Effect of Buffer Composition on Binding of Adenosine 3':5'-Cyclic Monophosphate by Bovine Adrenal Extracts

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The binding of cyclic [^3H]AMP by bovine adrenal extracts was studied in different buffer systems; 50 mM-Tris/HCl, pH 7.5, buffer containing 4 mM-EDTA (disodium salt) offered advantages such as low non-specific interference and high binding affinity. 2-Mercaptoethanol decreased, but EDTA and theophylline increased, the specificity of cyclic [^3H]AMP binding as expressed by the displacement of cyclic [^3H]AMP by a 1000-fold excess of unlabelled cyclic GMP. The study indicated heterogeneity of the binding material. Mg^{2+} -dependent binding may be eliminated in Tris/EDTA buffer. The heterogeneity and the specificity of intracellular cyclic AMP binders and their dependence in environmental factors should be considered in the evaluation of cyclic AMP binding and second-messenger systems.

Saturation assay techniques using specific proteins from various animal tissues have been widely applied for the determination of cyclic AMP and cyclic GMP in biological materials (Gilman, 1970; Murad *et al.*, 1971; Brown *et al.*, 1971; Tsang *et al.*, 1972; Wombacher & Körber, 1972; Tovey *et al.*, 1974; Nistrup-Madsen *et al.*, 1975; Aksnes & Sövik, 1976). Increasing attention has been paid to the role of cyclic nucleotide-dependent protein kinases in cyclic AMP- and cyclic GMP-mediated processes. However, the receptors for cyclic nucleotides have been less investigated. As regulatory subunits of protein kinases, the receptors play a key role in second-messenger systems (Kuo & Greengard, 1969; Gill & Garren, 1970; Rubin & Rosen, 1975). Moreover, it has been reported that a considerable amount of the cyclic AMP-binding proteins act separately from the regulatory subunits of cyclic AMP-dependent protein kinases (Prasad *et al.*, 1976). These proteins may influence cyclic AMP-mediated processes. The factors that change specificity of cyclic AMP binding have not been previously studied. Because the structure of cyclic AMP is closely related to that of cyclic GMP and the cellular functions of these nucleotides are mostly opposite, these factors may modify the response to cyclic AMP.

In the present study different buffer systems were tested to develop and optimize methods for measuring cyclic [^3H]AMP-binding activity and specificity.

Materials and Methods

Buffers

These were: 50 mM-Tris, adjusted to pH 7.5 with HCl (Tris buffer); 50 mM-Tris/250 mM-sucrose/25 mM-KCl/5 mM- MgCl_2 , adjusted to pH 7.4 with HCl

(Mg buffer); 50 mM-Tris/8 mM-theophylline/6 mM-2-mercaptoethanol, adjusted to pH 7.4 with HCl (TF-ME buffer); 50 mM-Tris/4 mM-EDTA (disodium salt), adjusted to pH 7.5 with HCl (EDTA buffer).

Preparation of extracts

Fresh bovine adrenals were cleaned, cut into small pieces and divided into two portions. One was homogenized in Mg buffer (Mg-extract) and the other in EDTA buffer (EDTA-extract) with an Ultra-Turrax homogenizer (3 vol. of buffer and 1 vol. of tissue). Extracts were centrifuged twice at 15000 rev./min for 20 min and at 20000 rev./min for 30 min. Protein concentrations (Lowry *et al.*, 1951) of the supernatants were adjusted to 15.0 mg/ml with the extraction buffer. All steps of the preparation were performed below 4°C. Extracts were stored at -20°C for no more than 1 month.

Other reagents

[8- ^3H]Adenosine 3':5'-cyclic monophosphate (27 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K.; for standard use 2 μl was added to 3.7 ml of assay buffer, giving 20 nmol/litre (1 pmol/50 μl). Cyclic AMP (acid) and cyclic GMP (monosodium salt) were from Boehringer, Mannheim, West Germany. Stock solutions of cyclic AMP and cyclic GMP contained 200 nmol/ml.

Coated charcoal suspension for separation of bound and free cyclic AMP was prepared by weighing 5 g of charcoal (Norit A; Amend Drug and Chemical Co., Irvington, NJ 07111, U.S.A.), 0.5 g of Dextran T-70 (Pharmacia, Uppsala, Sweden), and 0.125 g of bovine serum albumin (fraction V; Sigma, St. Louis, MO 63178, U.S.A.) into 50 ml of assay buffer.

For determination of radioactivity PCS scintillation liquid (Amersham/Searle, Arlington Heights, IL, U.S.A.) and a liquid-scintillation counter (Wallac, Turku, Finland) were used. The percentage bound was calculated from radioactivity as described by Brown *et al.* (1971) and Tsang *et al.* (1972). Scatchard (1949) plots were applied to characterize the binding of cyclic [^3H]AMP. Statistical comparisons were made by Student's *t* test.

Results and Discussion

Binding extracts

Both extraction procedures yielded almost equal amounts of cyclic [^3H]AMP-binding sites per mg of protein and similar binding affinities (Fig. 1). Mg-extract exhibited more efficient binding than EDTA-extract at low protein concentrations (Figs. 2, 3 and 4), when dilutions of the binder were made in

Fig. 1. Scatchard (1949) plots of the dialysed bovine adrenal extracts

Extracts were dialysed for 20 h against EDTA buffer at 4°C. The incubation mixture contained 50 μl of cyclic [^3H]AMP (0.25–4.0 pmol) in EDTA buffer, 100 μl of EDTA buffer and 150 μl of extract; protein concentration was adjusted to 1.5 mg/ml with EDTA buffer. The extract was not added to blanks. Incubation time was 1.5 h at 0°C. Then 100 μl of coated-charcoal suspension was added. After centrifugation (10 min at 2500 rev./min at 22°C) 200 μl of supernatant was mixed with 10 ml of scintillation liquid and its radioactivity counted for 120 s. O, Mg-extract; Δ , EDTA-extract.

Mg $^{2+}$ -free buffer. The difference between binding curves of EDTA-extract and Mg-extract at protein concentrations of 3.75 and 15.0 mg/ml was significant ($P < 0.001$, Fig. 2). The corresponding dose-response curves also differed significantly ($P < 0.001$, Fig. 3). These results may indicate the extraction of heterogeneous binding material.

Mg $^{2+}$ -EDTA interactions

Some buffer components used may affect binding of cyclic [^3H]AMP. Gilman (1970), using bovine skeletal muscle, reported that although Mg $^{2+}$ is not

Fig. 2. Cyclic [^3H]AMP-binding curves for two bovine adrenal extracts in different buffer systems

Incubation conditions were as in Fig. 1. Extracts were diluted in the assay buffer, except in ($\square$), (∇) and ($\blacktriangledown$), in which dilutions were made in extraction buffers. (a) EDTA-extract; (b) Mg-extract. Results are shown $\pm$ s.d. of three experiments. TF-ME buffer was used as assay buffer in (Δ) and ($\circ$), and EDTA buffer in ($\blacktriangle$), ($\bullet$) and ($\blacktriangledown$). EDTA buffer, to which 9.5 mM-MgCl $_2$ was added, was assay buffer in ($\square$), and EDTA buffer, in which the EDTA concentration was 8 mM, was assay buffer in (∇).

Fig. 3. Standard dose-response curves of cyclic AMP in different buffer systems

Incubation conditions were as in Fig. 1, except instead of 100 μ l, 50 μ l of assay buffer and 50 μ l of unlabelled cyclic AMP was added. Binding-protein concentration was 3.0 mg/ml. Binder dilutions were made in assay buffer. $\circ$, Mg-extract and TF-Me buffer as assay buffer; $\bullet$, Mg-extract and EDTA buffer as assay buffer; $\square$, Mg-extract and 50 mM-Tris/HCl (pH 7.4)/8 mM-theophylline as assay buffer; Δ , EDTA-extract and TF-ME buffer as assay buffer; $\blacktriangle$, EDTA-extract and 50 mM-Tris/HCl (pH 7.4)/8 mM-theophylline as assay buffer.

Fig. 4. Scatchard (1949) plots of cyclic $[^3\text{H}]\text{AMP}$ binding in different buffer systems

Incubation conditions were as in Fig. 1, except that assay buffer was varied. Protein concentration of the binders was 3.0 mg/ml. Binder dilutions were made in assay buffer. Affinity constants describing the binding kinetics were calculated from the slope of the plots. The affinity constants (M^{-1}) calculated from the number of points given in parentheses were: $\square$, Mg-extract, TF-ME buffer, 0.8×10^9 (6); $\circ$, Mg-extract (after 1 month), TF-ME buffer, 0.9×10^9 (7); $\bullet$, Mg-extract, EDTA buffer, 0.5×10^9 (6); Δ , EDTA-extract, TF-ME buffer, 1.0×10^9 (6); $\blacktriangle$, EDTA-extract, EDTA buffer, 1.0×10^9 (6).

required for binding, it significantly increases the forward and reverse reaction rates. Albano *et al.* (1974) reported that Ca^{2+} , Mg^{2+} , Mn^{2+} and Zn^{2+} appear to inhibit receptor binding of cyclic AMP.

Binding of cyclic $[^3\text{H}]\text{AMP}$ decreased with protein concentrations exceeding 7.5 mg/ml in Mg-extract, but not in EDTA-extract (Fig. 2). Mg^{2+} ions may account for this, because Mg-extract was diluted in an assay buffer containing no Mg^{2+} ions. On the other hand, when the Mg^{2+} concentration was not decreased by using extraction buffer as a diluent (∇ and $\blacktriangledown$, Fig. 2b), the binding curves had a less-steep slope, giving maximum binding at the lowest protein concentration. Thus cyclic $[^3\text{H}]\text{AMP}$ binding seemed to be enhanced by the presence of Mg^{2+} ions at low protein concentrations. At a protein concentration of 7.5 mg/ml the difference between the two upper curves ($\circ$ and $\bullet$) and the lowest curve ($\blacktriangledown$) was significant ($P < 0.001$, Fig. 2b). When the EDTA concentration in 50 mM-assay buffer was raised from

4 to 8 mM, the binding curve of Mg-extract was shifted upwards ($P < 0.005$, Fig. 2b). A downward shift was seen when 9.5 mM- MgCl_2 was added ($P < 0.005$, Fig. 2a). This excess of Mg^{2+} apparently decreased cyclic $[^3\text{H}]\text{AMP}$ binding.

Tsang *et al.* (1972) reported that EDTA increased total cyclic AMP binding. Our data agree with this. Chelation of Mg^{2+} by EDTA may explain the decreased binding affinity in Mg-extract when TF-ME buffer was replaced by EDTA buffer. This may also apply to EDTA-extract, when buffer was changed by dialysis against Mg buffer (Fig. 5). A slight excess of Mg^{2+} ions may overcome the effects of EDTA on these occasions. Heterogeneity of the extracted binders may explain why Mg-extract gave a steeper slope in binding curves than did EDTA-extract at low protein concentrations diluted in assay buffer (Fig. 2). The Mg^{2+} -dependent binders that apparently exist may be components of the phosphodiesterase complex (Barling *et al.*, 1974).

Specificity of [^3H]AMP binding

The specificity of cyclic [^3H]AMP binding was different in different buffers (Table 1). Displacement of cyclic [^3H]AMP was high with unlabelled cyclic AMP and low with unlabelled cyclic GMP when EDTA buffer was used as an assay buffer and diluent. In EDTA-extract the specificity of cyclic [^3H]AMP binding was lower than in Mg-extract, but the variation also seemed to be lower.

Fig. 5. Scatchard (1949) plots of dialysed Mg-extract and EDTA-extract

Mg-extract was dialysed against EDTA buffer and EDTA-extract against Mg buffer. Incubation conditions were as in Fig. 1. Protein concentration of the binders was 1.7 mg/ml. Binder dilutions were made in assay buffer. The affinity constants (M^{-1}) calculated from the number of points given in parentheses were: $\circ$, Mg-extract, EDTA buffer, 1.0×10^9 (5); $\triangle$, EDTA-extract, TF-ME buffer, 0.4×10^9 (7).

Effects of theophylline, mercaptoethanol and EDTA

Theophylline and EDTA both inhibit phosphodiesterase (Butcher & Sutherland, 1962; Cheung, 1967). Tovey *et al.* (1974), Nistrup-Madsen *et al.* (1975) and Aksnes & Sövik (1976) have used EDTA instead of theophylline in cyclic AMP assays. Binding curves (Fig. 2) and Scatchard plots (Fig. 4) indicated that with EDTA-extract, TF-ME buffer may give more cyclic [^3H]AMP binding than EDTA buffer. This may be caused by theophylline or mercaptoethanol (Fig. 3). EDTA increased the specificity of cyclic [^3H]AMP binding in both Mg-extract and EDTA-extract ($P < 0.05$ and $P < 0.025$ respectively), but theophylline increased it only in EDTA-extract ($P < 0.05$). These properties of EDTA and theophylline have apparently not been previously described. Mercaptoethanol seemed to facilitate binding in the system of Mg-extract and TF-ME buffer, but not in the system of EDTA-extract and TF-ME buffer (Fig. 3). However, it also lowered the specificity of cyclic [^3H]AMP binding in Mg-extract ($P < 0.005$).

Effect of storage

After 1 month storage at -20°C a considerable decrease in binding-site concentration was obtained in Mg-extract, but no decrease in EDTA-extract (Fig. 4). Mg^{2+} apparently facilitates the dissociation of cyclic AMP from the binding protein (Brostrom *et al.*, 1971), and some Mg^{2+} -dependent components in the heterogeneous binding material could have been destroyed during 1 month at -20°C .

Conclusions

We suggest that EDTA acts as a stabilizer of cyclic AMP binding and, as reported (Tovey *et al.*, 1974), keeps non-specific interference low.

Cyclic nucleotide binding is important in second-messenger systems, and may vary in pathological

Table 1. Specificity of cyclic [^3H]AMP binding in different buffer systems

Incubation conditions were as in Figs. 1 and 3, except that the amount of unlabelled cyclic AMP or cyclic GMP added to compete with cyclic [^3H]AMP was 1000 pmol. Results are means of three experiments $\pm$ s.d.

Extract	Assay buffer	Decrease in radioactivity (%) after addition of:	
		Cyclic AMP	Cyclic GMP
Mg-extract	Tris buffer	80.9 ± 0.5	31.7 ± 1.7
Mg-extract	EDTA buffer	97.8 ± 0.4	27.9 ± 1.5
Mg-extract	50 mM-Tris/HCl (pH 7.4)/8 mM-theophylline	97.0 ± 0.6	29.0 ± 2.0
Mg-extract	TF-ME buffer	96.6 ± 0.8	42.4 ± 2.6
EDTA-extract	Tris buffer	97.5 ± 0.5	56.2 ± 2.2
EDTA-extract	EDTA buffer	97.5 ± 0.4	50.7 ± 1.6
EDTA-extract	50 mM-Tris/HCl (pH 7.4)/8 mM-theophylline	93.7 ± 0.6	50.8 ± 2.1
EDTA-extract	TF-ME buffer	92.2 ± 1.0	51.5 ± 2.4

states such as malignancy (Granner, 1972; Cho-Chung & Clair, 1975; Goldberg *et al.*, 1975; Prasad & Sinha, 1976). The specificity of cyclic AMP binding, which had not been fully evaluated, could be a factor of some significance.

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