ADENYLATE CYCLASE, PHOSPHODIESTERASES AND PROTEIN KINASE OF RAT GLIAL CELLS IN CULTURE

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1. Introduction

Tumoral cells in culture from both glial [1, 2] and neuronal [3] origins are able to undergo part of the morphological and biochemical differentiation of the normal cells. Thus glial cells synthetize the S 100 protein characteristic of normal glial tissue [4]. Recently, it was shown that glial cells accumulate large amounts of 3′-5′-AMP in response to catecholamines [5–7]. These cells in culture may represent a very useful tool for studying the role of 3′-5′-AMP in nervous tissue. In this preliminary study some of the properties of the enzymes involved in the metabolism and action of 3′-5′-AMP in rat glial cells (clone C_6) are described. Glial cells homogenates contain adenylate cyclase, phosphodiesterase and proteine kinase activities.

The adenylate cyclase activity was stimulated by isoproterenol, epinephrine and norepinephrine. Isoproterenol was more effective than either epinephrine or norepinephrine; its action was blocked by propanolol but not by phentolamine suggesting that the adrenergic receptor present is of the β type. Dopamine was also able to stimulate adenylate cyclase activity. Serotonine, acetylcholine tyramine and tryptamine were inactive.

Phosphodiesterase activity can be described as the sum of the activities of two phosphodiesterases with different maximal velocities and affinities for 3′-5′-AMP. The larger part of the phosphodiesterases and 3′-5′-AMP dependent protein kinase activities was associated with the 5000 *g* sediment of glial cells homogenates.

2. Methods

Rat glial cells (clone C_6) were grown in 250 ml glass bottles on HAM F 10 medium supplemented with 10% foetal calf serum and collected during stationary phase of growth. The washed cells were dispersed in a cold hypotonic medium (25 mM, Tris-HCl pH 7.6, 5 mM EDTA: 0.1 ml per 10^6 cells) and homogenized in a glass Elvehjem potter (6 strokes). The homogenate was centrifugated at 5000 *g* for 10 min at 0°. The supernatant was separated and the pellet dispersed in the same volume of buffer. These two fractions were stored frozen in liquid nitrogen for 1 to 6 weeks with no apparent loss in the enzymatic activities tested.

Adenylate cyclase activity was measured by the conversion of [^{32}P]α-ATP into [^{32}P]3′-5′-AMP. The incubation medium (final volume 100 μl) contained: 100 mM Tris-HCl pH 7.6, 3 mM $MgCl_2$, 0.1 mM ATP, [^{32}P]α-ATP 2 μCi/ml, 1 mM 3′-5′-cyclic AMP, phosphocreatine 15 mg/ml and creatine kinase 5 mg/ml. The reaction was initiated by the addition of enzyme (10 to 75 μg); incubation was performed at 37° for 5 min. The reaction was stopped by dilution with 150 μl of a solution containing 50 mM Tris-HCl pH 7.6, 3 mM ATP, 5 mM 3′-5′-cyclic AMP and [^{3}H] 3′-5′-cyclic AMP 0.1 μCi/ml followed by immediate separation of 3′-5′-cyclic AMP on an aluminium oxyde column according to Ramachandran et al. [8].

Phosphodiesterase activity was measured by the conversion of [^{3}H]3′-5′-AMP into [^{3}H]5′-AMP. The aluminium oxyde chromatography provides a very convenient and rapid procedure for the separation of 3′-5′-AMP from 5′-AMP completely retained by the

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column. However, using this technique, it is necessary to completely block further hydrolysis of [^{3}H]5′-AMP by phosphatase present in tissue homogenates since adenosine would not be retained by the column. This condition was achieved by adding an excess of unlabelled 5′-AMP and inhibition of phosphatase by fluoride.

The incubation medium final volume (55 μl) contained 45 mM Tris-HCl pH 8.0, 25 mM $MgCl_2$, 50 mM NaF, 3.4 mM 5′-AMP, [^{3}H]3′-5′-AMP (0.11 μCi/ml), unlabelled 3′-5′-AMP (final concentrations ranging from 0.1 to 500 μM). The reaction was initiated by adding the enzyme (5.0 to 230 μg/ml) and the reaction mixture was incubated at 37° for 120 min. The reaction was stopped by dilution with 150 μl of a solution containing: 50 mM Tris-HCl pH 7.6, 1 mM 3′-5′-AMP and [^{14}C]3′-5′-AMP (0.01 μCi/ml). The diluted incubation medium was immediately applied to the top of a dry aluminium oxide column and 3′-5′-AMP eluted.

Control samples incubated with tracer amounts of [^{14}C]5′-AMP (0.1 μCi/ml) without labelled 3′-5′-AMP were run in parallel to test the blockade of phosphatase.

The amount of 3′-5′-AMP remaining at the end of the incubation period was calculated from the ^{3}H content of the column eluate corrected for the yield of the separation procedure deduced from the recovery of [^{14}C]3′-5′-AMP introduced with the diluting solution.

In each experiment, the velocity substrate concentration curves were drawn using the integrated form of the Michaelis equation:

$$\frac{2.303}{t}\log\frac{S_0}{S_t} = -\frac{1}{K_m}\left(\frac{(S_0 - S_t)}{t}\right) - V_m$$

in which t is the incubation time, S_0 and S_t the substrate concentrations at the beginning and the end of the incubation, respectively. The Michaelis constant (K_m) was deduced from the slope of the linear regression line obtained and the maximum velocity of the reaction (V_m) from the x intercept. The latter value was expressed as pmoles 3′-5′-AMP hydrolysed per min per mg protein.

Protein kinase activity was measured by incorporation of ^{32}P from [^{32}P]γ-ATP into lysine rich histone according to the procedure described by Myamoto et al. [9]. The incubation medium (final volume: 250 μl) contained: 60 mM sodium acetate pH 6.0, 3 mM $MgCl_2$, 0.3 mM EDTA, 8 mM β mercaptoethanol,

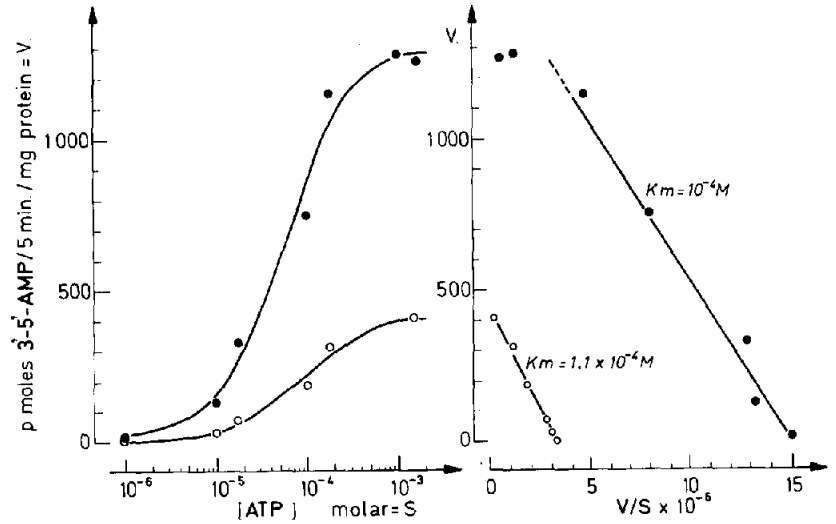

Fig. 1. Effects of ATP concentration on basal and isoproterenol sensitive adenylate cyclase activities. Adenylate cyclase was assayed as indicated under Methods. The protein content of the incubation medium was 0.017 mg. Key to symbols: (○) basal activity, (●) isoproterenol (10^{-5} M) sensitive activity. On the left part of the figure activity (V) is plotted as a function of ATP concentration (log scale). On the right part the same data are plotted according to Eadie. The experimental curves indicate that isoproterenol did not modify the apparent K_m for ATP. Inhibition by substrate was observed only in presence of isoproterenol.

0.04 mM ATP, histone 1 mg/ml, [^{32}P]γ-ATP: 0.6 μCi/ml. 3'-5'-AMP 0 or 1 μM and enzyme 10 to 90 μg protein. Incubation was performed at 30° for 20 min. The reaction was stopped by precipitation with 2 ml of 5% trichoracetic acid, 0.25% sodium tungstate pH 2.0. Proteins were determined by the method of Lowry [10].

3. Results and discussion

The rate of 3'-5'-AMP formation by the 5000 *g* pellet of glial cells homogenates was linear with time for incubation periods less than 10 min and with enzyme concentration from 60 to 500 μg protein per ml.

Adenylate cyclase activity was stimulated 4- to 10-fold by 10^{-5}M isoproterenol. The activation ratio was independant of ATP concentration in the incubation medium from 10^{-6} to 10^{-3} M. For the ATP concentration used in most experiments (0.1 mM) the activation was optimum at pH 7.5 and 3 mM Mg^{2+}. As indicated by fig. 1 isoproterenol stimulation resulted in an increase in the maximal velocity of the adenylate cyclase reaction with no change in the apparent affinity for ATP (the K_m values were close to 0.1 mM for both basal and isoproterenol sensitive activities). As indicated by Gilman and Nirenberg [5] and Schimmer [6] the adrenergic receptor present in glial cells is of the β type. Thus (fig. 2), the β agonist isoproterenol was more effective (apparent K value: 10^{-7} M) than epinephrine or norepinephrine (apparent K value: 8×10^{-7} M). Furthermore (fig. 3) the β blocker propanalol (1 mM) completely blocked the response to isoproterenol while in the presence of the α blocker phenoxybenzamine (1 mM) the stimulatory effect of isoproterenol was still present. However, it must be noted that the two blockers led to a significant inhibition of basal activity.

As indicated by table 1 dopamine was also able to activate the adenylate cyclase of glial cells. Serotonine,

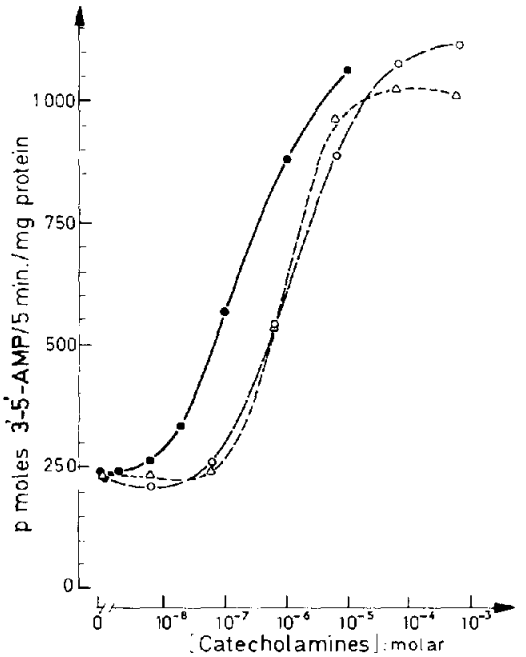

Fig. 2. Responses of glial cells adenylate cyclase to stimulation by catecholamines. Adenylate cyclase was assayed as indicated under Methods. The protein content of the incubation medium was 0.010 mg. Adenylcyclase activity is plotted as a function of isoproterenol (•—•—•), epinephrine (o—o—o) or norepinephrine (△—△—△) concentrations in the medium (log scale). Concentrations are expressed as molar concentrations of D–L catecholamines.

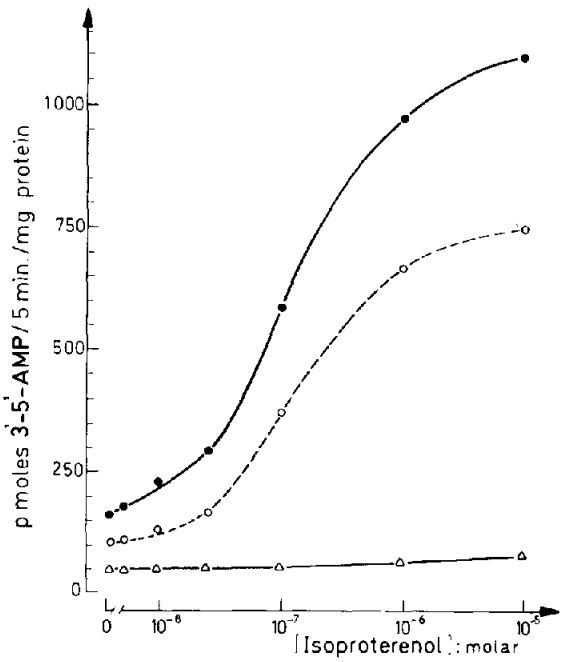

Fig. 3. Effects of adrenergic blockers on the response of glial cells adenylate cyclase to isoproterenol stimulation. Responses of glial cells adenyl cyclase to increasing amounts of isoproterenol were measured with the same enzyme (0.018 mg protein per tube) in three experimental conditions: in the presence of 1 mM propranolol (△—△—△), in the presence of 1 mM phenoxylbenzamine (o—o—o) and in the absence of adrenergic blockers (•—•—•).

Table 1
Specificity of glial cells adenylcyclase.

Pharmacological agent (M)	Adenyl cyclase activity	Pharmacological agent (M)	Adenyl cyclase activity
Isoproterenol		Serotonine	
0	216	0	216
10^{-6}	2320	10^{-7}	211
10^{-5}	2445	10^{-6}	227
10^{-4}	2503	10^{-5}	207
		10^{-4}	216
Dopamine		Acetylcholine	
0	216	0	216
10^{-7}	211	10^{-6}	201
10^{-6}	237	10^{-5}	200
10^{-5}	401	10^{-4}	202
10^{-4}	1112		
Tryptamine		Adenosine	
0	216	0	216
10^{-6}	201	10^{-4}	184
10^{-5}	190	10^{-3}	181
10^{-4}	195	10^{-2}	107
Tyramine		Theophylline	
0	216	0	216
10^{-6}	191	10^{-4}	190
10^{-5}	191	10^{-3}	185
10^{-4}	222	10^{-2}	166

Adenylcyclase was assayed as indicated under Methods. Activities are expressed as pmoles 3'-5'-AMP/10 min/mg protein.

acetylcholine, tryptamine and tyramine were inactive. Adenosine which is able to induce a large increase in the total 3'-5'-cyclic AMP content of cortex slices [11] was in fact inhibitory at high concentrations; similar results were obtained with theophylline.

Fluoride ions stimulated glial cells adenylate cyclase activity. However fluoride stimulation was very small as compared to stimulations obtained in other tissues (see for instance [16]). Furthermore, it was possible to block and finally to reverse the fluoride effect by lowering temperature of the incubation medium (fig. 4).

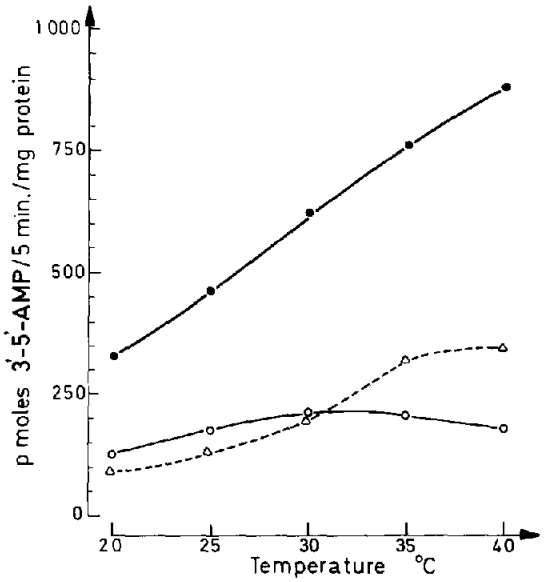

Fig. 4. Temperature dependence of the particulate glial cells adenylcyclase. Adenylcyclase was assayed as indicated under Methods. The protein content of the incubation medium was 0.052 mg. Key to symbols: (o—o—o) basal activity, (△—△—△) activity measured in presence of 10 mM sodium fluoride, (●—●—●) activity in presence of 10^{-5} M isoproterenol.

Both the 5000 *g* pellet and supernatant of glial cells homogenates were able to ensure the conversion of [^{3}H]3'-5'-cyclic AMP into 3'-5'-AMP. Measurements of [^{3}H]3'-5'-AMP conversion for substrate concentrations ranging from 10^{-7} to 5×10^{-4} M suggested the presence of two phosphodiesterases with different maximum velocities and affinities for 3'-5'-cyclic AMP. Thus (fig. 5) when $2.303/t \log S_0/S_t$ was plotted against $(S_0 - S_t/t$ the experimental points were clear-

Table 2
Kinetic parameters of glial cells phosphodiesterases.

Phosphodiesterase I		Phosphodiesterase II	
Apparent affinity K_m (M)	Maximal velocity pM/min/mg	Apparent affinity K_m (M)	Maximal velocity pM/min/mg
5000 *g* pellet 7×10^{-6}	300	1.35×10^{-4}	4060
5000 *g* pellet 3.7×10^{-6}	162	1.14×10^{-4}	1140

K_m and V_m values were deduced from graphical plots similar to that described by fig. 5. Values are the mean of two determinations.

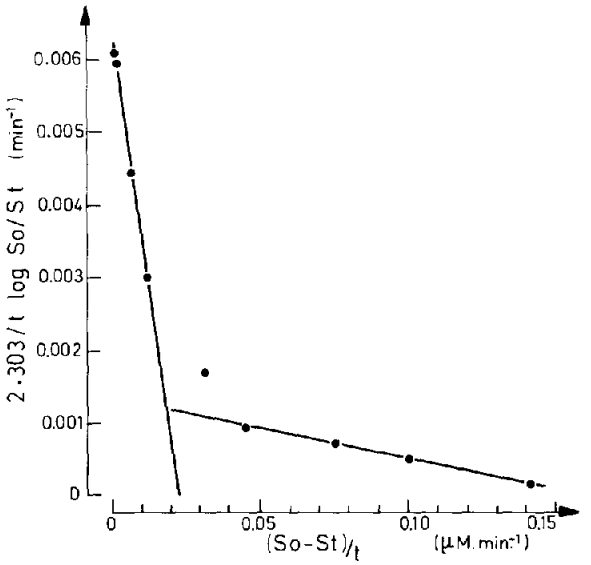

Fig. 5. Presence of two phosphodiesterases in the soluble fraction of glial cells homogenate. The conversion of a tracer amount of [^{3}H]3′-5′-AMP was measured as indicated under Methods in the presence of increasing amounts of unlabelled 3′-5′-AMP. The final nucleotide concentrations at the beginning of the incubation (S) were respectively: 0.2, 0.5, 2.0, 5.0, 20.0, 50.0, 100.0, 200.0 and 500.0 μM. Substrate concentrations at the end of the incubation period were calculated and the data plotted using the integrated form of the Michaelis equation:

$$\frac{2.303}{t} \log \frac{S_0}{S_t} = \frac{1}{K_m} \times \frac{(S_0 - S_t)}{t} - V_m$$

As shown by the graph the experimental points were distributed along two strainght lines indicating the presence of two phosphodiesterases with different affinities for 3′-5′-AMP and different maximal velocities. The protein content of the incubated samples was 36 μg.

ly distributed along two distinct straight lines. The K_m values and maximal velocities of these two phosphodiesterase activities are given in table 2.

Table 3 gives the distributions of adenylate cyclase, phosphodiesterases and protein kinase activities between the 5000 *g* pellet and supernatant of glial cells homogenates. In contrast with the situation encountered in most tissues except nervous tissue [12–15] the larger part of phosphodiesterase activities and more than 50% of the 3′-5′-AMP dependent protein kinase activity were recovered in the particulate fraction.

Table 3
Distribution of adenylate cyclase, phosphodiesterase and protein kinase in glial cells homogenates.

Enzyme activity	5000 *g* Pellet		5000 *g* Supernatant	
	Specific activity	Total activity (%)	Specific activity	Total activity (%)
Adenylate cyclase[1]				
basal	538	91.8	148	8.2
+ Isoproterenol (10^{-5}M)	1972	90.9	592	9.1
+ Fluoride (10^{-2}M)	672	88.0	274	12.0
Phosphodiesterases[2]				
Phosphodiesterase I	300	85	162	15
Phosphodiesterase II	4060	91.5	1140	8.5
Protein kinase[3] basal	116.5	60.4	310	39.6
+ 3′-5′-AMP (10^{-6}M)	420.5	77.5	492.3	22.5

Values are the means of 4 determinations. [1]Adenylate cyclase activity: pmoles 3′-5′-AMP formed/5 min/mg protein. [2]Phosphodiesterase activity: pmoles 3′-5′-AMP hydrolysed/min/mg protein. [3]Protein kinase activity: pmoles ^{32}P incorporated/min/mg protein.

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