and chlorpromazine·HCl were generous gifts from Endo Laboratories and Takeda Research Laboratories (Osaka), respectively. Dithiothreitol and *p*-chloromercuribenzoic acid were supplied by Wako Pure Chemicals (Osaka); 1-chloro-2,4-dinitrobenzene and phenoxybenzamine·HCl were from Tokyo Kasei Kogyo (Tokyo); and precoated silica gel F254 glass plates were from E. Merck (Darmstadt). [1-^{14}C]PGH_2 was prepared as described (2).

Preparation of Tissue Homogenates. Male Wistar rats (300–350 g) were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg), perfused with 200 ml of ice-cold saline, and killed by exsanguination. Eleven organs (whole brain, spinal cord, lung, heart, liver, stomach, small intestine, kidney, spleen, adrenal gland, and vesicular gland) were quickly removed. The brain was chilled on ice and separated into 11 parts—cerebral neocortex, cerebellum, pons and medulla oblongata, midbrain, hypothalamus, thalamus, bulbus olfactorius, hippocampus, caudoputamen, pineal body, and meninges. These tissues were weighed and homogenized with 2 vol of 10 mM potassium phosphate buffer (pH 6.0) containing 0.5 mM dithiothreitol in a Polytron homogenizer. Mouse neuroblastoma cells (NS-20 and N1E-115) and rat glioma cells (C6 BU-1) (see below) were sonicated with a Branson Sonifier model W 185D (output 4, for 1.5 min). Homogenates were centrifuged at 10,000 × *g* for 20 min at 4°C and the supernatant solutions were used as enzyme sources.

Assay of Enzyme Activity. The PGD synthetase activity was determined as described (2) with 50 μM [1-^{14}C]PGH_2 with a higher specific activity (165,000 cpm/2.5 nmol). Glutathione *S*-transferase was assayed according to the method of Habig *et al.* (3). Protein was determined by the method of Lowry *et al.* (4) with bovine serum albumin as a standard.

Cell Culture. N1E-115, NS-20 (5), and C6 BU-1 (6) cells were grown in 60-mm petri dishes, in Dulbecco–Vogt modification of Eagle's minimal essential medium containing 5% fetal calf serum, in a humidified atmosphere of 10% CO_2/90% air at 37°C. After confluency, cells were washed three times with the same medium without serum.

Assay of Cyclic AMP Accumulation in Intact Cells. N1E-115 cell monolayers (1.6 × 10^6 cells, about 2 mg of protein per dish) were preincubated for 30 min at 24°C with 2 ml of medium lacking $NaHCO_3$ but supplemented with 25 mM Hepes buffer (pH 7.4) and 0.5 mM IBMX. Reactions were started by the addition of 20 μl of PG or other compounds, or both. Cells were incubated for the time specified and then the medium was removed by aspiration. One milliliter of ice-cold 7% (wt/vol) trichloroacetic acid was added to terminate the reaction and to extract intracellular cyclic AMP. Dishes were washed with an additional 1 ml of trichloroacetic acid. The trichloroacetic acid extracts and washes were centrifuged at 10,000 × *g* for 10 min. Cyclic AMP was purified (7) and measured by radioimmunoassay according to the method of Honma *et al.* (8). Cell protein was measured in the 0.5 M NaOH-solubilized trichloroacetic acid precipitates.

RESULTS

Tissue Distribution of PGD Synthetase. The PGD synthetase activity was high in brain, spinal cord, and alimentary tract (Table 1). The activity was almost completely inhibited by the addition of 1 mM *p*-chloromercuribenzoic acid but not by 1 mM 1-chloro-2,4-dinitrobenzene, a substrate of glutathione *S*-transferase. Although serum albumin or glutathione *S*-transferase has been reported to convert PGH_2 to PGD_2 (9, 10), these proteins did not seem to participate in the observed ac-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: PG, prostaglandin; IBMX, 3-isobutyl-1-methylxanthine.

Table 1. Distribution of PGD synthetase in various tissues of rat

Tissue	Wet weight, g	Specific activity* PGD synthesis	+ 1 mM DNB†	+ 1 mM PCMB†
Whole brain	1.8 ± 0.1	2.54 ± 0.1	2.44 ± 0.09	0.03
Spinal cord	0.6 ± 0.2	1.99 ± 0.08	1.82 ± 0.04	0.02
Lung	2.6 ± 0.2	0.39 ± 0.03	0.10 ± 0	0.03
Heart	1.4 ± 0.3	0.03 ± 0	0.03 ± 0	0.02
Liver	12.6 ± 1.5	0.19 ± 0.03	0.08 ± 0.01	0.03
Stomach	4.8 ± 0.3	0.43 ± 0.04	0.38 ± 0.02	0.04
Intestine	1.4 ± 0.2	0.84 ± 0.06	0.69 ± 0.04	0.04
Kidney	3.0 ± 0.4	0.03 ± 0	0.02 ± 0.01	0.02
Spleen	0.8 ± 0.1	0.14 ± 0.01	0.05 ± 0.01	0.03
Adrenal gland	0.08 ± 0.01	0.02 ± 0	0.02 ± 0.01	0.02
Vesicular gland	0.9 ± 0.2	0.03 ± 0	0.03 ± 0.01	0.03

Values are expressed as mean ± SEM of five experiments. Five rats were used in each experiment.
* Shown as nmol/min per mg of protein at 24°C.
† Enzyme activity was determined when 1 mM 1-chloro-2,4-dinitrobenzene (DNB) or *p*-chloromercuribenzoic acid (PCMB) was added to the standard incubation mixture.

tivity of these crude tissue preparations. On the other hand, glutathione *S*-transferase activity was detectable in lung, liver and spleen (data not shown), suggesting that the apparent PGD synthesis of these organs was at least partly attributable to this enzyme. Rat platelets produced neither PGD_2 nor thromboxane B_2 under the present assay conditions.

The regional distribution of PGD synthetase in rat brain is shown in Table 2. The enzyme activity was present in all parts of the brain [1.5–5.4 milliunits/mg of protein except for meninges (0.11, 0.13) and white matter (0.24; 0.36)]. The specific activities were 2–3 times higher in hypothalamus and thalamus. The specific activities (means from two to five animals) found in other mammalian brains varied as follows: mouse, 2.8; guinea pig, 2.5; cow, 1.4; swine, 1.3; rabbit, 0.8; and cat, 0.3.

PGD Synthesis in Homogenates of Neuroblastoma Cells. To ascertain whether the high activity in brain is attributable to neuronal cells or glial cells, cultured cell lines were tested. Marked PGD synthetase activity was observed in neuroblastoma cells, whereas the activity in glioma cells was almost insignificant (Fig. 1). The major product in NS-20 cells (cholinergic clone) and N1E-115 cells (adrenergic clone) was PGD_2 (40–65% of total products from PGH_2) with minor products including PGE_2 (8–12%), $PGF_{2\alpha}$ (10–15%), 6-keto-$PGF_{1\alpha}$ (2–4%), and 12*L*-hydroxy-5,8,10-heptadecatrienoic acid (10–14%). Addition of 1 mM reduced glutathione did not affect the pattern of products other than a slight increment of PGE_2 synthesis (12–17%). The optimal pH for PGD synthesis was ≈8 and the activity was inhibited by 90% with 1 mM *p*-chloromercuribenzoic acid. These properties were similar to those of the purified PGD synthetase from rat brain (2).

Stimulation of Cyclic AMP Formation by PGD_2. When cultured neuroblastoma cells (N1E-115) were incubated in the presence of various PGs for the time indicated, PGD_2 caused a rapid and pronounced increase in the intracellular cyclic AMP concentration; the effect of PGE_1 was much more sluggish (Fig. 2*A*). However, neither PGE_2 nor $PGF_{2\alpha}$ had a significant effect. Dose-dependency of cyclic AMP formation is illustrated in Fig. 2*B*. A significant increase in intracellular cyclic AMP was observed with PGE_1 and PGD_2 at 10 nM but not with PGE_2 or $PGF_{2\alpha}$. The effect of PGE_1 and PGD_2 was almost the same between 1 and 100 nM. Above μM, the effect of PGD_2 seemed to level off, whereas that of PGE_1 was dose-dependent even at higher concentrations. The release of cyclic AMP into the medium was small (about 10% of the total cyclic AMP formed) when cells were incubated with 1 μM or 0.1 mM PGD_2 for 10 min.

The effect of PGD_2 was examined in the presence and absence of catecholamines or their antagonists. PGD_2-stimulated cyclic AMP synthesis was not blocked at all by dihydroergotamine, DL-propranolol, or chloropromazine at 10 μM (Table 3), indicating that the effect was not via α- or β-receptors of catecholamines or dopamine receptors. However, phenoxybenzamine (10 μM) inhibited both basal and PGD_2-dependent cyclic AMP formation by about 80%. Naloxone·HCl (50 μM),

Table 2. Regional distribution of PGD synthetase in rat brain

Region	Wet weight, mg	Specific activity* Per mg of protein	Per g of tissue
Cerebral neocortex	247.5 ± 14.8	2.95 ± 0.11	29.5 ± 3.1
Cerebellum	272.3 ± 2.7	1.61 ± 0.15	18.1 ± 2.2
Pons and medulla oblongata	210.3 ± 3.3	2.59 ± 0.15	28.2 ± 2.4
Midbrain	154.1 ± 7.1	2.02 ± 0.15	27.5 ± 1.1
Hypothalamus	18.4 ± 0.9	5.44 ± 0.47	110.6 ± 4.7
Thalamus	67.0 ± 3.6	3.15 ± 0.19	53.5 ± 4.4
Bulbus olfactorius	44.0 ± 3.0	1.53 ± 0.09	29.2 ± 2.1
Hippocampus	62.2 ± 1.3	2.04 ± 0.22	31.0 ± 2.7
Caudoputamen	49.4 ± 1.6	1.64 ± 0.14	20.2 ± 1.7
Pineal body†	0.17;0.16	2.22;2.39	28.4;29.5
Meninges‡	—	0.11;0.13	0.6;0.6

All values are means ± SEM of five experiments. In each experiment five rats were used.
* Shown as nmol/min at 24°C
† Two experiments (30 rats in each experiment).
‡ Two experiments (5 rats in each experiment).

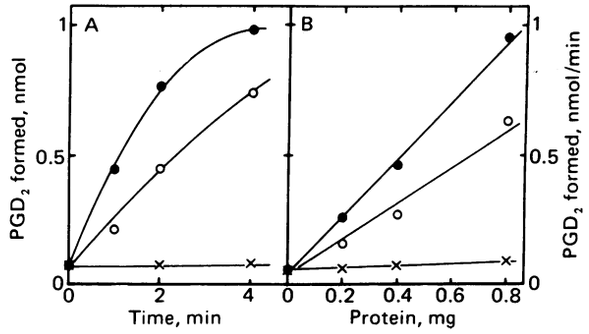

FIG. 1. PGD synthesis in mouse neuroblastoma cells (NS-20 and N1E-115) and rat glioma cells (C6 BU-1). (*A*) Time course of reaction. Cell homogenates (0.4 mg of protein) were incubated with [1-^{14}C]-PGH_2. (*B*) Effect of varying enzyme amounts on the rate of PGD_2 synthesis. ●, N1E-115; ○, NS-20; ×, C6 BU-1.

an opiate antagonist, reversed the inhibition almost completely (Exp. 2). Norepinephrine (3 μM) inhibited PGD_2-stimulated cyclic AMP synthesis by 20% without altering basal cyclic AMP levels, whereas dopamine (3 μM) had no effect.

DISCUSSION

PGD synthetase has been recently demonstrated with several other tissues (11–14) or cultured cells (15–17) in addition to rat brain. In this communication, we report quantitative data on enzyme distribution which indicate a marked difference in the amount of enzyme in different organs of the rat. High activity was apparent in the central nervous system as well as in the gastrointestinal tract, thereby reflecting the importance of PGD_2 in these systems. In brain, the activity was ubiquitously distributed with a relatively higher specific activity in hypothalamus and thalamus. The activity was at least 40 times higher in neuroblastoma cells than in glioma cells, suggesting a significant role of PGD_2 in neural function.

Biological functions of PGD_2 have not been well understood. Intravenous injection of PGD_2 into a monkey caused a sedative effect without significant cardiovascular events (18). PGD_2 was also reported to be an inhibitor of platelet aggregation (19). Although Abdel-Halim *et al.* (20, 21) described the presence of high amounts of PGD_2 in rat brain and its release from brain

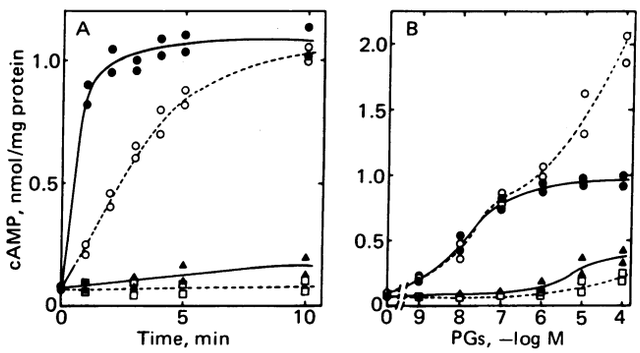

FIG. 2. Effects of various PGs on intracellular cyclic AMP (cAMP) accumulation in N1E-115 cells. (*A*) Duplicate petri dishes (60-mm diameter), each with about 2 mg of cell protein, were incubated in an atmosphere of 10% CO_2/90% air with 2 ml of medium per dish supplemented with 0.5 mM IBMX for 30 min at 24°C. Then, PGs at 3 μM were added and incubated for the indicated time, and intracellular cyclic AMP was determined. (*B*) Effects of PGs at various concentrations of intracellular cyclic AMP accumulation. Incubation was carried out for 10 min at 24°C. Final concentration of ethanol (used as a solvent of PGs) was <0.1%, which had no effect on cyclic AMP accumulation by itself (7). ●, PGD_2; ○, PGE_1; △, PGE_2; □, $PGF_{2\alpha}$.

Table 3. Effects of catecholamines and antagonists on intracellular cyclic AMP concentration in N1E-115 cells

Addition	Conc., μM	Intracellular cyclic AMP, pmol/mg cell protein: 0 PGD_2	3μM PGD_2
	Exp. 1		
None		52	1080
L-Norepinephrine	3	46	770
DL-Propranolol	10	43	980
Phenoxybenzamine	10	13	240
Dihydroergotamine	10	43	1040
Dopamine	3	44	960
Chloropromazine	10	35	1020
	Exp. 2		
None		61	920
Phenoxybenzamine	10	21	280
Phenoxybenzamine + naloxone	10 / 50	54	880
Naloxone	50	67	980

Duplicate petri dishes (60-mm diameter), each with about 2 mg of cell protein, were preincubated with 2 ml of medium without serum but supplemented with 0.5 mM IBMX, 0.1 mM ascorbic acid, and 0.1 mM pargyline-HCl for 30 min at 24°C. Catecholamines (dissolved in 20 μl of 0.1 mM ascorbic acid) or antagonists were then added in the presence or absence of 3 μM PGD_2. Dishes were incubated for an additional 10 min at 24°C, and then intracellular cyclic AMP was determined.

slices, the physiological function of PGD_2 in the central nervous system remains obscure. Our data show that PGD_2 is a specific and potent activator of adenylate cyclase of cultured neuroblastoma cells. Because the experiments were carried out in the presence of a phosphodiesterase inhibitor (IBMX), the observed increase in cyclic AMP concentration is most probably due to activation of adenylate cyclase rather than inhibition or inactivation of phosphodiesterase. It is interesting to note that PGD_2 caused a rapid increase in intracellular cyclic AMP, and no more synthesis was observed after about 1 min (Fig. 2*A*). Because the excretion of cyclic AMP from cells was negligible, the apparent cessation of cyclic AMP formation appeared to be due to either rapid degradation of PGD_2 or desensitization of membrane receptor, or both. Recently, we found a new type of PG dehydrogenase specific for PGD_2 in brain (unpublished data). Although PGE_1 was more active than PGD_2 at concentrations >1 μM, the effect is much slower than with PGD_2. Furthermore, the natural occurrence of PGE_1 in brain has been controversial because the precursor fatty acid (8,11,14-icosatrienoic acid) is absent in the brain of mice and rats (22, 23). Catecholamine receptors do not seem to be involved in the activation of adenylate cyclase by PGD_2. The effect of phenoxybenzamine, an α-blocker, may be ascribed to its agonistic effect on opiate receptors (24, 25) because activators of opiate receptors are known to inhibit adenylate cyclase stimulated by PGE_1 (7, 26). This interpretation is supported by the observation that naloxone completely reversed the inhibitory effect of phenoxybenzamine. These results, taken together, suggest that PGD_2 may interact with a specific receptor on neuroblastoma cells.

Although the functional roles of cyclic AMP in nervous systems remain unclear, it is reportedly related to cell differentiation or neurite extension. Furthermore, at certain types of synapses it regulates presynaptic release or postsynaptic actions of neurotransmitters (27, 28). Because our data were obtained with cultured neuroblastoma cells, it would be necessary to examine the effect of PGD_2 on neural tissues or cells *in vivo*. However, on the basis of the results presented here we propose,

as a working hypothesis, that PGD_2 is a neuromodulator and plays a role in the neural function.

We are grateful to Dr. M. Otsuka (Tokyo Medical and Dental University), Dr. K. Sasaki (Kyoto University), and Dr. K. Takai (our department) for useful discussions and to M. Ohara for critical reading of the manuscript. A portion of the early phase of this work was carried out in collaboration with Mrs. K. Watanabe. This work has been supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, and by grants from the Japanese Foundation on Metabolism and Diseases, Fujiwara Memorial Foundation, Japan Heart Foundation 1979, and the Research Foundation for Cancer and Cardiovascular Diseases.

1. Nugteren, D. H. & Hazelhof, E. (1973) *Biochim. Biophys. Acta* **326,** 448–461.
2. Shimizu, T., Yamamoto, S. & Hayaishi, O. (1979) *J. Biol. Chem.* **254,** 5222–5228.
3. Habig, W. H., Pabst, M. J. & Jakoby, W. B. (1974) *J. Biol. Chem.* **249,** 7130–7139.
4. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
5. Amano, T., Richelson, E. & Nirenberg, M. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 258–263.
6. Amano, T., Hamprecht, B. & Kemper, W. (1974) *Exp. Cell Res.* **85,** 399–308.
7. Sharma, S. K., Nirenberg, M. & Klee, W. A. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 590–594.
8. Honma, M., Satoh, T., Takezawa, J. & Ui, M. (1977) *Biochem. Med.* **18,** 257–273.
9. Hamberg, M. & Fredholm, B. B. (1976) *Biochim. Biophys. Acta* **431,** 189–193.
10. Christ-Hazelhof, E., Nugteren, D. H. & van Dorp, D. A. (1976) *Biochim. Biophys. Acta* **450,** 450–461.
11. Oelz, O., Oelz, R., Knapp, H. R., Sweetman, B. J. & Oates, J. A. (1977) *Prostaglandins* **13,** 225–234.
12. Ali, M., Cerskus, A. L., Zemechik, J. & McDonald, J. W. D. (1977) *Thromb. Res.* **11,** 485–496.
13. Friesinger, G. C., Oelz, O., Sweetman, B. J., Nies, A. S. & Data, J. L. (1978) *Prostaglandins* **15,** 969–981.
14. Christ-Hazelhof, E. & Nugteren, D. H. (1979) *Biochim. Biophys. Acta* **572,** 43–51.
15. Roberts, L. J., II, Lewis, R. A., Lawson, J. A., Sweetman, B. J., Austen, K. F. & Oates, J. A. (1978) *Prostaglandins* **15,** 717.
16. Jakschik, B. A., Parker, C. W. & Needleman, P. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37,** 384.
17. Fitzpatrick, F. A. & Stringfellow, D. A. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 1765–1769.
18. Ellis, C. K., Smigel, M. D., Oates, J. A., Oelz, O. & Sweetman, B. J. (1979) *J. Biol. Chem.* **254,** 4152–4163.
19. Nishizawa, E. E., Miller, W. L., Gorman, R. R., Bundy, G. L., Senensson, J. & Hamberg, M. (1975) *Prostaglandins* **9,** 109–121.
20. Abdel-Halim, M. S., Hamberg, M., Sjöquist, B. & Änggård, E. (1977) *Prostaglandins* **14,** 633–643.
21. Abdel-Halim, M. S. & Änggård, E. (1979) *Prostaglandins* **17,** 411–417.
22. Lunt, G. G. & Rowe, C. E. (1968) *Biochim. Biophys. Acta* **152,** 681–693.
23. Baker, R. R. & Thompson, W. (1972) *Biochim. Biophys. Acta* **270,** 489–503.
24. Cicero, T. J., Wilcox, C. E. & Meyer, E. R. (1974) *Bicohem. Pharmacol.* **23,** 2349–2352.
25. Sabol, S. L. & Nirenberg, M. (1979) *J. Biol. Chem.* **254,** 1913–1920.
26. Collier, H. O. J. & Roy, A. C. (1974) *Nature (London)* **248,** 24–27.
27. Daly, J. (1977) *Cyclic Nucleotides in the Nervous System* (Plenum, New York).
28. Bloom, F. E. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38,** 2203–2207.