A SIMULTANEOUS PROTEIN-BINDING ASSAY FOR ADENOSINE 3':5'-MONOPHOSPHATE AND GUANOSINE 3':5'-MONOPHOSPHATE IN BIOLOGICAL MATERIALS

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Summary: Adenosine 3':5'-monophosphate (cyclic AMP) and guanosine 3':5'-monophosphate (cyclic GMP) have been determined simultaneously by combining individual protein binding assays using different isotopically labeled cyclic nucleotides. Preparations of cyclic AMP-binding protein from beef adrenal cortex and cyclic GMP-binding protein from the fat body of silkworm pupae (Bombyx mori) have been used for the assay. The method allows the analysis of cyclic AMP and cyclic GMP levels in crude extracts without any purification. The assay has been applied to hormone-stimulated Mouse liver and phorbol ester-treated Rat embryo cells.

The protein-binding assays based upon competition of labeled and tissue-extracted unlabeled cyclic nucleotide for a specific binding protein site have been successfully developed for cyclic AMP (1,2) and cyclic GMP (3-6) and are routinely used in many laboratories. However, there is an increasing need for a simultaneous assay for both cyclic nucleotides, especially in view of their use in clinical diagnosis (6). Such an assay should be capable of being performed on unfractionated tissue extracts, and must be able to determine the amount of each cyclic nucleotide over a large range of the molar ratio cyclic AMP/cyclic GMP. In order to satisfy these requirements the binding proteins first, should have highest affinity for the homologous cyclic nucleotide and lowest affinity for the heterologous cyclic nucleotide and second, must be insensitive to potential interfering substances in the tissue extracts under the assay conditions. We describe herein an assay employing a cyclic AMP-binding protein from beef adrenal cortex and a cyclic GMP-binding protein from pupal fat body (Bombyx mori) which allows the simultaneous assay of both cyclic nucleotides with a high specificity.

The abbreviations used are : cyclic AMP, cyclic adenosine 3':5'-monophosphate ; cyclic GMP, cyclic 3':5'-monophosphate ; cyclic CMP, cyclic cytosine 3':5'-monophosphate ; PBS, phosphate buffered saline ; TPA, 12-0-tetradecanoyl-phorbol-13-acetate.

MATERIALS AND METHODS

Chemicals : (^{3}H) cyclic GMP (8.25 Ci/mmole) and (^{32}P) cyclic AMP (10-20 Ci/mmole) were obtained from New England Nuclear Corporation (Boston, Mas., U.S.A.). (^{3}H) cyclic AMP (27 Ci/mmole) and Scintix were purchased from the Radiochemical Centre (Amersham, Bucks, U.K.) ; Ficoll from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Unlabeled nucleotides and other chemicals were purchased from Boehringer (Mannheim, France SA) and glucagon from Lilly and Co (Indianapolis,Ind,U.S.A.). TPA was a gift from Professor Hecker, Biochemische Institute,Deutsches Krebsforschungszentrum, Heidelberg, RFA.

Preparation of the cyclic nucleotide binding proteins : Fresh beef adrenal glands were stripped of medulla and the cortex was homogenized in two volumes of 0.25 M sucrose, 50 mM KCl and I mM $MgCl_2$ in a 0.05 M Tris-HCl buffer (pH 7.4). The protein was obtained after ammonium sulfate precipitation, dialysis, and DEAE cellulose chromatography as described by Walton and Garren (2). After elution with a 0.01 M Tris-HCl buffer (pH 7.4) containing 0.2 M NaCl, the active fractions were pooled and dialysed against a 5 mM Tris-HCl buffer (pH 7.4) containing 6 mM mercaptoethanol. The protein solution was concentrated with Ficoll up to 2 mg of protein per ml and aliquots were stored at -20°C.

The fat body was removed from the pupae of Bombyx mori, rinced three times with the lepidopterian Ringer's solution and homogenized with 4 volumes of neutral 4 mM EDTA solution. The binding protein was prepared by acetic acid and ammonium sulfate precipitations according to the method of Kuo et al. (7). The final pellet was dissolved in 5 mM potassium phosphate buffer containing 2 mM EDTA (pH 7.0) and the resulting solution was dialysed against 20 volumes of the same buffer. After centrifugation (27,000 g for 30 min.) the supernatant was collected and brought up to a final concentration of about 11 mg per ml with a 5 mM potassium phosphate buffer (pH 7.0) containing 2 mM EDTA. The binding protein was stored in lyophilised aliquots.

All the steps were carried out at 0-4°C. The final preparations were analysed for protein content by the method of Lowry et al.(8). Cyclic AMP-dependent adrenal gland and cyclic GMP-dependent pupal fat body will be referred to as binding protein A and binding protein G respectively.

Individual assays for cyclic GMP and cyclic AMP : assays were typically conducted in 100 µl of 50 mM sodium acetate (pH 4.0) containing 1.2 pmoles of (^{3}H) cyclic GMP or 2 pmoles of (^{32}P) cyclic AMP, and sample or standard. All subsequent treatments were carried out on ice. Reactions were initiated by adding 224 µg of binding protein G or 10 µg of binding protein A, in the presence of bovine γ-globulines (450 µg) as carrier. The test mixture was gently mixed by pipetting and the reaction allowed to proceed for 90 min. Then 0.2 ml of an ice-cold 3.8 M ammonium sulfate solution was added and mixed carefully by tilting. The tubes were allowed to stand for 10 min and centrifuged for 2 min. at 8,000 g (Eppendorf centrifuge). The supernatant was aspirated and the precipitate resuspended in 0.2 ml of 2.7 M ammonium sulfate solution by tilting. The insoluble proteins were precipitated after 10 min. by centrifugation for 1 min. at 8,000 g and dissolved in 0.5 ml of distilled water. The solution containing the binding protein complex was quantitatively transferred to a scintillation vial containing 10 ml of a scintillation mixture (Scintix) and counted at about 30% efficiency in a liquid scintillation spectrometer (Intertechnique). All assays were performed in duplicate and tissue or cell extracts were usually assayed at two or three dilutions.

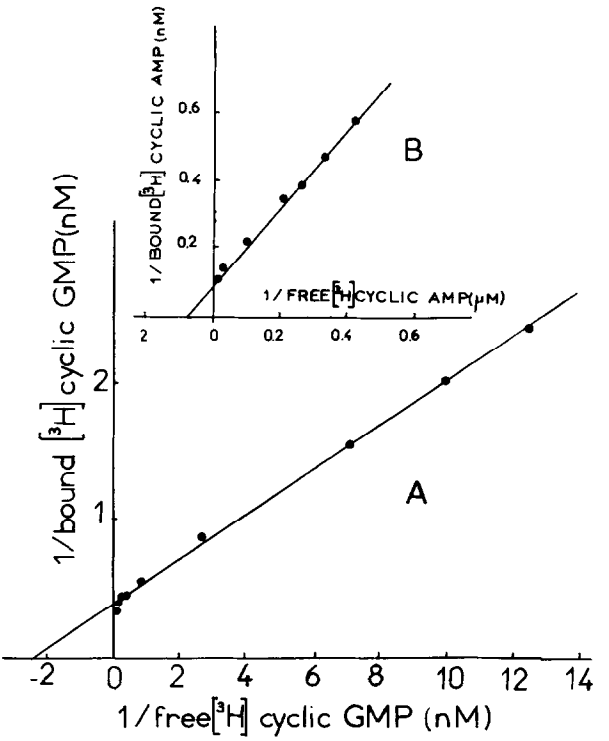

Fig. 1 : Double-reciprocal plots of cyclic GMP and cyclic AMP (insert) binding to protein G. Binding reactions were carried out as described in experimental section for the individual cyclic nucleotide assay, except that varied amounts of (^{3}H) cyclic GMP or (^{3}H) cyclic AMP for 448 /ug of binding protein were added.

Simultaneous cyclic nucleotide assay : The incubation mixture contained a final volume of 0.1 ml : 50 mM sodium acetate (pH 4.0), 1.2 pmole of (^{3}H) cyclic GMP, 2 pmoles of (^{32}P) cyclic AMP, and sample or standard. The reaction was started by adding the mixed binding proteins A and G. All subsequent treatments were similar to those described for the individual assays and the radioactivity was determined by the double-labeling technique using (^{32}P) and (^{3}H) internal standards.

Preparation of samples : The culture medium of Rat embryo cells growing in 60 mm Petri dishes was poured off and the cells were washed twice with 5 ml of warm PBS before the addition of 5 ml of ice-cold 6% trichloracetic acid. The acid-soluble supernatant was collected, extracted, evaporated to dryness and redissolved as described by Gilman (1). Mouse liver (about 0.5 g) was freeze-clamped, powdered and homogenized in 10 volumes of 6% trichloracetic acid. The acidified homogenates were allowed to stand for 15 min. at 0°C before centrifugation and the acid-soluble supernatant was treated as described for cultured cells. Recovery (70-80%) was monitored with 1 m/uCi of (^{3}H) cyclic AMP or (^{3}H) cyclic GMP. The acid-insoluble material from liver and cultured cells was dissolved overnight in 0,6 N NaOH and analysed for protein content by the method of Lowry et al. (8).

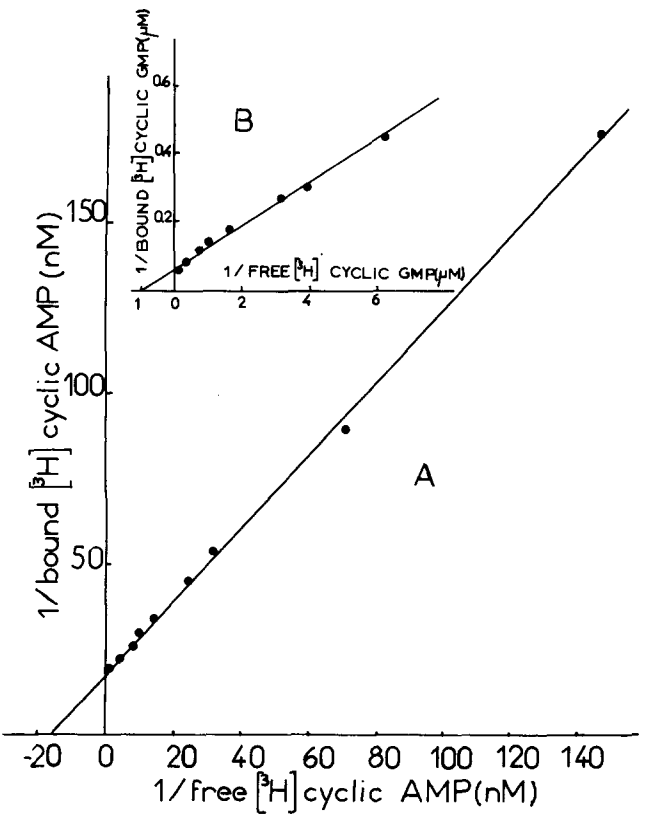

Fig. 2 : Double-reciprocal plots of cyclic AMP and cyclic GMP (insert) binding to protein A. Binding reactions were performed as described under "Materials and Methods" for the individual cyclic nucleotide assay, except that varied amounts of (^{3}H) cyclic AMP or (^{3}H) cyclic GMP for 40 μg of binding protein were added.

RESULTS

The precipitation of the cyclic nucleotide-protein complex at high ammonium sulfate concentration has been successfully substituted for adsorption on cellulose nitrate membrane, for the study of the binding of cyclic nucleotide to proteins A and G. The amount of complex was proportional to the amount of added binding protein preparations up to 120 μg for binding protein A and 1 mg for binding protein G under the incubation conditions.

Determination of binding and inhibition constants : The kinetics of cyclic AMP and cyclic GMP binding to their specific protein were analyzed by Klotz plotting and are illustrated in Fig. 1A and 2A. The apparent

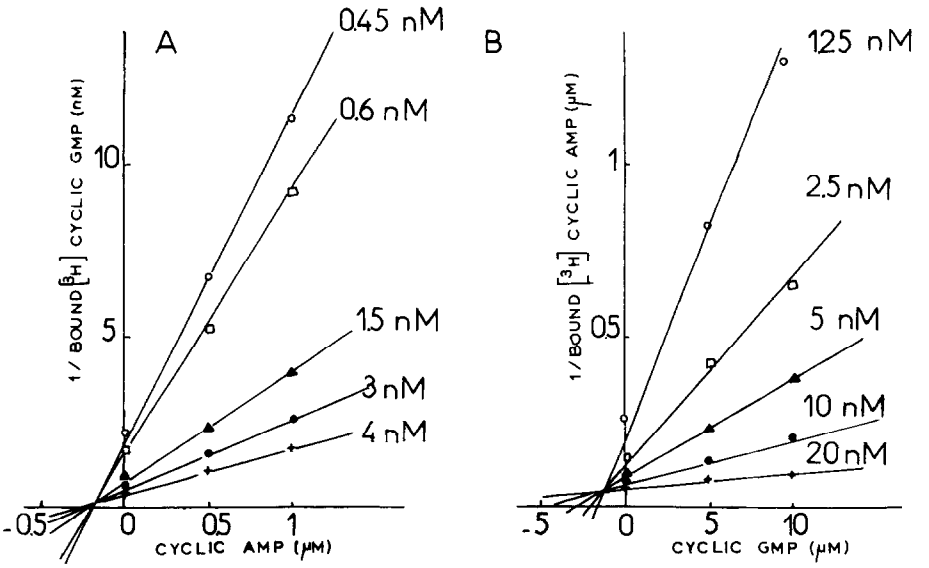

Fig. 3 : Dixon plots of the homologous cyclic nucleotide binding to protein G (A) and protein A (B) in the presence of the heterologous cyclic nucleotide. Binding reactions were carried out as described in legends 1 and 2. From the graph cyclic AMP inhibited cyclic GMP binding to protein G with a K_i of about 0.15 /uM and cyclic GMP inhibited cyclic AMP binding to protein A with a K_i of about 1.5 /uM.

Kd for the binding of cyclic AMP to protein A from adrenal gland was 7 nM and agreed closely with the reported value (2). The apparent Kd of cyclic GMP (0.5 nM) demonstrates the uncommonly high affinity of this nucleotide for the binding protein G from silkmoth pupa. The dissociation constants for the heterologous cyclic nucleotides were similarly determined and data are shown in Fig. 1B and 2B : cyclic AMP bound to protein G with an apparent Kd of 0.1 to 0.2 /uM and cyclic GMP bound to protein A with an apparent Kd of about 1 /uM. Direct inhibition constants were determined according to Dixon's procedure and data are presented in Fig. 3.

Individual cyclic AMP and cyclic GMP assays : The standard binding reactions were conducted at saturating concentrations of labeled cyclic AMP or cyclic GMP in such a way that protein G and protein A bound 20 and 2% of the total nucleotide respectively. The dose-binding curves have been done by competition with increasing amounts of unlabeled homologous (standard curve) and heterologous cyclic nucleotides. Actually the heterologous cyclic nucleotide does not compete with the labeled specific one up to 0.8 to 1 /uM. The third naturally occurring cyclic nucleotide, cyclic CMP, does

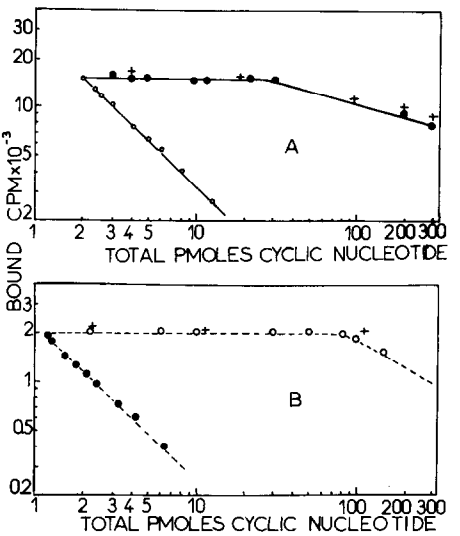

Fig. 4 : A : Dose-binding curves for cyclic nucleotides on protein A. 2 pmoles of (^{32}P) cyclic AMP competed with increasing amounts of cyclic AMP (o——o), cyclic GMP (o——o) or cyclic CMP (——).
B : Dose-binding curves of cyclic nucleotides on protein G. 1.2 pmole of (^{3}H) cyclic GMP competed with increasing amounts of cyclic GMP (o----o), cyclic AMP (o----o) or cyclic CMP (----).

not compete either with the homologous nucleotide (data shown in Fig. 4).

The assay was carried out on unfractionated cell extracts and the specifity checked by the cyclic phosphodiesterase test. Table 1 illustrates a typical experiment on cultured cell extracts which demonstrates that the cyclic nucleotides specifically competed with the labeled cyclic nucleotides, and that no acid-soluble substance interfered with the binding reaction, since the enzyme treatment restores the full labeled nucleotide binding. Identical data have been obtained with tissue extracts (not shown) Also known amountsof standard cyclic AMP or cyclic GMP added to any biological test sample before assay, were as a rule, quantitatively recovered.

Simultaneous cyclic AMP and cyclic GMP assay : When added in the combined assay mixture, proteins A and G did not bind any detectable amount of (^{3}H) cyclic GMP and (^{32}P) cyclic AMP respectively, and the binding affinity for the specific cyclic nucleotide was not altered. Unlabeled cyclic AMP and GMP in a single test tube competed exclusively with (^{32}P) cyclic AMP and (^{3}H) cyclic GMP respectively, for the binding site of the specific binding protein. Consequently when data were plotted logarithmically, the linear standard curves which were obtained for each nucleotide were superimposable upon those which were obtained when the reactions were carried

TABLE 1

Effects of the cyclic nucleotide phosphodiesterase treatment on cyclic AMP and cyclic GMP contents in cell extracts :

Extracts from Rat embryo cells (about 6 x 10^6 cells and 1.3 to 1.4 mg of protein per dish) were prepared as described in "Materials and Methods", and cyclic nucleotides were concentrated by dissolving the dried extracts in a volume of acetate buffer one-tenth that of the original extract. Aliquots were diluted to a 0.1 ml final volume containing 20 µg of cyclic nucleotide phosphodiesterase from beef heart, 70 mM Tris-HCl buffer (pH 8.0) and 2 mM $MgCl_2$ and incubated for 1 hour at 30°C. The reaction was stopped by boiling for 3 min. and aliquots (equivalent to 55 µg of cell protein) were assayed in parallel with control samples for cyclic AMP and cyclic GMP contents using the combined binding assay. The values are the mean of four determinations.

Addition in assay	Bound (^{32}P) cyclic AMP cpm ± SE	Unlabeled cyclic AMP per assay pmoles ± SE	Bound (^{3}H) cyclic GMP cpm ± SE	Unlabeled cyclic GMP per assay pmole ± SE
50 mM acetate buffer	15,284 ± 300	0	1,814 ± 30	0
Cell extracts -control	7,218 ± 86	2.3 ± 0.1	1,300 ± 30	0.41 ± 0.01
-treated	14,832 ± 250	0	1,837 ± 88	0

out separately (Fig. 4). The sensitivity of the assay which has been calculated on the basis of the amount of cyclic nucleotide causing a 5% decrease of total cpm bound was 0.1 pmole for cyclic GMP and 0.2 pmole for cyclic AMP under the conditions described.

Cyclic AMP and cyclic GMP levels have been measured in cultured cells and in liver using individual and simultaneous assays. Embryo Rat cells have been used during the stationary phase and after treatment with TPA which was reported to increase cyclic GMP levels in vitro (9,10). Cyclic nucleotide contents were assayed also in control and glucagon-stimulated liver (table 2).

TABLE 2

Cyclic AMP and cyclic GMP levels in control and treated liver and cultured cells as determined by individual and simultaneous binding assays.

Cultured Rat embryo cells were collected either at zero-time or six hours after the addition of TPA (0.5 ug per ml). Four-month-old female mice (strain IC) were injected intraperitoneally with saline or 40 ug of glucagon and killed by decapitation 5 and 10 min. later. Samples were prepared as detailed in the experimental section and aliquots (equivalent to 30-130 ug and 0.2-0.8 mg of protein for cells and liver respectively) were assayed for cyclic AMP and cyclic GMP contents using both individual and combined protein binding assays. The values are expressed in pmoles of cyclic nucleotide per mg of protein.

		cyclic AMP		cyclic GMP		cyclic AMP/cyclic GMP	
		individual	combined	individual	combined	individual	combined
Mouse liver	Control	4.2	4.1	0.61	0.50	7	8
	Glucagon-treated						
	5 min	11.6	12.3	0.73	0.66	16	19
	10 min	16.5	17.2	0.81	0.80	20	21
Rat embryo cell	Control	59.9	62.1	8.9	9.1	7	7
	TPA-treated	46.3	50.9	23.1	22.0	2	2

DISCUSSION

The cyclic AMP-binding protein from beef adrenal cortex and the cyclic GMP-binding protein from silkworm pupa have been selected from a number of other binding proteins because of their relative affinity for the homologous and heterologous cyclic nucleotide. The apparent K_D ratio for the heterologous over the homologous cyclic nucleotide was over 200 (Fig. 1 and 2). Under the assay conditions, no interference of the heterologous cyclic nucleotide was detectable between a molar ratio of cyclic AMP/ cyclic GMP of 0.03 to 80 in the assay mixture. Since the assay is based on a logarithmical decrease of the bound radioactivity, the determination was only accurate within the limits : 0.1-5 pmoles for cyclic GMP and 0.2-10 pmoles for cyclic AMP. Therefore the molar ratio cyclic AMP/ cyclic GMP in the sample can be determined between 0.04 to 100, although the actual range in the assay mixture was between 0.4 to 10 if the amount of labeled cyclic nucleotide contained therein is taken into account. It must be stressed that depending on the range and the sensitivity required, the concentration of either one or both labeled cyclic nucleotide in the assay can be modified as long as the condition of saturation is satisfied. Thus the procedure is compatible with the simultaneous determination of both cyclic nucleotides in most biological materials with a strict specificity.

Murad and Gilman (11) have previously described a combined protein-binding assay which failed to specifically assay cyclic GMP when both nucleotides were present in an equimolar ratio, and did not succeed in determinating cyclic AMP and cyclic GMP levels in biological materials excepturine without purification. The major improvement in the described assay is due to the substitution of the cyclic GMP-binding protein from fat body pupa (Bombyx mori) for the cyclic GMP-binding protein from lobster tail muscle. This binding protein from silkmoth pupa has a quite unusually high affinity for cyclic GMP relative to cyclic AMP and is rather insensitive to interfering substances in samples. Furthermore it is easily prepared from pupal fat body which is devoid of cyclic AMP-binding protein (12) and as previously mentionned, the separation of free from bound nucleotide by ammonium sulfate precipitation allows the use of a crude binding protein preparation.

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