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Radioimmunoassay for the Cyclic Nucleotides*

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THE use of competitive binding techniques for the measurement of cyclic nucleotides in tissues and body fluids has facilitated investigations of the roles of these compounds in cell function. Radioimmunoassay methods have been developed for four cyclic nucleotides (adenosine 3′,5′-monophosphate (cAMP), guanosine 3′,5′-monophosphate (cGMP), inosine 3′,5′-monophosphate (cIMP), and uridine 3′,5′-monophosphate (cUMP)) (10, 11). This method uses antibodies which have been raised in rabbits or goats after conjugation of a succinylated derivative of the cyclic nucleotide to protein. High specific activity iodinated derivatives of the cyclic nucleotides are used as markers in the assay system. A non-immunological competitive binding method to measure cAMP and cGMP also allows the determination of these nucleotides with ease and sensitivity (6, 9). In the latter method, specific binding proteins for cAMP and cGMP from bovine skeletal muscle and lobster muscle, respectively are used as the specific agent and ^{3}H-cyclic nucleotides serve as marker. By either radioimmunoassay or competitive binding assay, multiple numbers of samples for cyclic nucleotide analysis can be assayed weekly because chromatographic steps are either unnecessary or have been minimized. The radioimmunoassay method for cyclic nucleotide determination has been published in detail (10, 11, 14) and has been reviewed recently (12, 13). This discussion will succinctly review the methodology and will concentrate on some practical considerations of the selection of antibodies, possible problems in assay, and the use of these antibodies in cyclic nucleotide immunocytochemistry.

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I. Preparation of Reagents for Radioimmunoassay

1. Succinyl 2′0 Cyclic Nucleotides

The cyclic nucleotides are succinylated at the 2′0 position with succinic anhydride in anhydrous pyridine (4). cAMP, cGMP, and cIMP are solubilized in pyridine by the addition of equal molar quantities of 4′-morpholine N,N′-dicyclohexylcarboxylcarboxyamine or triethylamine to form either the morpholinium or triethylammonium salt of the cyclic nucleotide. The guanosine nucleotides are particularly insoluble in most organic solvents, but partial solubilization of cGMP is achieved by forming the trioctylammonium salt in anhydrous pyridine. An excess of succinic anhydride is added to the suspension of cyclic nucleotide in pyridine, and the reaction is carried out at room temperature for 18 hr with constant stirring. Water is then added to convert unreacted succinic anhydride to succinic acid, and the pyridine is removed by rotary evaporation at 40°C under reduced pressure. Succinylated cAMP (ScAMP) is isolated by Dowex 50 column (H+ form) chromatography with distilled water as the eluant. The fractions containing ScAMP are pooled and lyophilized. Succinyl cGMP (ScGMP), succinyl cIMP (ScIMP), and succinyl cUMP (ScUMP) are purified by thin layer chromatography on cellulose with the solvent system butanol: glacial acetic acid:H_2O (12:3:5, v/v). In this system, the succinylated cyclic nucleotides run ahead (R_F = 0.42) of the unreacted cyclic nucleotide (R_F = 0.30).

2. Preparation of Immunogen

The succinylated cyclic nucleotide is coupled to a large molecular weight protein, and the reaction conditions have not been changed from that previously described (10, 11). Rabbits are immunized with 1 mg of the cyclic nucleotide protein conjugate which has been emulsified in complete Freund's adjuvant. Booster injections with small amounts of conjugate are given at 4- to 6-week intervals and the animals bled 10 to 14 days later. A relatively high titer antibody is found in a majority of animals after one booster injection, but the titer does increase with repeated injections of conjugate.

3. Synthesis of 2'O-Succinyl Cyclic Nucleotide Tyrosine Methyl Ester

While ^{3}H-cyclic nucleotide can be used as a marker in the cyclic nucleotide radioimmunoassay (15), greater sensitivity is achieved when high specific activity iodinated derivatives are employed. These derivatives are made by tyrosination of the succinylated compounds and subsequent iodination of the tyrosine moiety. The synthesis of the tyrosine derivatives of 2'O-succinyl cAMP and cGMP (ScAMP-TME and ScGMP-TME) by the mixed carboxylic carbonic acid reaction with ethyl chloroformate is recommended (11). Cailla and Delaage (3) have modified the synthesis of 2'O succinyl AMP methyl ester by this reaction and have described the reaction conditions and products in detail. The tyrosinated derivatives are then iodinated by the method of Hunter and Greenwood (7), and are purified by column chromatography on Sephadex G-10 or by thin layer chromatography on cellulose. Either ^{131}I or ^{125}I derivatives may be used in the immunoassays and for the simultaneous assays of cAMP and cGMP it is convenient to make ^{131}- and ^{125}-labeled derivatives of the cyclic nucleotides respectively (14).

II. Tissue Analysis

Tissue samples for cyclic nucleotide immunoassay are homogenized in trichloroacetic acid (TCA) and the supernatants are extracted three times with ethyl ether and evaporated over a stream of air. The residue is then resuspended in 0.05 M sodium acetate buffer, pH 6.2, and used directly in the immunoassay. Plasma for cAMP assay is extracted with an equal volume of either 0.6 N perchloric acid or 10% TCA and purified by Dowex column chromatography (13). Purification is not necessary for cGMP assay of plasma. Urine samples are added directly into the immunoassay.

The immunoassay procedure is unchanged from the conditions described previously (11). cAMP and cGMP immunoassays are performed in 0.05 M sodium acetate buffer, pH 6.2, in a final reaction volume of 600 μl. It is convenient to add an amount of antibody which will bind 50% to 60% of the labeled marker, approximately 15,000 cpm of ^{125}I-labeled marker and 500 μg of rabbit γ-globulin. After 2 to 18 hr of incubation, 2.5 ml of 60% $(NH_4)_2SO_4$ are added and the tubes centrifuged at 4°C for 15 min and the precipitate is then counted in a *gamma* spectrometer.

In the simultaneous assays of cAMP and cGMP (14), the immunoassay procedure is identical, except that specific cAMP and cGMP antibodies are added in the 100 μl antibody aliquot. ^{131}I-ScAMP-TME and ^{125}I-ScGMP-TME (approximately 15,000 cpm each) are added in a 100 μl aliquot. The precipitate is then counted in a dual channel spectrometer equipped with a punched paper tape printout. A computer program for the use on a NCR Century 200 system with intermediate FORTRAN has been written for analysis for both the single and simultaneous radioimmunoassays.

III. Cyclic Nucleotide Immunoassay

A standard curve for cAMP immunoassay is shown in figure 1. The sensitivity of both the cAMP and cGMP radioimmunoassays is in the range of 0.025 to 0.05 picomoles in an assay volume of 600 μl. A significantly greater sensitivity can be achieved by reducing assay volume, but this tends to decrease precision in the assay. The sen-

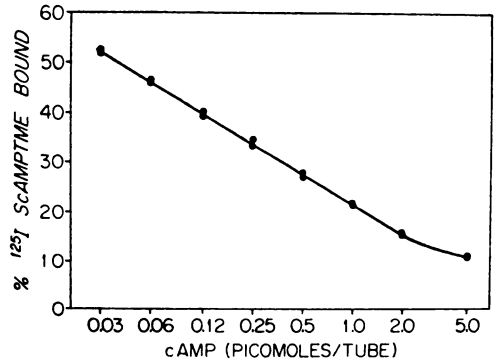

FIG. 1. Standard immunoassay curve for cAMP. Reaction conditions described in text. Reaction volume was 600 μl. Antibody was kindly supplied by Schwarz-Mann Bioresearch, Orangeburg, N.Y.

sitivity achieved allows the measurement of cAMP and cGMP in triplicate on 1 to 2 mg and 10 to 15 mg of tissue respectively. Since 2'O-succinyl cAMP reacts approximately 200 times more avidly with the antibody than cAMP (11), sensitivity can be increased over 100-fold by succinylation of samples. This reaction has been described by Cailla and Delaage,[1] and is advantageous when only small amounts of tissue are available.

To avoid or minimize chromatographic preparation of tissue samples, it is important to select antisera that minimally cross react with other nucleotides. The antibody for cAMP assay that is currently employed in our laboratory shows no significant interference by adenosine triphosphate (ATP), adenosine monophosphate (AMP), or adenosine diphosphate (ADP) at 1 million fold or greater concentration of these nucleotides (fig. 2). The cGMP antibody requires greater than a 300,000-fold concentration of ATP before there is significant cross reaction in the assay. Cross reactivity of both of these antibodies with the corresponding cyclic nucleotide is 0.01%. Since the concentration of cAMP in tissues is usually an order of magnitude greater than cGMP, antibodies to cAMP with 1% or more cross reactivity can be used without purification of samples.

[1] H. Cailla and M. Delaage: Personal communication.

Certain antibodies will show some interference by high concentrations of theophylline.

A convenient check on the reliability of a cyclic nucleotide determination is by measurement of the amount of immunologically reactive cyclic nucleotide before and after hydrolysis by cyclic nucleotide phosphodiesterase. After such treatment, virtually all of the immunological reactive cAMP is hydrolyzed in various rat tissues and in human urine and plasma. In certain rat tissues (liver, skeletal muscle, kidney cortex) a blank of 15% to 40% is found. Tissue values for cGMP are corrected by subtracting the blank from the total cGMP measured. It would appear that nucleotide contamination is not the cause of the blank in the cGMP assay since the addition of the mono-, di-, and trinucleoside phosphates of adenosine and guanosine at the concentration found in these tissues does not cause interference in the assay.

IV. Simultaneous Assay of cAMP and cGMP

Tissue measurements of cAMP and cGMP are identical when performed by simultaneous or individual cyclic nucleotide immunoassay (14). To avoid chromatographic steps, it is helpful to select a cGMP antibody which shows minimal cross reactivity with cAMP. As seen in table 1, the addition of cAMP in 1000-fold excess compared to cGMP causes no significant displacement in the cGMP immunoassay.

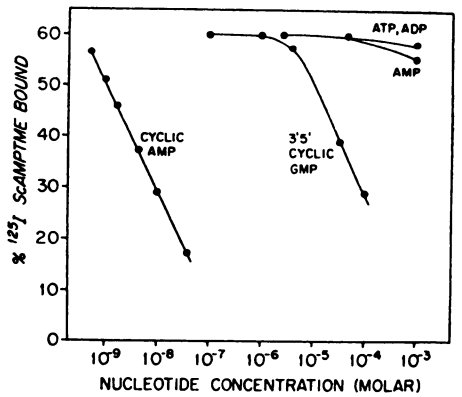

FIG. 2. The inhibition of ^{125}I ScAMP-TME binding to cAMP antibody by varous nucleotides.

TABLE 1*

Effect of increasing concentrations of cAMP on the measurement of cGMP by simultaneous radioimmunoassay

cAMP	cGMP	
	Added	Measured
pmoles/tube	*pmoles/tube*	
1	0	0.00
10	0	0.00
100	0	0.00
1000	0	0.44 ± 0.03
1	0.5	0.53 ± 0.02
10	0.5	0.52 ± 0.06
100	0.5	0.51 ± 0.02
1000	0.5	0.88 ± 0.05
1	2.0	1.81 ± 0.13
10	2.0	1.76 ± 0.10
100	2.0	2.06 ± 0.23
1000	2.0	1.95 ± 0.16

* Reproduced from A. L. Steiner, R. E. Wehmann, C. W. Parker and D. M. Kipnis: Radioimmunoassay for the measurement of cyclic nucleotides. *In* Advances in Cyclic Nucleotide Research, vol. 2, pp. 51-61, Raven Press, New York, 1972.

Since tissue levels of cAMP are frequently an order of magnitude greater than cGMP values, the utility of the simultaneous assay can be increased by choosing a cAMP antibody with slightly reduced sensitivity. Such an alteration would be helpful in certain cases of hormonal stimulation where the concentration of cAMP increased substantially while the concentration of cGMP remains constant.

V. Problems Related to Measuring cAMP and cGMP in Tissues and Body Fluids

Since the cyclic nucleotides can be hydrolyzed by cyclic nucleotide phosphodiesterase in the process of obtaining tissues and body fluid samples, it is important to freeze tissues quickly and to prevent thawing during homogenization. Recovery of added cyclic nucleotide is greater than 90% when such precautions are taken. Certain substances in the ether extracted TCA supernatants of tissues and body fluids can interfere in both the cAMP and cGMP immunoassays. This is checked by measuring the cyclic nucleotide concentration before and after hydrolysis by cyclic nucleotide phosphodiesterase. Such substances include residual TCA, Ficol hypaque, or, rarely, are unknown tissue compounds. Since certain human plasma samples will occasionally give a false high value we routinely purify plasma supernatant by Dowex column chromatography (see above). An enhancement of binding in the cAMP radioimmunoassay by extracts from cerebral cortex and lipocytes has been described by Weinryb (15). The "antibody binding enhancing factor" was removed by Dowex 50 H^+ column chromatography, but not by 12% TCA or heating to 90° C. The phenomenon has not been observed by this author with extracts from these tissues, and it might be a property of the separation technique employed (Millipore filter).

VI. Measurement of Adenylate and Guanylate Cyclase Activity by Radioimmunoassay

Since antibodies to both cAMP and cGMP can be obtained which have minimal cross reactivity with the corresponding trinucleoside phosphate, the radioimmunoassay method is particularly useful in the measurement of adenylate and guanylate cyclase. In addition expensive isotopes are not required. The reactions are run in the presence of millimolar concentrations of ATP or guanosine triphosphate (GTP), added cation, and millimolar concentrations of theophylline. After the assay is terminated by boiling, an aliquot of the reaction mixture is then added directly into the cAMP or cGMP immunoassay. When assays for adenylate cyclase by radioimmunoassay are compared with the isotopic method of Krisha *et al.* (8), the results are identical. Since theophylline can interfere in the immunoassays for both cAMP and cGMP, antibodies should be checked for cross reactivity with this methyl xanthine.

VII. Cyclic Nucleotide Immunocytochemistry

The use of rabbit sera that were made against 2'0 succinyl cAMP for the localization of cAMP in brain tissue has recently been reported (1a, 16). cGMP has also been localized in thyroid follicular cells after the addition of cGMP antibodies (5). In this immunocytochemical technique, cAMP or cGMP serum from rabbits is added to unfixed tissue after which goat anti-rabbit γ-globulin conjugated to fluorocene isocyanate is added. After washing with buffer, the preparation is examined under ultraviolet light and the fluorescence visualized is compared with tissue preparations that have been treated with non-specific rabbit serum. With this technique, Bloom *et al.* (2) have shown in rat cerebellum that the administration of norepinephrine increases cAMP selectively in Purkinje cells. Since it appears that both cAMP and cGMP have divergent roles in neurotransmission, the localization of these cyclic nucleotides in specific cells should be helpful in determining which nucleotides are involved in the action of specific transmitters. In addition intracellular pools of the cyclic nucleotides can be determined by immunocytochemistry. Bloom *et al.* (1) have presented a more complete discussion of this technique.

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