STIMULATION BY PROSTAGLANDIN E_1 OF ADENOSINE 3':5'-CYCLIC MONOPHOSPHATE FORMATION IN NEUROBLASTOMA CELLS IN THE PRESENCE OF PHOSPHODIESTERASE INHIBITORS

Bernd HAMPRECHT

Max-Planck-Institut für Biochemie, 8033 Martinsried bei München, Germany

and

Joachim SCHULTZ

Institut für Toxikologie der Universität, 7400 Tübingen, Germany

Received 10 May 1973

1. Introduction

Cultured cells from mouse neuroblastoma C-1300 display a set of properties, which are characteristic of neurons. They resemble neurons in morphology [1–4], their content of enzymes involved in neurotransmitter synthesis [1, 2, 4, 5], their electrical properties [6–8] and their response to neurohormones [6, 9, 10]. The latter findings imply that the neuroblastoma cells carry the receptors necessary to recognize neurohormones like acetylcholine and prostaglandin E_1 (PGE_1).

The levels of adenosine 3':5'-cyclic monophosphate (cyclic AMP) in neuroblastoma cells are increased in the presence of PGE_1, albeit to different extent with different clonal lines [10]. The slight stimulation seen with some of the lines [10] could be due either to a reduced sensitivity to PGE_1 (e.g. lack of PG-receptors) or to a very rapid breakdown of the cyclic AMP formed. As the main point, the present report shows that PGE_1 increases the levels of cyclic AMP up to several hundred fold above the control levels, provided inhibitors of cyclic nucleotide phosphodiesterase like papaverine (pap) [11, 12] and isobutylmethylxanthine (IBMX) [12, 13] are present; furthermore it demonstrates that this is also true for lines, which are hardly stimulated by PGE_1 alone.

2. Materials and methods

2.1. Cell culture

The neuroblastoma cells used were either clonal lines isolated from mouse neuroblastoma C-1300 by Dr. T. Amano [4] or mutant lines derived from these. NS 20 [4] and N4TG3 [14] were obtained from Dr. T. Amano, N18TG2 [15] from Dr. J. Minna and N12 and N115 [4] from Dr. M. Nirenberg. N4TG3 and N18TG2 are 6-thioguanine resistant mutants. The cells were grown at 37°C in Falcon plastic flasks (75 cm^2) or plastic dishes (diameter 60 mm) containing Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, in an atmosphere of 90% air, 10% CO_2 and 100% humidity. The medium for N4TG3 and N18TG2 contained 0.1 mM 6-thioguanine. The lines N12, NS20, N115 and N18TG2 were subcultured by using a medium free of divalent cations [16], N4TG3 by exposure to 0.05% trypsin [16]. The viability of the cells was determined by exclusion of nigrosin.

2.2. Experimental incubation

The conditions for labeling of cyclic AMP with [^{14}C]adenine, for the determination of total adenine

nucleotides and for the incubations (10 min) with the hormones and phosphodiesterase inhibitors were performed as described elsewhere [12, 17]. The final concentrations of these substances in the incubation were: 2.9 μM PGE_1, 0.1 mM norepinephrine (NE), 0.5 mM papaverine and 1 mM IBMX. The cyclic AMP content of the cells was measured using the method of Gilman [18]. Cyclic AMP was purified prior to the assay [19]. Protein was determined with the biuret method [20, 21].

3. Results

The increase of the cyclic AMP levels in the lines NS20 and N4TG3 in presence of PGE_1 (table 1) confirms a previous report [10]. The stimulation of the mutant line N18TG2 is much higher than that previously found for the wild type N18 [10]. The elevation of the cyclic AMP content of lines N12 and N115 is very modest. Papaverine alone potently raises cyclic AMP levels in N4TG3 (17-fold), N18TG2 (10-fold) and N115 (39-fold). The other lines are stimulated less markedly. In comparison, the effect of IBMX alone is less pronounced. When PGE_1 is combined with either papaverine or IBMX or with both, cyclic AMP levels amount to values, which are up to about 25 times higher than the highest values previously found in neuroblastoma lines stimulated by PGE_1 and theophylline [10]. The combination of PGE_1 with IBMX is generally more effective than that with papaverine. When both inhibitors are accompanying PGE_1 the cyclic AMP levels are further increased above those found with only one inhibitor fortifying the effect of PGE_1. The highest concentrations of intracellular cyclic AMP amount to values between 10 and 60% of the levels of the free adenine nucleotides. The maximal levels of cyclic AMP are reached within 10–15 min of exposure to PGE_1 and a phosphodiesterase inhibitor (fig. 1).

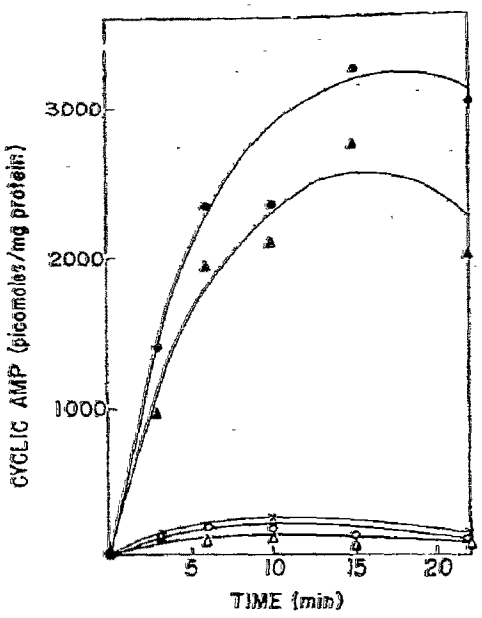

Fig. 1. Time course of cyclic AMP formation by the neuroblastoma line N4TG3 in the presence and absence of phosphodiesterase inhibitors. 8.4×10^5 cells (per 60 mm dish) were exposed for varying lengths of times to 3 ml of solutions of PGE_1 (X–X–X), papaverine (△—△—△), IBMX (○—○—○), PGE_1 + papaverine (▲—▲—▲) and PGE_1 + IBMX (●—●—●). For experimental details see Methods.

Table 1
Effect of PGE_1 and phosphodiesterase inhibitors on the levels of cyclic AMP in neuroblastoma cell lines.

Cell line	(pmoles cyclic AMP/mg protein) Control	pap.	IBMX	PGE_1	PGE_1 + pap.	PGE_1 + IBMX	PGE_1 + pap. + IBMX	Adenine nucleotides (pmoles/mg protein)	Total cells per flask $\times 10^{-6}$	Protein per flask (mg)	Viability of cells (%)
N4TG3	11	181	63	86	940	1750	2220	9200	10	7	91
N12	13	31	37	32	1200	1630	2280	23300	11	13	86
N18TG2	10	97	42	177	1860	2500	3660	6500	18	8	94
N115	5	193	24	13	512	686	1150	6400	–	13	–
NS20	17	44	35	41	695	1730	2620	–	21	11	67

The cells grown in plastic flasks (75 cm^2) were exposed for 10 min to the substances indicated in the table. For experimental details see Methods. pap. = papaverine; – = not measured.

The formation of cyclic AMP by the intact cells can also be followed by measuring the incorporation of [^{14}C]adenine into cyclic AMP [12]. After labeling the intracellular adenine nucleotide pool, the cells were exposed to the same compounds as in table 1. With all cell lines the controls showed only a slight conversion of the intracellular label into cyclic AMP (table 2). In the presence of PGE_1 or one of the phosphodiesterase inhibitors some of the cell lines incorporated somewhat more label into cyclic AMP than the controls. However, it was only when PGE_1 and at least one of the inhibitors were present that the labeling of cyclic AMP increased strongly. These results are qualitatively similar to those shown in table 1. Yet, there are quantitative differences. In most cases the stimulations observed with the levels of cyclic AMP are by far stronger than with the incorporation of intracellular label into cyclic AMP.

Catecholamines have been reported not to increase cyclic AMP levels in neuroblastoma cells [10]. On the background of the strong effect of PGE_1 in the presence of the phosphodiesterase inhibitors, it appeared interesting to see, whether or not NE would elevate the levels of cyclic AMP when the inhibitors were applied simultaneously. As seen in table 3, neither of the two cell lines tested showed a substantial increase of its cyclic AMP content above the respective reference values in the presence of NE or NE and the inhibitors.

Table 2

Incorporation by neuroblastoma lines of [^{14}C]adenine into cyclic AMP in the presence and absence of PGE_1 and phosphodiesterase inhibitors.

	(%) Conversion							
Cell line	Control	pap.	IBMX	PGE_1	PGE_1 + pap.	PGE_1 + IBMX	PGE_1 + pap. + IBMX	[^{14}C]adenine uptake into the cells (cpm × 10^{-3}/mg protein)
N4TG3	0.1	1.1	0.3	0.3	4.6	6.3	7.5	19.1
N12	0.1	0.3	0.3	0.2	2.5	4.2	4.9	8.9
N18TG2	0.1	0.4	0.1	0.5	3.6	3.8	5.6	14.6
N115	0.1	0.8	0.2	0.1	2.8	4.1	6.4	6.4
NS20	0.1	0.3	0.1	0.1	1.5	2.7	3.8	9.7

Cells labeled with [^{14}C]adenine for 40 min [12] were exposed to the substances indicated in the table for 10 min. Percent conversion = 100 × (radioactivity of cyclic AMP isolated)/(total radioactivity in the cells). For cell densities, viabilities and cell protein per flask see table 1. The cells used were parallel cultures of those used in table 1. The data on the uptake of radioactive adenine are the mean of 7 values.

Table 3

Effect of NE and phosphodiesterase inhibitors on cyclic AMP levels in 2 neuroblastoma cell lines.

		(pmoles cyclic AMP/mg protein)								
Cell line		Control	pap.	pap. + IBMX	NE	NE + pap.	NE + pap. + IBMX	Protein per flask (mg)	Total cells per flask × 10^{-6}	viability of cells (%)
N4TG3	Expt. 1	35	–	95	52	–	85	0.5*	–	–
	Expt. 2	15	153	–	16	162	73	–	4	99
N18TG2	Expt. 1	32	10	–	8	17	–	19	48	82
	Expt. 2	14	–	–	10	–	–	7	24	91

* Per 60 mm plastic Petri dish.

For experimental details see Methods; – = not measured.

4. Discussion

The experiments show that the increase in levels of cyclic AMP due to stimulation by PGE_1 is different with various neuroblastoma lines. This elevation of the cellular content of cyclic AMP is strongly potentiated when the phosphodiesterase inhibitors papaverine and/or IBMX are present. The conclusion is that the neuroblastoma cell lines tested have an active PGE_1 receptor–adenyl cyclase system and that the large differences found in the responses to PGE_1 alone are mainly due to differences in phosphodiesterase activities. On the other hand, there are still differences in the cyclic AMP levels even in the presence of the phosphodiesterase inhibitors. They could be attributed to differences in the rates of synthesis of cyclic AMP, different levels of intracellular nucleotides and/or different isozymic forms of cyclic AMP phosphodiesterase, which are only partially or not at all inhibited by the inhibitors used.

Despite the fact that incorporation of [^{14}C]adenine into cyclic AMP parallels the cyclic AMP levels observed, the changes seen with the radioactive method are less pronounced than those detected by measuring the content of cyclic AMP. The reasons for this quantitative difference between the results obtained with the 2 methods are probably differences in uptake of [^{14}C]-adenine into the cells and differences in intracellular pools of precursors. The comparison of the methods demonstrates that measuring adenine incorporation into cyclic AMP may be useful for finding qualitative differences. For quantitation, however, measuring of cyclic AMP levels is necessary.

Exposure of neuroblastoma cells to dibutyryl cyclic AMP [22], PGE_1 [23] or papaverine [24] initiates the outgrowth of neurites, an effect which is probably mediated by increased levels of intracellular cyclic AMP. In the light of the results presented here, a failure of neuroblastoma lines to express differentiated functions in the presence of PGE_1 might be overcome by inhibiting the cyclic nucleotide phosphodiesterase activity of the cells.

It appears difficult to differentiate between a slightly stimulatory effect of PGE_1 or some other neurohormone on the cyclic AMP content of a neuroblastoma culture and the biological variation among parallel cultures. In such a case it is helpful to see whether or not phosphodiesterase inhibitors like papaverine or IBMX will potentiate the effect of PGE_1 (or some other neurohormone acting on the adenyl cyclase system). Since NE does not even cause an increase in the cyclic AMP levels in the presence of the inhibitors, we conclude that the cells are probably not equipped with adrenergic receptors.

All neuroblastoma lines thus examined for their ability to be stimulated by PGE_1 are derived from the same tumor [4]. Nevertheless do they vary in their response to PGE_1 and the inhibitors (alone and in combination). Such variations have also been observed with their content of enzymes characteristic of neurones, their morphology, size and karyotype [4]. Therefore, the different genetic constitution of the cells is very likely to be the origin of such variations.

Despite the high concentrations of the phosphodiesterase inhibitors used, the effects of the two inhibitors are partially additive. These results could be explained by the existence of two or more isozymic forms of phosphodiesterase, which are affected by the inhibitors to a different extent. Isozymic forms of phosphodiesterase have been detected in various tissues from rats and rabbits [25], in glioma and neuroblastoma lines [26].

Acknowledgements

We like to thank Dr. J. Pike (Upjohn Company) for a gift of PGE_1 and Drs. T. Amano, J. Minna and M. Nirenberg for cells.

References

[1] Augusti-Tocco, G. and Sato, G. (1969) Proc. Natl. Acad. Sci. U.S. 64, 311.
[2] Schubert, D., Humphreys, S., Baroni, C. and Cohn, M. (1969) Proc. Natl. Acad. Sci. U.S. 64, 316.
[3] Seeds, N.W., Gilman, A.G., Amano, T. and Nirenberg, M. (1970) Proc. Natl. Acad. Sci. U.S. 66, 160.
[4] Amano, T., Richelson, E. and Nirenberg, M. (1972) Proc. Natl. Acad. Sci. U.S. 69, 258.
[5] Rosenberg, R.N., Vandeventer, L., de Francesco, L. and Friedkin, M.E. (1971) Proc. Natl. Acad. Sci. U.S. 68, 1435.
[6] Harris, A.J. and Dennis, M.J. (1970) Science 167, 1253.
[7] Nelson, P., Ruffner, W. and Nirenberg, M. (1969) Proc. Natl. Acad. Sci. U.S. 64, 1004.
[8] Nelson, P.G., Peacock, J.H., Amano, T. and Minna, J. (1971) J. Cell. Physiol. 77, 337.

[9] Nelson, P.G., Peacock, J.H. and Amano, T. (1971) J. Cell. Physiol. 77, 1353.
[10] Gilman, A.G. and Nirenberg, M. (1971) Nature 234, 356.
[11] Rufeger, U., Tellheim, B. and Frimmer, M. (1971) Naunyn-Schmiedebergs Arch. Pharmak. 270, 428.
[12] Schultz, J., Hamprecht, B. and Daly, J.W. (1972) Proc. Natl. Acad. Sci. U.S. 69, 1266.
[13] Beavo, J.A., Rogers, N.L., Crofford, O.B., Hardman, J.G., Sutherland, E.W. and Newman, E.V. (1970) Mol. Pharmacol. 6, 597.
[14] Amano, T., Hamprecht, B. and Kemper, W. submitted for publication.
[15] Minna, J., Glazer, D. and Nirenberg, M. (1972) Nature New Biol. 235, 225.
[16] Blume, A., Gilbert, F., Wilson, S., Farber, J., Rosenberg, R. and Nirenberg, M. (1970) Proc. Natl. Acad. Sci. U.S. 67, 786.
[17] Schultz, J. and Hamprecht, B. (1973) Naunyn-Schmiedebergs Arch. Pharmak., in press.
[18] Gilman, A. (1970) Proc. Natl. Acad. Sci. U.S. 67, 305.
[19] Schultz, J. and Daly, J.W. (1973) J. Biol. Chem. 248, 843.
[20] Beisenherz, G., Bolte, H.J., Bücher, Th., Czok, R., Garbade, K.H., Meyer-Arendt, E. and Fleiderer, G. (1953) Z. Naturforsch. 8b, 555.
[21] Jacobs, E.E., Jacobs, M., Samadi, D.R. and Bradley, L.B. (1956) J. Biol. Chem. 223, 147.
[22] Prasad, K.N. and Hsie, A.W. (1971) Nature New Biol. 233, 141.
[23] Prasad, K.N. (1972) Nature New Biology 236, 49.
[24] Prasad, K.N. and Sheppard, J.R. (1972) Exptl. Cell Res. 73, 436.
[25] Monn, E. and Christiansen, R.O. (1971) Science 173, 540.
[26] Uzonov, P., Shein, H.M. and Weiss, B. Fifth Intern. Congress on Pharmacology, San Francisco, (1972) Abstr. 1431.