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REVERSIBLE INHIBITION OF ADENYLATE CYCLASE ACTIVITY OF RAT BRAIN CAUDATE NUCLEUS BY OXIDIZED GLUTATHIONE

Akemichi Baba, Eibai Lee, Toshio Matsuda, Tetsuroh Kihara and Heitaroh Iwata

Department of Pharmacology, Faculty of Pharmaceutical Sciences, Osaka University, Suita, Osaka, Japan

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SUMMARY

Basal and dopamine-stimulated adenylate cyclase (EC 4.6.1. 1.) activities were strongly inhibited by GSSG, but not by GSH. Adenylate cyclase that had been inactivated by GSSG was reactivated by incubation with various sulfhydryl compounds including GSH. Formation of mixed disulfides by reaction between GSSG and protein-SH groups increased on incubation with GSSG and returned to the normal level on subsequent incubation with DTT.

The regulation of adenylate cyclase activity by various endogenous substances has been studied in a variety of membrane preparations (see ref. 1). Some studies have shown that sulfhydryl reagents affect adenylate cyclase activity (2-9), but little is known about how sulfhydryl groups are actually involved in regulation of the enzyme.

The regulation of enzyme activity by disulfide exchange has been clearly demonstrated in fructose diphosphatase (10), pyruvate kinase (11) and glycogen synthetase (12), and the functional significance of glutathione disulfide in disulfide exchange has been proposed (see ref. 13). This paper reports a mechanism for regulation of adenylate cyclase activity of rat

Abbreviations: EGTA; ethylene glycol bis(β-aminoethyl ether)-N,N',-tetraacetic acid, DTT; dithiothreitol.

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brain caudate nucleus by disulfide exchange between the sulfhydryl group(s) of the enzyme and GSSG.

METHODS

Preparation of enzyme

Male Sprague-Dawley rats, weighing about 150 to 200 g, were killed by decapitation. The caudate nuclei were homogenized in 50 volumes of 2 mM Tris-maleate buffer (pH 7.4) containing 2 mM EGTA. Unless otherwise indicated, this homogenate was used as an enzyme source.

Assay of adenylate cyclase activity

The standard assay system (final volume 0.5 ml) for measurement of adenylate cyclase activity contained (in mmol/liter): Tris-maleate (pH 7.4), 80; ATP, 0.3; $MgSO_4$, 1.2; isobutylmethylxanthine, 1; EGTA, 0.6; plus 50 µl of homogenate. The reaction mixture without ATP was preincubated at 0° for 20 min and the reaction was started by adding ATP. Unless otherwise indicated, test substances were added to the preincubation medium. The reaction was carried out for 2.5 min at 30° and stopped by placing the tube in a boiling-water bath for 3 min. Then the mixture was centrifuged at 5,000 × g for 10 min to remove insoluble material. The amount of cyclic AMP formed in each tube was measured on duplicate or triplicate 50-µl aliquots by the method of Brown et al. (15, 16). Interference of test substances in the binding protein assay of cyclic AMP was negligible. Under the experimental conditions used, enzyme activity was proportional to the time of incubation and enzyme concentration. The effects of GTP and guanyl-5'-yl imidodiphosphate on the striatal adenylate cyclase reported by Clement-Cormier et al. (14, 17) were also observed in this study.

Assay of mixed disulfide

The amount of mixed disulfide formed between protein-SH and GSSG (GSS-protein) (18) was determined as follows: The suspension was incubated with or without GSSG, centrifuged at 10,000 × g for 15 min and washed twice to remove free GSSG. The resulting pellets were then treated with sodium borohydride to reduce disulfide bonds (18), and centrifuged, and the resulting supernatant was assayed for GSH (Total GSH) (19). The amount of GSSG remaining in the precipitate after washing was also determined (19). The blank value was determined in the same way, but without the step of reduction with sodium borohydride (Free GSH). This blank value represents the amount of GSH that did not form disulfide bonds with protein-SH. The amount of GSH derived from GSS-protein was calculated by subtracting the amount of free GSH and GSSG from the total GSH (18).

Protein concentration was determined by the method of Lowry et al. (20).

RESULTS

The effects of various concentrations of dopamine on the adenylate cyclase activity of a homogenate of the caudate nucle-

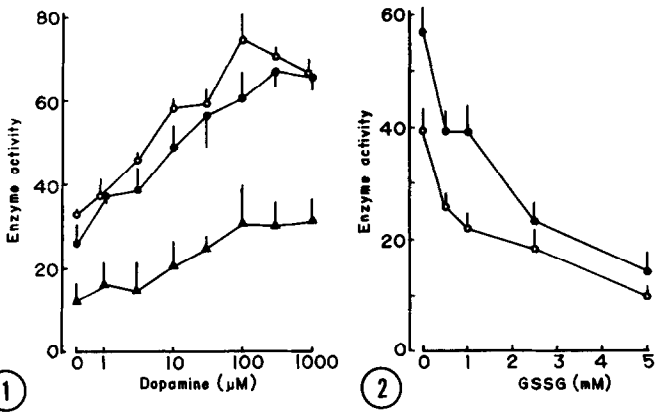

Fig. 1. (left) Effect of glutathione on adenylate cyclase activity of rat brain caudate nucleus. Glutathione was added to the medium during preincubation. Adenylate cyclase activity (pmol/mg protein/min) was determined in the presence of 10 μM GTP. Each point represents the mean ± S.E. of 4 separate experiments. Control; (o), 5 mM GSH; (●), 2.5 mM GSSG; (▲).

Fig. 2. (right) Dose-response to GSSG of adenylate cyclase activity. Adenylate cyclase activity was determined in the absence of GTP. Each point represents the mean ± S.E. of 5 separate experiments. Basal; (o), Dopamine-stimulated; (●).

us were examined (Fig. 1). In the presence of 10 μM GTP, adenylate cyclase activity was stimulated by a very low concentration of dopamine, maximum stimulation occuring with 100 μM dopamine. Addition of 2.5 mM GSSG to the preincubation medium significantly inhibited the enzyme activity both in the absence (basal) and presence of dopamine (dopamine-stimulated) ($P<0.01$), whereas 5 mM GSH had no effect. The percentage stimulation of enzyme activity by dopamine was not affected by GSSG. DTT, cysteine, cysteamine and 2-mercaptoethanol at concentration of up to 5 mM had no effect on the enzyme activity, whereas 2.5 mM cystine, cystamine and homocystine definitely inhibited the enzyme activity (data not shown). The effect of GSSG on adenylate cyclase activity was dose-dependent (Fig. 2), 30 to 40% inhibition being obtained with 0.5 mM GSSG. This dose-response effect of GSSG was determined in the assay system without GTP, but a similar

Table 1. Effect of pretreatment of GSSG and addition of sulfhydryl compounds on adenylate cyclase activity of the membrane fraction

Incubation 1st.	2nd.	Basal activity	Dopamine-stimulated activity
None	None	84.8 ± 5.1	135.5 ± 12.8
GSSG	None	36.0 ± 4.8**	74.8 ± 11.1*
GSSG	GSH	76.8 ± 11.6	98.6 ± 13.5
GSSG	DTT	88.1 ± 5.0	132.8 ± 13.6
GSSG	Cysteine	89.6 ± 11.0	119.4 ± 15.9
GSSG	Cysteamine	68.2 ± 6.3	99.0 ± 8.6
GSSG	2-Mercaptoethanol	82.3 ± 7.9	105.8 ± 7.4

After preincubated with or without GSSG as described in the Methods, the suspension was centrifuged for 15 min at 10,000 × g to remove free GSSG in the medium. The resulting pellet was washed twice with homogenizing buffer. The final precipitate was assayed for enzyme activity in the presence of 10 μM GTP after preincubation for 20 min at 0° with or without various sulfhydryl compounds. Each value represents the mean ± S.E. of 4 to 6 separate experiments. Enzyme activity; pmol/mg protein/min. DTT, dithiothreitol.
* $P<0.05$, ** $P<0.01$

dose-dependent effect of GSSG was observed even in the presence of 10 μM GTP or guanyl-5'-yl imidodiphosphate (data not shown). Furthermore, when the membrane fraction (M_1) (21) from the caudate nucleus was used as an enzyme source, GSSG also strongly inhibited the adenylate cyclase activity.

The effect of GSSG on adenylate cyclase was reversible. In Table 1 the specific activity of the enzyme shown is that of the particulate fraction, which was higher than that of the homogenate. Pretreatment with 2.5 mM GSSG markedly inhibited both the basal and dopamine-stimulated activity ($P<0.01$). Furthermore, enzyme that had been inactivated by GSSG was reactivated by incubation with various sulfhydryl compounds including GSH.

Table 2. Changes of protein-SSG mixed disulfide (GSS-protein) concentration in the particulate fraction on pretreatment with GSSG and subsequent addition of DTT

Incubation 1st.	2nd.	Total GSH[a]	Free GSH[a]	Free GSSG[b]	GSS-protein[a]
None	None	3.0 ± 0.2	0.1 ± 0.1	N.D.[c]	2.9 ± 0.2
GSSG	None	6.3 ± 0.2	0.6 ± 0.1	0.3 ± 0.1	5.2 ± 0.3**
GSSG	DTT	4.6 ± 0.1	1.0 ± 0.1	0.1 ± 0.1	3.5 ± 0.2

Experimental conditions were as for Table 1. After incubation with or without DTT, the suspension was centrifuged and washed once. The resulting precipitate were assayed for GSH and GSSG. Each value represents the mean ± S.E. of 4 separate experiments. a; nmol GSH/mg protein, b; nmol GSSG/mg protein, c; Not detected. ** $P<0.01$; Compared with "None-None".

The amount of GSS-protein in the enzyme preparation was determined (Table 2). The amount was significantly increased by incubating the preparation with 2.5 mM GSSG ($P<0.01$), and the levels of GSS-protein returned to the control range on further incubation with DTT.

DISCUSSION

Several sulfhydryl reagents inhibit adenylate cyclase from various sources (2-9). Most previous studies have been made with sulfhydryl blocking reagents and have indicated the requirement for free thiol groups for adenylate cyclase activity. In the present study, we found that GSSG but not GSH inactivated adenylate cyclase in rat brain caudate nucleus. The involvement of disulfide exchange as a possible regulatory mechansim for sulfhydryl enzymes has been extensively studied with rabbit liver fructose diphosphatase (10). The idea that a disulfide exchange also occurs during inactivation of adenylate cyclase by GSSG is supported by the present finding that when adenylate cyclase has been inactivated by GSSG it can be almost fully re-

activated by a wide range of sulfhydryl compounds. This idea is also supported by our results showing that the amount of GSS-protein increased when the enzyme preparation was incubated with GSSG and then decreased to normal level when it was incubated with DTT. These results indicate that the inactivation of adenylate cyclase are explained by the oxidation of sulfhydryl groups in the enzyme. We also found that GSSG inactivated adenylate cyclase from rat cerebral cortex and cardiac sarcolemma (data not shown). However, pretreatment with GSSG had no effect on the Mg^{++} and Na^{+}-K^{+}-ATPase activities in the caudate membrane fraction, showing that GSSG does not inactivate all sulfhydryl enzymes.

It is difficult to demonstrate the physiological significance of disulfide exchange in the regulation of adenylate cyclase. Isaacs and Binkley (22) proposed that formation of GSS-protein is a mechanism for maintenance of a disulfide-sulfhydryl ratio such that the integrity of the membrane is maintained during oxidative and reductive stresses. As shown in Table 2, a small amount of GSS-protein was detected even in an intact preparation. GSS-protein is possibly involved in the maintenance of the integrity of nerve membrane.

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