PROBENECIDE SENSITIVE 3'-5'-CYCLIC AMP SECRETION BY ISOPROTERENOL STIMULATED GLIAL CELLS IN CULTURE

J. PENIT, S. JARD and P. BENDA

Laboratoire de Physiologie cellulaire,
College de France,
11, Place Marcelin Berthelot, Paris 75005, France

Received 25 January 1974

1. Introduction

Very little is known at the present time about the biological functions of glial cells in the central nervous system (CNS). The close anatomical relationships between glial cells and neurones has been taken as evidence for a functional or metabolic coupling between these two types of cells [1, 2]. The small volume of extracellular fluid between glial cells and neurones raises the possibility that glial cells control the chemical composition of this extracellular compartment. On the other hand, it has recently been shown that glial cells in culture are able to accumulate large quantities of 3'-5'-cyclic AMP in response to catecholamines stimulation [3, 4]. Thus it seems likely that the glial cells are at least partly responsible for the increase in the 3'-5'-cyclic AMP content of CNS slices observed after incubation in presence of catecholamines or other neurotransmitters [5–7]. Besides its role as an intracellular messenger for the action of neurotransmitters on glial cells per se, 3'-5'-cyclic AMP could exert a regulatory action on the neurone if the glial cell is able to secrete 3'-5'-cyclic AMP and to modulate the nucleotide concentration in the interspace between neurones and glial cells. The existence of 3'-5'-cyclic AMP secretion has been clearly demonstrated for at least one cell type. Davoren and Sutherland [8] reported that pigeon erythrocytes respond to epinephrine stimulation by increased cyclic AMP production and secretion into the extracellular fluid. The nucleotide secretion occurred against a concentration gradient and was partially blocked by probenecide.

In this paper we show that actively growing glial cells in culture are also able to secrete 3'-5'-cyclic AMP into the external medium and that this secretion is sensitive to probenecide.

2. Methods

2.1. *Biological material*

Glial cells of the clone C_6 were derived from glial tumors induced by repeated injections of *N*-nitrosomethylurea in Wistar rats [9, 10]. Cells were seeded in 60 mm Petri dishes at a concentration of 2×10^5 cells per dish. They were grown at 37°C in 4 ml HAMF 10 medium supplemented with 10% foetal calf serum in an atmosphere of 93% air and 7% CO_2. The culture medium contained penicillin G (50 U/ml) and streptomycin sulfate (50 μg/ml). The medium was changed 3,5 and 6 days after plating. Except where otherwise indicated the cells were harvested on the 7th day.

2.2. *Experimental procedures*

Just before the experiments, the HAMF medium was removed by aspiration and the culture dishes rinsed three times with 2 ml of the same medium without serum and antibiotics. All cells used in any one experiment came from the same source and were seeded at the same time. All determinations were performed in duplicates using two different dishes.

 North-Holland Publishing Company – Amsterdam

The incubation in presence of isoproterenol and/or probenecide were performed at 37°C. For the probenecide experiments the incubation medium was buffered with 10 mM Tris–HCl pH 7.4.

At the end of the incubation period, the external medium was rapidly collected by aspiration and cells fixed by 1.4 ml of trichloroacetic acid (TCA), 10% (w/v), and allowed to stand at room temperature for 1 hr. The cells were scraped and collected together with the TCA solution. Each extract received a tracer dose of [^{3}H] cyclic AMP (0.5 pM) for nucleotide recovery determination and were centrifuged. TCA in the supernatant fraction was extracted with diethyl ether (4 ml repeated 5 times). In control experiments, 3′-5′-AMP hydrolysis in the extracellular medium was measured by incubating cells, as indicated above, for 5 and 120 min in presence of [^{3}H] cyclic AMP (0.2 μCi/ml) and unlabelled cyclic AMP (final concentrations 5×10^{-8} M and 10^{-6} M). The external medium was collected at the end of the incubation period and treated with TCA as indicated for cellular extracts.

2.3. *Analytical procedures*

3′-5′-Cyclic AMP was determined using a modification of Gilman's receptor radioassay [11]. The source of specific cyclic AMP binding protein was a preparation of bovine kidney 3′-5′-cyclic AMP dependent protein kinase, purified according to the procedure described by Myamoto Kuo and Greengard, up to the DEAE cellulose chromatography step [12]. The incubation medium (final volume 250 μl) contained: 50 mM sodium acetate pH 4.0, 4.8×10^{-10} M [^{3}H] 3′-5′-cyclic AMP (21 Ci/mM), 40 μg receptor protein and 50 μl of 3′-5′-cyclic AMP standards (0.05 to 12.5 pmoles) or unknown samples added under a volume of 50 μl. Each sample was incubated for 90 min at 0°C. Bound cyclic AMP was separated by filtration on Millipore HAWP (0.45 μ–25 mm). The millipore filters were dried and their radioactivity content measured by liquid scintillation in 8 ml of Toluol, (2-2′Phénylen-bis (4-methyl-5 phenyloxazol), 0.4 mg, and (2.5 Diphenyloxazol) 32 mg. To test the absence of substances interferring with [^{3}H] cyclic AMP binding, a parallel set of determinations was performed using biological samples in which a known amount (1.2 pM) of unlabelled cyclic AMP was added.

Proteins in the TCA pellet of cellular extracts were determined by Lowry's method [13] using bovine serum albumin as a standard. Cyclic AMP thin layer chromatography was performed on cellulose plates in an ammonium acetate M, absolute ethanol (3–7 v/v) system.

2.4. *Products used*

[^{3}H] Cyclic AMP (21 Ci/mM) was purchased from the C. E. N. Saclay, and cyclic AMP from Schwartz Bioresearch. Isoproterenol (D.L. isopropylnorepinephrine) from Fluka was dissolved in water together with an equal amount of ascorbic acid. The isoproterenol solution (10^{-3} M) was prepared immediately before the experiment. Probenecide (Theraplix) was dissolved in NaOH 0.25 N at a concentration of 10^{-2} M. The solution was neutralized with dry KH_2PO_4.

3. Results

3.1. *Control experiments*

The growth cycle of glial C_6 cells used in this study is depicted in fig. 1.

Experiments were performed using rapidly growing cells collected 7 days after plating. Over the whole of the initial part of the growth cycle, the total protein content can be taken as a good index of cell number. Cellular 3′-5′-cyclic AMP contents were therefore expressed as pmoles/mg protein.

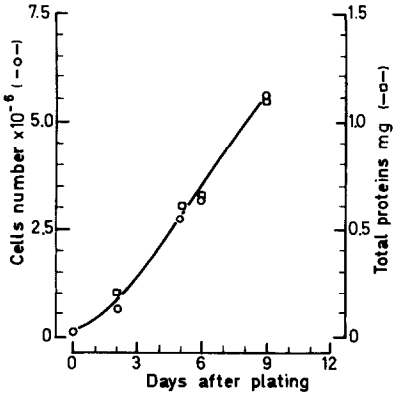

Fig. 1. Growth cycle of C_6 glial cells. C_6 glial cells were seeded in 60 mm Petri dishes at a concentration of 2×10^5 cells per dish and grown as indicated under methods. 3, 5, 6 and 9 days after plating trypsinised cells were collected for numeration. Total protein determinations were obtained from separate dishes.

Table 1
Hydrolysis of extracellular 3′-5′-cyclic AMP

Extracellular 3′-5′-cyclic AMP (M)	% 3′-5′-Cyclic AMP hydrolysis after:	
	5 min	120 min
5×10^{-8}	0.2	12.7
10^{-6}	0	2.8

Seven-day-old glial cells (about 4.5×10^6 cells per dish) were incubated for 5 or 120 min in 2 ml of HAMF 10 containing [^{3}H] 3′-5′-cyclic AMP (0.2 μCi/ml) and unlabelled 3′-5′-cyclic AMP: final concentration 5×10^{-8} M or 10^{-6} M. At the end of the incubation period the external medium was collected and treated with TCA (10% final concentration). Aliquots of the TCA extract were submitted to thin layer chromatography. The % hydrolysis was calculated from the ratio: radioactivity in the 3′-5′-cyclic AMP spot/total radioactivity. The experimental values were corrected for the radiochemical purity (97%) of the labelled nucleotide.

Table 1 shows that 3′-5′-cyclic AMP added to the external medium is very slowly hydrolysed. The % hydrolysis of 3′-5′-cyclic AMP was measured at two concentrations in the external medium (5×10^{-8} M and 10^{-6} M). These concentrations correspond to those measured in the extracellular medium after a 90 min incubation period of glial cells in the absence or presence of isoproterenol (see below). At low external concentration (5×10^{-8} M) 87% of the added nucleotide was still present after a 120 min incubation period. Raising the concentration to 5×10^{-6} M further reduced the % conversion. The rate of 3′-5′-cyclic AMP secretion was not corrected for extracellular hydrolysis.

3.2. *Time course of intra and extracellular 3′-5′-cyclic AMP accumulation following isoproterenol stimulation*

The results of two typical experiments are shown in fig. 2 (a and b).

Glial cells were stimulated by a near maximal dose of isoproterenol (10^{-6}). Stimulation resulted in a large and rapid increase in cellular 3′-5′-cyclic AMP content from about 20 pM/mg protein to 3000 (fig. 2a) or 6000 (fig. 2b) pmoles/mg protein. The time course of nucleotide accumulation varied from one experiment to another. In experiment 2a, a maximum was reached after 15 min incubation in presence of isoproterenol, the cellular content decreasing rapidly thereafter. In

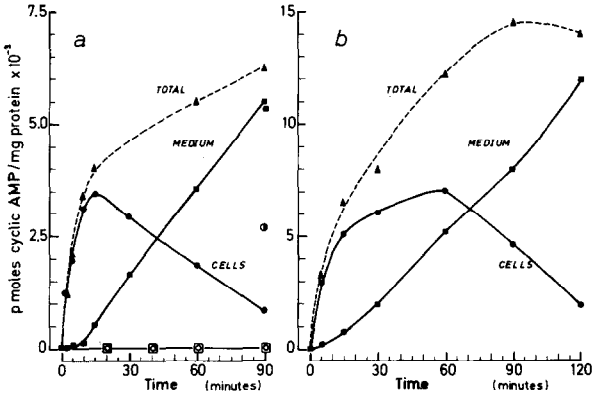

Fig. 2. Time course of 3′-5′-cyclic AMP accumulation by C_6 glial cells. For each of the two experiments described (a and b) 7-day-old cells were used. For one group of 4 dishes, the incubation medium contained isoproterenol (10^{-6} M final concentration). 2, 5, 10, 15, 30, 60 and 90 min later the medium of two separated dishes was collected and the cells rapidly fixed for 3′-5′-cyclic AMP determinations. For a second group of 10 dishes, the medium did not contain isoproterenol; cells and extracellular medium were collected at 0, 20, 40, 60, 90 min. In a third group of two dishes, the incubation medium containing isoproterenol was renewed every 15 min and the cells collected at 90 min. Intracellular and extracellular 3′-5′-cyclic AMP contents are expressed as pM/mg protein. Values on the figure are the mean of two determinations. The figure describes the time course of intracellular (●) extracellular (■) and total (▲) cyclic AMP accumulation by isoproterenol stimulated cells. The open symbols refer to the control non-stimulated cells. When the medium was renewed every 15 min the extracellular cyclic AMP secretion was obtained by adding the medium nucleotide contents at the end of each of the 6 successive 15 min incubation periods (■). The corresponding final intracellular content is indicated by (●).
Experiment b was conducted in a similar way except that it included only isoproterenol stimulated cells.

experiment 2b, the maximum level, was not reached until 60 min. When the external medium (fig. 2a) containing isoproterenol was renewed every 15 min, the decrease in the cellular content observed in the terminal phase of the experiment was strongly reduced (fig. 2a). Thus the differences observed in the time course of 3′-5′-cyclic AMP accumulation could reflect differences in the rate of inactivation of isoproterenol in the external medium, rather than alteration in cell structure (partial lysis) or activity.

In both of the two experiments described, a large amount of 3′-5′-cyclic AMP was secreted into the ex-

ternal medium. After 45 to 75 min incubation, more cyclic nucleotide was found in the external medium than in the intracellular compartment. After 10–15 min incubation, 3′-5′-cyclic AMP secretion proceeded at a linear rate, despite the large variations observed in the intracellular content. Both in the two experiments, the total nucleotide content increased up to 90 min. The possibility that the amount of 3′-5′-cyclic AMP found in the incubation medium could have been liberated by cell lysis, can be reasonably excluded. The maximal amount of phosphodiesterase in the incubation medium sufficient to account for the observed extracellular cyclic nucleotide hydrolysis (see control experiments) corresponds to the activity contained in less than 0.01% of the total cell population [14]. Elimination of soluble proteins or proteins associated with cellular debris would have been eliminated by removal of the incubation medium. In fact, the total cellular protein content measured after 120 min incubation was identical to that determined at 0 time on the control samples. Renewal of the incubation medium containing isoproterenol did not modify the amount of cyclic nucleotide secreted into the external medium after 90 min (fig. 2a). In control non stimulated cells, the intracellular 3′-5′-cyclic AMP content remained constant, the extracellular nucleotide concentration was less than 30 pM/mg protein after 90 min incubation period (fig. 2a).

From a rough estimation of the intracellular volume (1.6 μl/10^6 cells), it can be calculated that under isoproterenol stimulation the 3′-5′-cyclic AMP concentration within the cell approaches values as high as 10^{-4} M. Due to the large extracellular/intracellular volume ratio (about 350 in our experimental conditions), the 3′-5′-cyclic AMP secretion occurred down a concentration gradient (maximal extracellular concentration 5×10^{-6} M). Assuming that glial cells are spherical, one can estimate the permeability coefficient for cyclic AMP from the nucleotide secretion rate and the mean intracellular concentration. The estimated value was in the range of 10^{-6} cm sec^{-1}. This is high enough to ensure that under in vivo conditions, the concentration of 3′-5′-cyclic AMP in the interspace between neurones and glial cells would rapidly reach equilibrium with the concentration within the glial cells. The observed 3′-5′-cyclic AMP secretion rate by glial cells is about twice that reported by Davoren and Sutherland for pigeon erythrocytes [8].

3.3. *Effects of probenecide on 3′-5′-cyclide AMP secretion by glial cells*

Probenecide did not modify the intracellular 3′-5′-cyclic AMP content of control glial cells and did not induce nucleotide secretion into the external medium (table 2). It strongly reduced the secretion of 3′-5′-cyclic AMP by isoproterenol stimulated cells. The details of these experiments are shown in table 3. Cells were exposed to probenecide (5×10^{-4} or 10^{-3} M) for 30 to 45 min, and stimulated by isoproterenol (10^{-6} M) 15 min after probenecide addition.

Table 2
Effect of probenecide on 3′-5′-cyclic AMP content of non stimulated glial cells

Treatment	3′-5′-Cyclic AMP (pmoles/mg protein)	
	Cells	Medium
Control	9.6	8*
Probenecide (5×10^{-4} M)		
5 min	12.5	7
15 min	12.0	5
45 min	10.0	6
60 min	11.5	11

Seven-day-old glial cells were incubated for 5, 15, 45 and 60 min in 2 ml of HAMF 10 containing probenecide 5×10^{-4} M. At the end of the incubation period the medium and the cells were collected for 3′-5′-cyclic AMP determinations. Values in the table are the mean of two determinations. Concentrations in the external medium fell at the lower limit of the sensitivity of the Gilman's method. This may account for the large dispersion of the experimental values. *Value measured 60 min after renewal of the incubation medium.

3′-5′-Cyclic AMP accumulation was measured after 15 min treatment with isoproterenol. A 3.7-fold decrease in nucleotide secretion was observed in probenecide treated cells while the intracellular content was not changed (table 3, exp. 1). 30 min after isoproterenol stimulation, the reduction of 3′-5′-cyclic AMP secretion caused by probenecide resulted in an increase of the intracellular content (exp. 2), indicating that the exit of 3′-5′-cyclic AMP is a separate event which occurs after intracellular production.

A further reduction in 3′-5′-cyclic secretion was ob-

Table 3
Effect of probenecide on intracellular and extracellular 3′-5′-cyclic AMP accumulation by isoproterenol stimulated glial cells

Exp N°	Treatment		3′-5′-Cyclic AMP content (pM/mg protein)		
	Probenecide	Isoproterenol (10^{-6} M)	Cells	Medium	Total
1	0	15 min	5488	954	6442
	5×10^{-4} M (30 min)	15 min	5530	233	5766
2	0	30 min	6102	3295	9397
	5×10^{-4} M (45 min)	30 min	8921	1822	10743
3	0	15 min	–	848	–
	5×10^{-4} M (30 min)	15 min	–	233	–
	10^{-3} M (30 min)	15 min	–	149	–

Glial cells, 7-day-old (4–5 10^6 cells per dish), were incubated in 2 ml of HAMF 10 medium containing either isoproterenol (10^{-6} M) alone or isoproterenol plus probenecide (5×10^{-4} or 10^{-3} M). The duration of the contact periods with these two agents are indicated in the first two columns of the table. The 3′-5′-cyclic AMP accumulated within the cells and in the extracellular medium is expressed as pmoles/mg of total cellular proteins. Values in the table are the mean of four determinations. Experiments 1 and 2 were performed using the same batch of cells.

tained by raising the probenecide concentration to 10^{-3} M. The inhibition by probenecide suggests that 3′-5′-cyclic AMP secretion is not a simple diffusional process. Further experiments are needed to decide if this secretion involves an active or a passive transport systeem.

Acknowledgement

This work was supported by the grant no. 72.7.0676 from the Delegation Generale à la Recherche Scientifique et Technique.

References

[1] Scheibel, M. E. and Scheibel, A. (1958) In: The Biology of Neuroglia (Windle, W. F., eds.) p. 111, Charles C. Thomas, Springfield.
[2] Galambos, R. (1961) Proc. Natl. Acad. Sci. U.S., 47, 129.
[3] Gilman, A. G. and Nirenberg, M. (1971) Proc. Natl. Acad. Sci. U.S., 68, 2165.
[4] Clark, R. B. and Perkins, J. P. (1971) Proc. Natl. Acad. Sci. U.S., 68, 2157.
[5] Rall, T. W. and Sattin, A. (1970) In: Role of cyclic AMP in cell function, Advances in Biochemical Psychopharmacology (Greengard, P. and Costa, E., eds.) Vol. 3, Raven Press, New York.
[6] Shimizu, H., Creveling, C. R. and Daly, J. W. (1970) In: Role of cyclic AMP in cell function, Advances in Biochemical Psycho-pharmacology (Greengard, P. and Costa, E., eds.), Vol. 3, p. 135, Raven Press, New York.
[7] Krishna, G., Forn, J., Voigt, K., Paul, M. and Gessa, G. L. (1970) In: Role of cyclic AMP in cell function, Advances in Biochemical Psycho-pharmacology (Greengard, P. and Costa, E., eds.) Vol. 3, p. 155, Raven Press, New York.
[8] Davoren, P. R. and Sutherland, E. W. (1963) J. Biol. Chem., 238, 3009.
[9] Benda, P., Someda, K., Messer, J. and Sweet, W. H. (1971) J. Neurosurg. 34, 310.
[10] Benda, P., Lightbody, J., Sato, G., Levine, L. and Sweet, W. H. (1968) Science 161, 370.
[11] Myamoto, E., Kuo, J. F. and Greengard, P. (1969) J. Biol. Chem, 244, 6395.
[12] Gilman, A. G. (1970) Proc. Natl. Acad. Sci. U.S., 67, 305.
[13] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265.
[14] Jard, S. Premont, J. and Benda, P. (1972) Febs Letters 26, 344.