Biochemical Pharmacology, Vol. 24, pp. 947–950. Pergamon Press, 1975. Printed in Great Britain.

COMPARTMENTATION OF AN ATP SUBSTRATE POOL FOR HISTAMINE AND ADRENALINE SENSITIVE ADENYLATE CYCLASE IN RAT SUPERIOR CERVICAL GANGLIA*

T. LINDL, M. C. B. HEINL-SAWAYA and H. CRAMER†

Department of Neurology, University of Freiburg D-78 Freiburg i.Br., Hansastr. 9, G.F.R.

(*Received* 14 *February* 1974; *accepted* 3 *December* 1974)

Abstract—Incubation with adrenaline or histamine induces a rapid accumulation of adenosine 3',5'-monophosphate (cyclic AMP) in the isolated superior cervical ganglion of the rat. The concentration of cyclic AMP is significantly elevated in the incubation medium after 4 min incubation with either amine. When ganglia are incubated with [^{14}C]adenine ATP and cyclic AMP are labelled. The formation of [^{14}C]cyclic AMP is enhanced by a subsequent incubation with histamine or adrenaline. Within the stimulation period of 10 min about 40 per cent of the radioactive cyclic AMP is released into the incubation medium. Endogenous ATP levels are not altered by incubation with adrenaline or histamine. Cultivation of ganglia for 4 hr increases their total ATP content, but the level of ATP falls to about 5 per cent when ganglia are incubated in a glucose-free medium. Liberation of ATP into the incubation medium is not observed under normal conditions, but after transfer of ganglia from cold medium into 37° Krebs–Henseleit medium considerable amounts of ATP are released into the incubation fluid within the first hour. This leakage of ATP is less pronounced when ganglia are incubated in the presence of adrenaline or histamine. Antimycin A_1 inhibits the formation of [^{14}C]ATP from [^{14}C]adenine, while the conversion rate of [^{14}C]ATP to [^{14}C]cyclic AMP is unaffected. Compartmentation of the ATP precursor pool for cyclic AMP is suggested by the difference in ATP/cyclic AMP ratios for endogenous and labelled compounds, and by the ineffectiveness of antimycin A_1 in decreasing the conversion rate of labelled adenine nucleotides to [^{14}C]cyclic AMP.

Electrical stimulation [1], catecholamines [2] and histamine [3] induce a rapid accumulation of adenosine 3',5'-monophosphate (cyclic AMP) in isolated superior cervical ganglia of the rabbit or the rat. By use of specific blocking agents adenylate cyclase receptors for catecholamines and histamine can be clearly distinguished [3]. As was found earlier with brain slices [4], exogenous [^{14}C]adenine is taken up into isolated ganglia and converted to [^{14}C]ATP, which serves as a substrate for adenylate cyclase [5]. Evidence has been obtained for the existence of a special ATP pool in or near cell membranes for several other tissues, namely muscle cell cultures and brain slices [6–8] suggesting specialized precursor pools of cyclic AMP. We report experiments which extend these studies with measurements of both ATP and cyclic AMP in superior cervical ganglia and in the incubation medium. In addition, the effect of Antimycin A_1, a blocker of mitochondrial ATP synthesis [9], on cyclic AMP formation is described which supports the concept of compartmentation of substrate pools for adenylate cyclase.

MATERIAL AND METHODS

[^{14}C]Adenine (sp. act. 59 mCi/m-mole) and [^{3}H]adenosine 3',5'-monophosphate (sp. act. 27·5 Ci/(m-mole) were obtained from Amersham-Buchler, Braunschweig. Antimycin A_1 was obtained from the Sigma Company. All other chemicals used were obtained from commercial sources and were reagent grade. Male Sprague–Dawley rats were a gift from Goedecke AG., Freiburg.

Rats weighing 250–300 g were stunned, the superior cervical ganglion on both sides was rapidly dissected and desheathed and groups of 2–10 ganglia were incubated as described previously [2, 3]. Cyclic AMP was determined in triplicate samples by the method of Gilman [10]. The formation of labelled cyclic AMP from [^{14}C]adenine was measured according to Shimizu [11]. The separation of labelled nucleotides was performed by two other thin layer chromatography methods [12, 13]. Endogenous ATP was determined in neutralized trichloroacetic acid extracts of ganglia and in the incubation medium by the luciferin–luciferase method [14] with the aid of a Dupont Luminescence Biometer. Lactate dehydrogenase was measured according to Wroblewski and LaDue [15] and protein content according to Lowry *et al.* [16] with bovine serum albumin as a standard.

RESULTS

Accumulation and release of endogenous cyclic AMP induced by histamine or adrenaline in rat superior cervical ganglia. Upon incubation with histamine or adrenaline, cyclic AMP accumulated rapidly in superior cervical ganglia and in the incubation medium

* Supported by the Deutsche Forschungsgemeinschaft (SFB 70).

† Reprint requests to Dr. H. Cramer.

Table 1. Effect of adrenaline (10^{-4}M) and histamine (10^{-3}M) on the cyclic AMP content in rat superior cervical ganglia and in the incubation medium (pmoles/cyclic AMP ± S.D. per mg tissue protein)

Addition	0 min	4 min	10 min	30 min
None (ganglia)	26·5 ± 3·5			22·2 ± 4·9
None (medium)	2·0 ± 0·4			1·7 ± 0·6
Adrenaline (ganglia)		211 ± 51	105 ± 16	40·0 ± 4·0
Adrenaline (medium)		4·2 ± 1·4†	11·1 ± 2·4‡	1·7 ± 1·3*
Histamine (ganglia)		184 ± 15	104 ± 13	27·4 ± 4·1
Histamine (medium)		3·8 ± 0·8†	4·5 ± 0·3*	2·8 ∓ 1·1*

* = N.S.
† = $P < 0.05$ compared to none.
‡ = $P < 0.01$ compared to none.

(Table 1). Release of the cyclic nucleotide started almost concomitantly with the rise in cyclic AMP within the tissue. With longer periods of incubation, the concentrations of cyclic AMP decreased both in the tissue and in the medium. Under control conditions only 0·2–0·5 pmoles cyclic AMP per ganglion were detected in the medium, which apparently was released immediately after transfer of ganglia or change of medium.

Concentration and release of endogenous ATP in rat superior cervical ganglia with respect to the time of incubation, the stimulation of adenylate cyclase with histamine or adrenaline and the temperature of preincubation. Freshly dissected ganglia which were kept in ice-cold Krebs–Henseleit solution pH 7·4 during preparation, showed a marked release of endogenous ATP after transfer into medium at 37° within the first hour of incubation (Fig. 1). Upon stimulation of adenylate cyclase with histamine (10^{-3} M) or adrenaline (10^{-4} M) less ATP appeared to be released into the medium during the first 5 min of incubation at 37° than in control medium with unstimulated ganglia. In either condition the concentration of ATP in the incubation medium fell after 60 min to values near the detection limit, indicating a rapid cleavage or reuptake of ATP. The ATP content of the ganglia

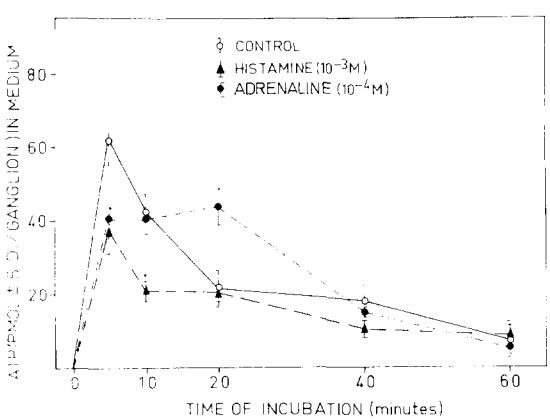

Fig. 1. Effect of histamine (10^{-3} M) and adrenaline (10^{-4} M) on the appearance of ATP in the incubation medium upon incubation *in vitro* of rat superior cervical ganglia. After dissection ganglia were immediately transferred into the medium without the otherwise used preincubation period of 1 hr. ATP was determined as described under Methods. Results are expressed as mean ± S.D., n = 6. * Significantly different from control value, $P < 0.05$.

did not change significantly within 1 hr of incubation without or with stimulation by histamine or adrenaline (Table 2). Incubation for 4 hr doubled the content of ATP, while incubation in a glucose-free medium decreased the content of ATP by about 95 per cent. No significant change in total ATP within the first hour of incubation was observed, when mitochondrial ATP synthesis was blocked by 10^{-7} M Antimycin A_1.

Formation and release of [^{14}C]cyclic AMP after labelling adenine nucleotides with [^{14}C]adenine. After incubating ganglia with [^{14}C]adenine for 1 hr about 0·6–1·6 per cent of the total radioactivity in the tissue could be detected as [^{14}C]cyclic AMP under control conditions. About 40 per cent of the labelled cyclic nucleotide was found outside the ganglia in the incubation medium after 10 min of incubation without [^{14}C]adenine. This percentage of released radioactive cyclic AMP was not changed by incubation in the presence of the amines. However, the conversion rate to [^{14}C]cyclic AMP rose about three-fold upon histamine (10^{-3}M) and six-fold upon adrenaline (10^{-4} M) stimulation for 10 min (Table 3), while the [^{14}C]ATP/[^{14}C]cyclic AMP ratio decreased from 2·6 to 1·3 and 1·0, respectively.

Effect of Antimycin A_1 on the content of [^{14}C]ATP and [^{14}C]cyclic AMP of superior cervical ganglia after preincubation with [^{14}C]adenine for various times. Antimycin A_1 which blocks *de novo* synthesis of mitochondrial ATP [9], caused no significant reduction of total ATP content of the ganglia during incubation for 1 hr. However, after prelabelling with [^{14}C]adenine, 10^{-7} M Antimycin A_1 caused a significant reduction in the incorporation of radioactivity into ATP (Table 4). This effect could be observed after 20 min incubation, and was more pronounced after 1 hr. In contrast

Table 2. Concentration of ATP in rat superior cervical ganglion at longtime incubation with adrenaline (10^{-4} M). histamine (10^{-3} M) and antimycin A_1 (10^{-7} M) and in glucose-free medium

Group	Number of samples	Incubation time (min)	nmoles ATP/mg protein ± S.D.
Control	6	60	20·8 ± 0·2
Adrenaline	5	60	13·2 ± 2·8*
Histamine	5	60	27·7 ± 8·4 N.S.
Antimycin A_1	7	60	19·7 ± 3·5 N.S.
Control	5	240	43·2 ± 6·3†
Control–glucose	5	240	2·1 ± 0·7†

* = $P < 0.05$ for difference from control values.
† = $P < 0.001$.

Table 3. Effect of adrenaline (10^{-4}M) and histamine (10^{-3}M) on the content of [^{14}C]ATP and [^{14}C]cyclic AMP in tissue and in the incubation medium and on the conversion rate to [^{14}C]cyclic AMP after prelabelling with [^{14}C]adenine (0·075 μCi [^{14}C]adenine per ganglion preincubation for 60 min)

	[^{14}C]ATP (cpm/ganglion)	[^{14}C]cyclic AMP (cpm/ganglion)	Conversion rate to [^{14}C]cyclic AMP (mean % of total radioactivity)	[^{14}C]cyclic AMP in the medium (mean % of total [^{14}C]cyclic AMP)
Control (tissue)	185 ± 15	45 ± 15	1·6	
Control (medium)	—	27 ± 3		37
Adrenaline (tissue)	145 ± 20	65 ± 25	8·5	
Adrenaline (medium)	—	50 ± 15		42
Histamine (tissue)	160 ± 16	90 ± 12	5·1	
Histamine (medium)	—	70 ± 10		40

Incubation time = 10 min.
Data are expressed as the mean of four experiments ± S.D.

to [^{14}C]ATP, the incorporation of radioactivity into [^{14}C]cyclic AMP was not influenced under these conditions. The ratio [^{14}C]ATP/[^{14}C]cyclic AMP which after labelling with [^{14}C]adenine reached 16 under control conditions, was decreased by Antimycin A_1 to 5 (Table 4).

DISCUSSION

Considerable amounts of cyclic AMP were measured in rat superior cervical ganglia. Upon stimulation with adrenaline or histamine levels of endogenous cyclic AMP as well as the conversion rate of [^{14}C]labelled adenine nucleotides to [^{14}C]cyclic AMP rose rapidly, indicating a fast increase in the synthesis rate of the cyclic nucleotide. Almost concomitantly significant amounts of endogenous or labelled cyclic AMP appeared in the incubation medium. There is only one early report of release of cyclic AMP from central nervous tissue [17], while in most studies of metabolism of cyclic AMP in nervous tissues possible release or leakage of the nucleotide was neglected. Pull and McIlwain [18] described bulk release of radioactive adenine nucleotides from stimulated brain slices. In our experiments ATP was not detected in the incubation medium under normal conditions. However, after transfer of ganglia from an ice-cold environment to a medium of 37° release of ATP occurred. This liberation of ATP was significantly decreased during adrenaline or histamine stimulation of adenylate cyclase. Further investigations could answer the question if binding of ATP plays a role in the adenylate cyclase–receptor interaction in membranes. Triphosphates in liver plasma membranes are involved in coupling between the receptor and the adenylate cyclase [19]. The leakage of nucleotides is also of interest with respect to the common methods of slicing and handling nervous tissues in studies of cyclic AMP. It has been shown previously that transfer of tissue slices from cold environments to media at 37° induces acute alterations in a number of biochemical parameters [20].

After prelabelling adenine nucleotides with [^{14}C]adenine for 60 min only 0·6–1·6 per cent of the total radioactivity was converted into [^{14}C]cyclic AMP confirming findings with brain slices [4]. While the endogenous ATP and cyclic AMP concentrations were 20·8 nmoles/mg protein and 26·5 pmoles/mg protein, respectively, showing a 775-fold difference, the ratio of [^{14}C]ATP to [^{14}C]cyclic AMP was between 3 and 16 (varying according to the concentration of [^{14}C]adenine and the duration of the preincubation period). When cyclic AMP formation was stimulated with histamine or adrenaline, the ratio of [^{14}C]ATP to [^{14}C]cyclic AMP decreased from a control value of 3 to below 2. These findings suggest the presence of a preferentially labelled ATP pool which serves as a substrate pool for adenylate cyclase and which perhaps is exhaustible upon stimulation of adenylate cyclase *in vitro*.

Our findings with Antimycin A_1, which significantly inhibits the incorporation of radioactivity into ATP, support this hypothesis, since the formation of [^{14}C]cyclic AMP was not influenced by Antimycin A_1. The high proportion of labelled cyclic AMP in the medium points to the fact, that cyclic AMP, owing to its molecular structure and/or its compartmentation, is capable of leaving cells, in contrast to

Table 4. Effect of Antimycin A_1 (10^{-7}M) on the content of [^{14}C]ATP and [^{14}C]cyclic AMP (in ganglia and medium) and on the conversion rate to [^{14}C]cyclic AMP after prelabeling superior cervical ganglia of the rat with [^{14}C]adenine for 20 and 60 min (0·3 μCi/ganglion)

Group	Time (min)	[^{14}C]ATP (cpm/ganglion ± S.D.)	[^{14}C]cyclic AMP (cpm/ganglion ± S.D.)	Total radioactivity (cpm/ganglion ± S.D.)	Conversion rate to [^{14}C]cyclic AMP (% of total radioactivity in ganglia and medium)	[^{14}C]ATP/[^{14}C]cyclic AMP
Control	20	415 ± 23	59 ± 10	6384 ± 260	0·9 ± 0·2	7
Control	60	1290 ± 150	78 ± 12	12700 ± 1050	0·6 ± 0·1	16
Antimycin A (10^{-7}M)	20	320 ± 20	40 ± 3	5540 ± 770	0·7 ± 0·1	8
Antimycin A (10^{-7}M)	60	430 ± 90	90 ± 10	13650 ± 770	0·7 ± 0·1	5

n = 3 for each group.

ATP which under identical conditions was nearly absent in the medium. Previous attempts to study pool sizes of ATP and cyclic AMP in stimulated nervous tissue have neglected release of nucleotides [21]. The observed release of a "second" messenger from intact nervous tissue moreover might be of additional interest since it is reminiscent of the mode of inactivation of 'first' messengers, e.g. catecholamines, by diffusion of agonists from receptor sites. In spite of the generally low concentrations of cyclic AMP in most tissues, high clearance values and excretion rates for cyclic AMP were found in the blood and in the urine [22, 23] and there is a marked flow of cyclic AMP through the cerebrospinal fluid compartment [24, 25]. Further studies of the extracellular levels and the transport of cyclic AMP appear to be warranted to answer questions concerning the mechanism of metabolic regulation and physiological roles of cyclic AMP.

REFERENCES

1. D. A. McAfee, M. Schorderet and P. Greengard, *Science* **171,** 1156 (1971).
2. H. Cramer, D. G. Johnson, I. Hanbauer, S. D. Silberstein and I. J. Kopin, *Brain Res.* **53,** 97 (1973).
3. T. Lindl and H. Cramer, *Int. Res. Comm. Syst.* **73–8,** 3-5-12, (1973).
4. H. Shimizu, J. W. Daly and C. R. Creveling, *J. Neurochem.* **16,** 1609 (1969).
5. T. Lindl and H. Cramer, *Biochim. biophys. Acta* **343,** 182 (1974).
6. M. Reporter, *Res. Commun.* **48,** 598 (1972).
7. H. Shimizu and H. Okayama, *J. Neurochem.* **20,** 1279 (1973).
8. J. Schultz and J. W. Daly, *J. biol. Chem.* **248,** 843 (1973).
9. H. R. Mahler and E. H. Cordes, *Biological Chemistry*, 2nd Edition, p. 686. Harper & Row, New York (1971).
10. A. G. Gilman, *Proc. natn. Acad. Sci. U.S.A.* **67,** 305 (1970).
11. H. Shimizu and J. W. Daly, in *Methods in Neurochemistry* (Ed. R. Fried) Vol. **2,** p. 147. Marcel Dekker, New York (1972).
12. P. K. Dighe, D. N. Pahuja and D. H. Sha, *J. Chromat.* **40,** 449 (1969).
13. M. Szabo and G. Burke, *Biochim. biophys. Acta* **264,** 289 (1972).
14. O. Holm-Hansen and C. R. Booth, *Limnol. Oceanogr.* **11,** 510 (1966).
15. F. Wroblewski and J. E. LaDue, *Proc. Soc. exp. Biol. Med.* **90,** 210 (1955).
16. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
17. S. Kakiuchi and T. W. Rall, *Molec. Pharmac.* **4,** 367 (1968).
18. I. Pull and H. McIlwain, *J. Biochem.* **130,** 975 (1972).
19. G. Brooker, *J. biol. Chem.* **246,** 7810 (1971).
20. H. F. Bradford, in *Methods in Neurochemistry* (Ed. R. Fried) p. 155. Marcel Dekker, New York (1972).
21. G. Krishna, J. Forn, K. Voigt, M. I. Paul and G. L. Gessa, *Adv. Biochem. Psychopharmacol.* **3,** 155 (1970).
22. A. F. Broadus, N. I. Kaminsky, J. G. Hardman, F. W. Sutherland and G. W. Liddle, *J. Clin. Invest.* **49,** 2222 (1970).
23. M. I. Paul, H. Cramer and W. E. Bunney, *Science* **171,** 300 (1971).
24. H. Cramer, K. Y. Ng and T. N. Chase, *J. Neurochem.* **19,** 1601 (1972).
25. H. Cramer and T. Lindl, *Psychopharmacologia* **26,** Suppl., 49 (1972).