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An Enzyme-Linked Immunosorbent Assay for the Rapid Quantification of Intracellular and Extracellular Guanosine 3′,5′-cyclic Monophosphate in Cultured Glial Cells*

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A sensitive enzyme-linked, indirect immunosorbent assay (ELISA) for the determination of guanosine 3′,5′-cyclic monophosphate (cGMP) in glial cells is described. The assay uses an antiserum produced against succinylated cGMP and is based on the competition between endogenous cGMP and a fixed amount of immobilized antigen. The antibody exhibited a high degree of specificity with negligible cross reactivity with other nucleotides or related compounds. The standard curve, linearized using a logit–log transformation, had an operating range of 1 fmol/50 µl to 5 pmol/50 µl. The sensitivity of the assay was significantly increased by acetylation of standards and samples. Recoveries of cGMP from samples of cultured cells ranged from 85% to 105% with a mean recovery of 97% ± 7%. Levels of cGMP measured with the ELISA were in agreement with the corresponding values obtained using radioimmunoassay. The present method provides for a cheap, sensitive and simple alternative to the commercially available cGMP assays.

KEY WORDS: cell culture; CNS; cyclic GMP; enzyme-linked immunosorbent assay; glia.

INTRODUCTION

Guanosine 3′,5′-cyclic monophosphate (cGMP) is a key intracellular second messenger molecule which mediates a multitude of cellular signaling events in the nervous system (1). The synthesis of cGMP from GTP is catalyzed by the enzyme guanylate cyclase (GC), which exists in both membrane and cytosolic forms. The membrane forms of GC are activated in response to the presence of a range of endogenous bioactive peptides or heat stable enterotoxins (2), whilst the soluble form of GC is activated in response to gaseous messenger molecules such as nitric oxide and carbon monoxide (3).

The ubiquitous nature and pivotal regulatory role of cGMP in cellular responses has necessitated the production of methods for the convenient and accurate determination of cGMP. Prior to 1970, the cyclic nucleotide content in biological samples was quantified using a number of accurate, yet intricate experimental approaches (4–6). The introduction of the radioimmunoassay (RIA) for cyclic nucleotides by Steiner et al. (7) and the protein-binding assay by Murad et al. (8) replaced these more cumbersome approaches. The accuracy and detection limits of the RIA were significantly increased to readily detect femtomole amounts of cGMP by acetylating the cyclic nucleotide with acetic anhydrides (9).

Whilst the uses of immunoassays involving radioactive labels are both extremely sensitive and precise, they have several drawbacks that limit their use for large-scale analysis. Of major significance

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are the health risks associated with the use of radioactively labeled compounds, combined with the necessity for the training of staff in the safe handling of such compounds, storage of waste and the need for specialized equipment for quantitizing radioactivity. Furthermore, the use of commercially produced RIA kits is limited by the half-life of the isotope used.

The need for a cyclic nucleotide assay, which has the high degree of sensitivity and precision of the radioimmunoassay, without the complications associated with the use of radioactive isotopes, has led to the development of a range of non-isotopic assays using high performance liquid chromatography (10), fluorescent indicators (11), mass spectrometry (12) and non-isotopic immunoassays based on the enzyme-linked immunosorbent assay (ELISA) method first reported by Engvall et al. (13). The quantification of cellular antigens by the ELISA method is comparable to the RIA in sensitivity, reproducibility and application in all cases where a direct comparison between RIA and ELISA has been executed (14–17). Moreover, the ELISA is quick, easily performed, does not require excessive pre-treatment of sample and is well suited for the screening of a large number of samples.

A number of commercially available non-isotopic immunoassays are available for cGMP quantification and have been used extensively to measure cGMP levels in a range of biological preparations (for examples see 18–22). Despite their simple format and reproducible results, the cost of these kits rules out their use in large-scale experimentation. Consequently, a number of groups have developed their own "in house" cGMP-specific ELISAs to suit their own purposes (17, 23–25). However, a number of these assays require the synthesis of specialized components such as acetylcholinesterase- (23) or biotin-coupled ScGMP (17) or the use of alkaline phosphatase-coupled protein A rather than a traditional secondary antibody (24).

Here we describe an ELISA method for the analyses of cGMP using an indirect ELISA utilizing immobilized antigen and a commercially produced alkaline-phosphatase-coupled secondary antibody. One of the major aims of this report is to provide sufficient experimental details regarding the execution of the assay so as to allow researchers to reproduce the assay in their work place. Samples of antisera would be made available to researchers attempting to reproduce this assay for their own experimental purposes.

EXPERIMENTAL PROCEDURE

Materials. Dulbecco's modified Eagle's Medium (DMEM) and fetal calf serum (FCS) were purchased from Life Technologies (Eggenstein, Germany). Atrial natriuretic peptide-(1–28) (ANP), Human brain natriuretic peptide-(1–32) (BNP), C-type atrial natriuretic polypeptide (CNP) and Freund's adjuvant were obtained from Calbiochem-Novabiochem (Schwalbach, Germany). Bovine serum albumin (BSA), rabbit serum albumin (RSA), 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide, gelatin, 2′-O-monosuccinyl-cGMP (ScGMP) and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma (Deisenhofen, Germany). Sodium nitroprusside (SNP) was from Merck Eurolab (Bruchsal, Germany). Penicillin G and streptomycin were from Serva (Heidelberg, Germany). "Falcon" cell culture dishes, 35 mm in diameter, were from Becton Dickinson (Schubert Medizinprodukte, Wackersdorf, Germany). Microtiter plates (Maxisorb F96) were purchased from Nunc (Wiesbaden, Germany). Acetic anhydride and triethanolamine were from Fluka (Buchs, Switzerland), ethylenediaminetetraacetic acid (EDTA) was from Roth (Karlsruhe, Germany) and Tween 20 was from Serva (Heidelberg, Germany). *p*-Nitrophenylphosphate was from Roche Applied Science (Mannheim, Germany). All bases, nucleosides and nucleotides were purchased from Roche Applied Science or Sigma. The secondary antibody (anti-rabbit IgG-alkaline phosphatase conjugate) was purchased from Sigma. The cGMP-[^{3}H]Biotrak Assay system was from Amersham Biosciences (Freiburg, Germany).

Solutions. Phosphate-buffered saline (PBS; 4.0 g KH_2PO_4, 4.0 g KCl, 28.8 g $Na_2HPO_4 \cdot 2H_2O$, 160 g NaCl per liter of double-distilled water) was made as a 20-fold concentrated solution and stored at +4°C until required. This 20-fold stock solution of PBS was used to prepare all of the other solutions used during the assay. To dilute the samples prior to assay a 1.33-fold concentrated PBS (1.33-PBS) was made from the 20-fold PBS. For washing steps and dilution of the secondary antibody PBS containing 0.05% (w/v) Tween 20 (PBS-T) was prepared. PBS containing 0.1% (w/v) Tween 20, 2.0 g/l BSA, 2.0 g/l NaN_3, 0.8 g/l EDTA (PBS-2TAAE) was used for the dilution of antisera. The substrate buffer for the indicator reaction consisted of 0.1 M glycine-NaOH-buffer, pH 10.4, containing 1 mM $ZnCl_2$ and 1 mM $MgCl_2$.

Preparation of immunogens. Coupling of 2′-O-succinyl-derivatives of cGMP to albumin was performed according to the method of Brooker et al. (26) using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide as a coupling agent. In brief, 13 mg of ScGMP was dissolved in 5 ml of 50 mM potassium phosphate buffer, pH 5.5, and combined with 74 mg RSA. Subsequently, 50 mg of the carbodiimide-coupling agent was added and the reaction mixture was gently shaken overnight (approximately 20 h) at 20–24°C. At the completion of the incubation step, the reaction mixture was dialyzed over a period of 36 h at 4°C using three changes of 2 l of 100 mM NH_4HCO_3-solution. The dialyzed solution was aliquoted and then lyophilized. From the extinction measured at the absorption maxima of ScGMP (254 nm) and RSA (277 nm), the average content of cGMP molecules bound per albumin molecule was calculated, yielding three molecules of cGMP per molecule of albumin (27).

Immunization. Antibodies against cGMP were raised by repeated immunizations following a modified version of the protocol of Friedl (28). The lyophilized protein-conjugated cyc-

lic nucleotide was reconstituted with sterile PBS on the day of injection and emulsified with an equal volume of complete Freund's adjuvant. An emulsion containing 0.2 mg of immunogen was injected intracutaneously at multiple sites on the animals back. The animals were boosted once every 4 weeks with 150–200 μg conjugate (diluted in PBS) in incomplete Freund's adjuvant. Antibody titer was determined 14 days after each immunization step. Sixteen days after the final booster injection, the animals were bled and the separated sera were portioned and stored in liquid nitrogen until required. Two lots of antisera were isolated, both of which have been described in detail elsewhere (27). From this original characterization, one polyclonal antibody was selected for use in the ELISA described here.

Antigen Coating and Blocking of Non-specific Binding. To prepare stock solutions of antigen for the coating process, 2.8 mg of lyophilized succinyl cGMP-albumin (rabbit) conjugate was dissolved in 10 ml PBS, frozen in liquid nitrogen and then stored at −20°C. Stock solutions of RSA were prepared via the addition of 50 mg RSA to 10 ml PBS. Aliquots were frozen in liquid nitrogen and stored at −20°C. The coating solution was prepared via the addition of 50 μl of RSA and 120 μl antigen to 15 ml PBS, giving final concentrations of 2.24 μg ScGMP-RSA conjugate and 16.67 μg RSA per ml PBS. The coating solution (125 μl per well) was added to the microtiter plate, and the coated plate was covered and incubated overnight in a humidified chamber at 4°C. Upon completion of the incubation period, the plate was washed twice with cold PBS-T and three times with cold PBS. Non-specific binding was blocked via the addition of 300 μl per well of a 1% (w/v) gelatin solution in PBS-T that had been prewarmed to 37°C. The plate was incubated at 37°C for 2 h in a humid chamber. At the completion of the incubation period, the plate was washed twice with PBS-T (37°C) and three times with cold PBS.

Preparation of Standards and Samples. cGMP standards were prepared from a stock solution of 100 nmol ScGMP in PBS and diluted in PBS to give standard cGMP solutions of 5, 1, 0.3, 0.1, 0.05, 0.03, 0.01, 0.005, 0.003 and 0.001 pmol/50 μl. Aliquots of each of the respective standards were added to microcentrifuge tubes for acetylation. Approximately 200 μl of standard was required to perform each point of the standard curve in triplicate.

A 50 μl aliquot of the respective samples was added to 150 μl of 1.33-PBS and mixed. The diluted samples, standards and a suitable volume of PBS were acetylated following the method of Harper and Brooker (9). For each 200 μl of standard, sample or buffer, 5 μl of a freshly prepared acetylation mixture (2:1 triethylamine: acetic acid anhydride) was added and the sample immediately mixed. After the acetylation of all samples and standards 50 μl of each were added to wells of the coated microtiter plate. To determine the maximum extinction (E_m; the binding in absence of cGMP) and the minimum extinction (E_O; the unspecific binding) 50 μl aliquots of acetylated PBS were added to the microtiter plate. The samples, standards and buffer were followed by 50 μl of rabbit anti-cGMP antibody diluted 1:10,000 in PBS-2TAAE. The antibody solution was omitted from the wells designated to determine the unspecific binding and 50 μl of PBS-2TAAE was added instead. At the completion of the pipetting steps, the plates were covered and incubated overnight in a humidified chamber at 4°C. The incubation was stopped after two washes with cold PBS-T, followed by three washes with cold PBS.

Secondary Incubation. A 125 μl aliquot of an alkaline phosphatase-coupled secondary antibody (anti-rabbit-IgG-AP, diluted 1:2000 in PBS-T) was added to each well of the microtiter plate which was then incubated at 37°C for 2 h in a humidified chamber. On completion of the incubation period the plates were washed twice with PBS-T, four times with cold PBS and once with cold water.

Indicator Reaction. A solution of 4-nitrophenylphosphate in substrate buffer (1 mg/ml) was prepared and prewarmed to 37°C. A 125 μl aliquot of the pre-warmed solution was added to each well of the microtiter plate, which in turn was incubated at 37°C for 70 min. The indicator reaction was stopped by the addition of 200 μl of 3 M NaOH. The extinction was measured at 405 nm.

Analysis of Antibody Specificity. As with any immunological test system the specificity of the antiserum towards the antigen in question is more often than not the major cause of error. Thus the question arises as to what extent the anti-cGMP used in the ELISA cross reacts with bases, nucleosides, nucleotides and similar compounds. The cross reactivity of the cGMP antisera was determined by the 50% displacement method. The relative binding of compounds that may potentially interfere with the assay was expressed as the percentile fraction of the concentration of cGMP required to displace 50% of the antibody from the immobilised antigen, divided by the concentration of tested nucleotide required to cause 50% displacement of the antibody in the same assay. To determine the relative binding values, a range of concentrations of the various competing agents (10; 100; 1000; 10,000; 100,000 pmol/50 μl sample volume) were prepared in PBS. A 200 μl aliquot of each agent was acetylated according to the method above and 50 μl of the acetylated sample was added to a microtiter plate, followed by 50 μl aliquots of the cGMP antisera. The plates were then processed as described above.

Cell Culture. Astroglia-rich primary cultures (APC) were prepared from the brains of newborn Wistar rats as previously described (29). Microglia-rich secondary cultures (MGSC) were prepared from the astroglia-rich cultures according to the method of Giulian and Baker (30), with modifications as described in Hirrlinger et al. (31). In brief, flasks with confluent astroglia-rich cultures were hit 10 times with the flat hand to detach microglial cells. The microglia-containing medium was collected and the cells deposited by centrifugation (500 *g*, 15 min, 4°C). Resuspended cells derived from several flasks were combined and cell number and viability determined (29). For the quantification of cGMP levels, between 500,000 and 750,000 viable cells were seeded in culture dishes 35 mm in diameter. After incubation for 20 min, non-attached cells were removed by washing three times with culture medium. The attached cells were then incubated in a medium consisting of 50% glia-conditioned medium (0.2 μm filtered supernatant of the harvesting procedure) and 50% fresh culture medium (DMEM/10%FCS) for 3 days.

Neuroblastoma × glioma hybrid cells 108CC15 were cultured in DMEM supplemented with 10% FCS, 0.1 mM hypoxanthine, 1 μM aminopterin and 16 μM thymidine as previously described (32).

Experimental Incubation. Stimulation of the cells was carried out according to previously described methods (33). To begin, the culture medium was removed from the dishes and the cells were washed at 37°C with 2 ml of incubation medium containing 145 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$,

1.0 mM $MgCl_2$, 2.0 mM Na_2HPO_4, 20 mM glucose and 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid adjusted to pH 7.4 with tris(hydroxymethyl)-aminomethane. Cells were then incubated with 1 ml of incubation medium in the presence or absence of 500 μM of the phosphodiesterase inhibitor IBMX for 30 min. To initiate the stimulation, the preincubation medium was aspirated and replaced with 1 ml of pre-warmed incubation medium containing stimulation agents at the concentrations indicated. To prepare samples for quantification of cGMP levels, the incubation medium was removed from the cells at the completion of the incubation period and 1 ml of ice-cold ethanol was added immediately to the cell monolayer to extract the cyclic nucleotides. The ethanol extract and another 1 ml of ethanol used to wash the plate were combined and evaporated in a Speed Vac concentrator. The resulting residue was resuspended in an appropriate volume of H_2O. After centrifugation (10 min at 3000 g) to remove cell residue and denatured proteins, samples from the supernatant were taken for cGMP analysis.

cGMP Radioimmunoassay. Additional quantification of cGMP was performed using two separate methods of radioimmunoassay. One was performed according to the method of Brooker et al. (26) as described elsewhere (32), whilst additional experiments were carried out with comparable results using a commercially available cGMP-RIA kit (Amersham Biosciences) as previously described (33).

Data Analysis. To effectively linearise the relationship between known amounts of cGMP and the measured absorbance, the data were transformed using the logit–log mode (34). When using the logit–log concentration relationship in competitive binding assays, the response is defined by logit $(y) = \log [y/1-y]$, where $y = b/b_0$ with b = fraction of the tracer bound and b_0 = value of b with no unlabelled ligand in the system (35). This type of linearisation has been extensively employed in the analysis of RIA data, and is directly applicable for use in ELISAs, yielding straight-line concentration–response curves that are amenable to statistical analysis (36,37).

For the purpose of this assay, the data were transformed using the equation: logit $(y) = \log [y/1-y]$ where $y = (E_s-E_0)/k_c$. In this case E_s = absorbance of the sample, E_0 = absorbance in the absence of antibody (non-specific binding) and $k_c = E_m-E_0$, where E_m is the maximum absorbance in the absence of cGMP (=0 pmol/50 μl cGMP standard). Plotting the transformed data vs. the logarithm ($\log_{10}$) of the concentration of cGMP in the standards results in a linear relationship between logit (y) and log cGMP which can be analyzed via the help of a regression line calculated according to the method of "least squares". The least squares regression line for such a plot provides the formula of logit $(y) = a + b$ * log[cGMP], where a and b are the intercept of the ordinate and the slope, respectively.

All measurements for standard and samples were made in triplicate and the results were interpolated from the standard curve obtained in the same plate. For statistical analysis, the values were expressed as the mean ± standard deviation (SD) from between three to six individual determinations per point. For the stimulation experiments, each experiment was performed at least twice on independent cultures with comparable results. In the figures, the bar representing the standard deviation was omitted if it was smaller than the symbol representing the data point.

Determination of Protein Content. Protein content was determined according to the method of Lowry et al. (38), using bovine serum albumin as the standard.

RESULTS

Calibration Curve

Plotting of absorbance vs. cGMP concentration resulted in a typical sigmoidal concentration–response curve, which could be linearized using the logit–log function (Fig. 1). Analysis of 20 independent calibration curves, performed on separate microtiter plates, on separate days using freshly prepared cGMP standards showed a high degree of reproducibility and accuracy with an average squared Pearson correlation coefficient (r) of 0.99 ± 0.02.

The process of acetylation, whereby the cyclic nucleotide is converted to 2′-O-acetyl cGMP, significantly increases the affinity of the nucleotide for the cGMP antibody (9). This increased affinity results in a concomitant increase in the sensitivity of the cGMP calibration curve (Fig. 2). Acetylation of the cGMP standards between 10 and 0.01 pmol/50 μl resulted in a typical sigmoidal curve with an associated change in absorbance of approximately 0.8 units. This was in contrast to the non-acetylated samples which exhibited an absorbance change of less than 0.2 absorbance units over the same range of cGMP concentrations. A similar sigmoidal

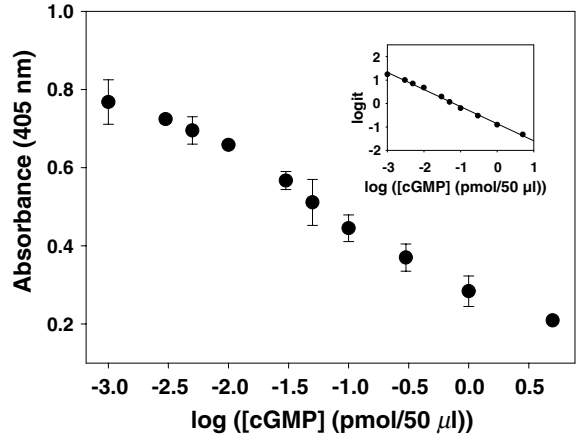

Fig. 1. A typical standard curve for the immunoassay of cGMP. The inset shows the accompanying logit–log transformation and associated linear regression that returns a correlation coefficient (r) of 0.99 over the cGMP concentrations tested. All data points are expressed as the mean ± SD of five determinations.

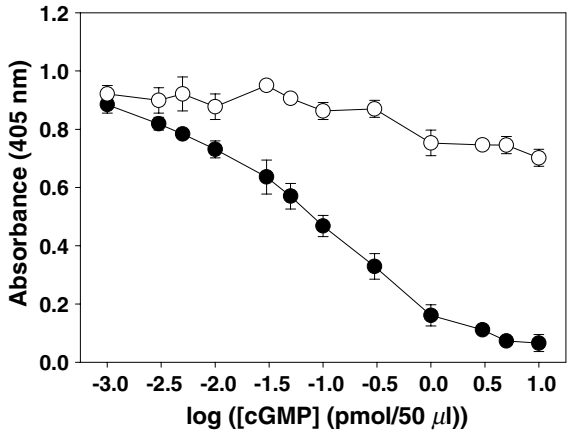

Fig. 2. A typical standard curve for the immunoassay of cGMP, showing the increased sensitivity of the assay after sample acetylation (●) compared to the non-acetylated samples (○). All data points are expressed as the mean ± SD of five determinations.

relationship between non-acetylated sample and an absorbance range of 0.8 was observed at cGMP concentrations between 10 pmol/50 µl and 1 µmol/50 µl, suggesting that the process of acetylation under these particular conditions increased the sensitivity of the assay by approximately 1000-fold.

Cross Reactivity

To determine the specificity of the rabbit cGMP antisera used in this assay, the cross reactivity of the antibody with various bases, nucleosides, nucleotides and related compounds was determined and compared (Table I). The cross reactivity was determined by the ability of the cross reacting compound in question to displace 50% of the antibody from the immobilised antigen. Consequently, the higher the amount of compound required to displace the antibody, the lower the cross reactivity. The percentage cross reactivity was expressed as the percentage of the concentration of cGMP required to displace 50% of the antibody from the immobilised antigen, divided by the concentration of interfering compound required to displace 50% of the antibody from the immobilised antigen. As expected, the antibody showed the highest degree of cross reactivity with cGMP. Of the other compounds tested, only cAMP and inosine 2′, 3′-cyclic monophosphate (cIMP) exhibited any meaningful cross reactivity with the antisera with values of 0.1 and 1%, respectively. The guanine-based nucleotides had little effect on the ability of the cGMP antisera to bind to immobilised antigen (0.0002%). The specificity of the cGMP antisera was best reflected in the fact that of the 41 compounds tested (excluding cGMP, cAMP and cIMP), 39 were shown to have cross reactivities of 0.0005% or less whilst cytidine 3′,5′-cyclic monophosphate (cCMP) and uridine 3′,5′-cyclic monophosphate (cUMP), which were the most effective in displacing the cGMP antisera from the immobilised antigen, were found to have cross reactivities of only 0.001% and 0.005%, respectively.

Recovery of Added Cyclic Nucleotide

For the investigation of interfering sample matrix effects, analytical recoveries for the measurement of known quantities of cGMP were performed in cell extracts taken from cultured 108CC15 cells. Known amounts of cGMP were added to diluted samples of cell extract where the amount of endogenous cGMP had been predetermined. The efficiency in recovering cGMP was calculated as a percentage of the amount of cGMP measured in the sample using the assay divided by the sum of the endogenous cGMP and the exogenously added cGMP. The recovery of cGMP from the samples ranged between 85% and 105% with a mean recovery of 97% ± 7% (Table II).

A tissue extract dilution series was performed to demonstrate the linear relationship between volume of tissue extract and amount of intracellular cGMP as measured by the ELISA (Fig. 3). Ethanolic extracts were prepared from APC and MGSC that had been stimulated with ANP (500 nM) in the presence of the phosphodiesterase inhibitor IBMX. A tight linear relationship was observed between the volume of cell extract and amount of cGMP measured for both preparations, with linear regression analysis yielding *r*-values of 0.989 (APC) and 0.998 (MGSC).

Comparison of cGMP–ELISA and cGMP–RIA

Validation of the ELISA method for the measurement of cGMP in neural cells was performed by a direct comparison of cGMP levels in cellular extracts from cultured cells measured with the ELISA and then by RIA using either the method described by Brooker et al. (26) or a commercial

Table I. Determination of the Cross Reactivites of the Rabbit Anti-cGMP Antisera with Related Compounds

Compound tested	Cross reactivity (%)	Compound tested	Cross reactivity (%)	Compound tested	Cross reactivity (%)
Adenosine	< 0.0001	dC	***	IBMX	0.0001
2′,3′-cAMP	< 0.0001	dCMP	< 0.0001	NAD^+	0.0002
cAMP	0.1	dCTP	***		
AMP	< 0.0001	dG	< 0.0001	$NADP^+$	< 0.0001
ADP	0.0002	dGMP	0.0002	NADH	0.0003
ATP	< 0.0001	dGTP	0.0005	NADPH	0.0001
Cytidine	< 0.0001	Guanosine	< 0.0001	Thymidine	< 0.0001
2′,3′-cCMP	< 0.0001	2′,3′-cGMP	0.0005	cTMP	0.0003
cCMP	0.001	cGMP	100	TMP	< 0.0001
CMP	0.0001	GMP	0.0002	TTP	< 0.0001
CDP	< 0.0002	GDP	0.0002	Uridine	< 0.0001
CTP	< 0.0001	GTP	0.0002		cUMP 0.005
dA	***	Hypoxanthine	***	UMP	< 0.0001
dAMP	< 0.0001	cIMP	1.0	UDP	0.0002
dATP	< 0.0001	IMP	< 0.0001	UTP	< 0.0001

Note: The relative binding was calculated using the formula [cGMP]/[cross reacting species] × 100, where [cGMP] and [cross reacting species] are the concentrations of cGMP and listed nucleotide, nucleoside or base required to displace 50% of the antibody from the immobilized antigen. *** Signifies that the tested compound was unable to displace the antibody at a concentration ≥100 nmol/50 µl of acetylated sample. 2′,3′-cAMP, adenosine 2′,3′-cyclic monophosphate; AMP, adenosine-5′-monophosphate; ADP, adenosine-5′-diphosphate; ATP, adenosine-5′-triphosphate; 2′,3′-cCMP, cytidine 2′,3′-cyclic monophosphate; cCMP, cytidine 3′,5′-cyclic monophosphate; CMP, cytidine-5′-monophosphate; CDP, cytidine-5′-diphosphate; CTP, cytidine-5′-triphosphate; dA, 2′-deoxyadenosine; dAMP, 2′-deoxyadenosine-5′-monophosphate; dATP, 2′-deoxyadenosine-5′-triphosphate; dC, 2′-deoxycytidine; dCMP, 2′-deoxycytidine-5′-monophosphate; dCTP, 2′-deoxycytidine-5′-triphosphate; dG, 2′-deoxyguanosine; dGMP, 2′-deoxyguanosine-5′-monophosphate; dGTP, 2′-deoxyguanosine-5′-triphosphate; 2′,3′-cGMP, guanosine 2′,3′-cyclic monophosphate; GMP, guanosine-5′-monophosphate; GDP, guanosine-5′-diphosphate; GTP, guanosine-5′-triphosphate; cIMP, inosine 2′,3′-cyclic monophosphate; IMP, inosine-5′-monophosphate; NAD^+, β-nicotinamide adenine dinucleotide; $NADP^+$, β-nicotinamide adenine dinucleotide phosphate; NADH, β-nicotinamide adenine dinucleotide (reduced); NADPH, β-nicotinamide adenine dinucleotide phosphate (reduced); cTMP, thymidine 3′,5′-cyclic monophosphate; TMP, thymidine-5′-monophosphate; TTP, thymidine-5′-triphosphate; cUMP, uridine 3′,5′-cyclic monophosphate; UMP, uridine-5′-monophosphate; UDP, uridine-5′-diphosphate; UTP, uridine-5′-triphosphate.

RIA kit (Amersham Biosciences) as previously described (33). A random selection of 60 samples, both diluted and undiluted, demonstrated a good correlation between the two assays in the range of 0–15 pmol cGMP/50 µl sample (Fig. 4). The relationship between the cGMP levels as determined with the ELISA and the corresponding values determined with the RIA were amenable to fitting with a straight line yielding a regression coefficient of 0.95 and a slope of 0.94.

The correlation between cGMP quantification using RIA and ELISA was also shown at high levels of cGMP, such as those found in the incubation medium of cultured microglial cells following stimulation with ANP (Table III). A comparison between the extracellular cGMP levels as measured by RIA and the extracellular levels as measured by ELISA showed little variation between the two methods of quantification. Furthermore, dilution of the incubation medium by a factor of 1: 10 with PBS had no effect on the accuracy or efficiency of the ELISA with the final value corresponding to that of the value determined with the RIA.

Interassay Variability

Interassay variability was determined by the method of repeated measurement of cGMP levels in samples from the same experiment. Astroglia-rich primary cultures were stimulated with ANP and the

Table II. Recoveries of cGMP from Cellular Extracts of Glial Cells

	cGMP (pmol/50 µl)	Recovery (%)
Sample 1 (undiluted)	0.432 ± 0.180	–
Sample 2 (Sample 1 diluted 1:2 with PBS)	0.216 ± 0.014	100
Sample 2 + 5 fmol cGMP	0.187 ± 0.019	85
Sample 2 + 10 fmol cGMP	0.216 ± 0.014	96
Sample 2 + 30 fmol cGMP	0.238 ± 0.029	97
Sample 2 + 100 fmol cGMP	0.314 ± 0.022	99
Sample 2 + 300 fmol cGMP	0.544 ± 0.032	105
Sample 2 + 500 fmol cGMP	0.703 ± 0.025	98

Note: Known amounts of cGMP were added to cellular extracts of the neuroblastoma × glioma hybrid cell line 108CC15 that had been stimulated with bradykinin (500 nM). Extraction and assay of cGMP was performed as described in the text. Values are expressed as the mean ± SD of three–five determinations, each of which was assayed in triplicate.

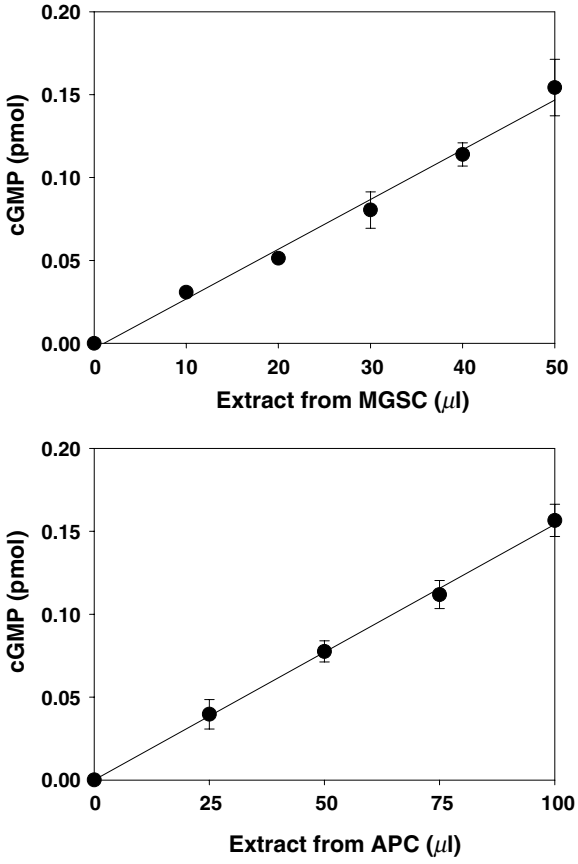

Fig. 3. A tissue extract dilution series was performed from samples of microglial secondary culture (MSGC) and astroglia-rich primary culture (APC) to demonstrate the linear relationship between volume of acetylated tissue extract and amount of intracellular cGMP, as measured by the ELISA, and to confirm a lack of non-specific inhibitory effect of the extract. Linear regression analysis yielded correlation coefficients of 0.989 (APC) and 0.998 (MSGC). All data points are expressed as the mean ± SD of five determinations.

cGMP was extracted as described above. The cGMP content in the respective samples was then determined repeatedly with three independent ELISAs. The assays were performed on different days over a period of 3 weeks. Each of the assays was carried out using the same sample, but with a different microtiter plate, freshly prepared buffers, freshly prepared standards and freshly diluted primary and secondary antibodies. Comparison of the results of three repeated measurements of cGMP showed that whilst the presence of ANP significantly increased the intracellular levels of cGMP in astroglia-rich cultures, there was no statistically significant difference amongst the results of the three control or ANP-stimulated groups (Fig. 5). This was reflected in the dimensions of the calculated means and standard deviations for the three assays, with cells incubated in the absence of ANP having an average cGMP content of 5.0 ± 0.6 pmol/mg protein, whilst cells stimulated with ANP had an average cGMP content of 54 ± 2 pmol/mg protein. The standard deviations in this case were 12% and 4% of the average values, respectively, demonstrating excellent reproducibility of results between assays.

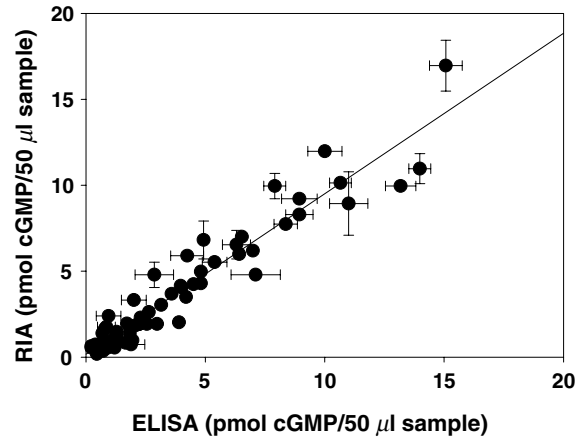

Fig. 4. Correlation of ELISA and RIA determinations of cGMP levels in cellular extracts from the neuroblastoma × glioma hybrid cell line 108CC15. Sixty random samples were diluted and cGMP levels were quantified in each sample using both assay methods. The line is a least squares linear regression fit to the data yielding a correlation coefficient of 0.95 and a slope of 0.94. All data points are expressed as the mean ± SD of five determinations for both assays.

Measurement of Intracellular and Extracellular cGMP levels in Astroglia-Rich Primary Cultures

The cGMP–ELISA was used to quantify changes in intracellular and extracellular levels in astroglia-rich primary cultures following stimulation of the particulate and soluble forms of guanylate

Table III. Correlation Between ELISA and RIA Determinations of cGMP Levels in the Incubation Media of Microglial Secondary Cultures Following Stimulation with ANP (500 nM)

Sample	Method of Measurement	cGMP (pmol/mg)	Recovery (%)
MGSC (no dilution)	RIA	845 ± 6	–
MGSC (no dilution)	ELISA	766 ± 28	92
MGSC (1:10 dilution)	ELISA	853 ± 2	101

Note: The stimulation of microglial cells was carried out on 3-day-old cultures at 37°C for 20 min in the presence of IBMX (500 μM). The values expressed are the cGMP levels as measured in samples of diluted and undiluted media that had been frozen at −20°C prior to the assay process.

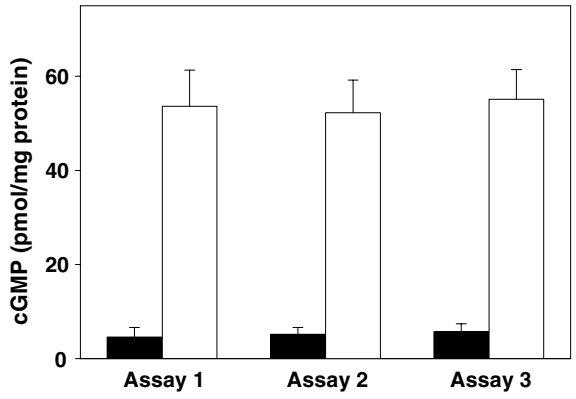

Fig. 5. Interassay variability was tested via the repeated measurement of cGMP levels in astroglia-rich primary cultures that had been stimulated with ANP (500 nM) for 20 min at 37°C. The black bars represent non-stimulated samples that were incubated in the presence of IBMX (500 μM). The white bars represent stimulated samples that were incubated in the presence of both IBMX and ANP. The assays were performed on separate days using assay reagents and standards prepared on the specific assay day. All data points are expressed as the mean ± SD of five determinations.

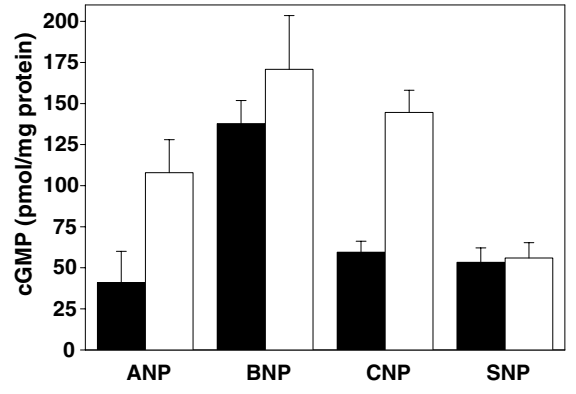

Fig. 6. Alterations of intracellular (black bars) and extracellular (white bars) cGMP levels in rat astroglia-rich primary cell cultures following exposure of cells to 500 nM ANP, 500 nM BNP, 500 nM CNP or 500 μM SNP. The stimulation of cells was performed on 14-day-old cultures for 20 min at 37°C in the presence of IBMX (500 μM). The intracellular and extracellular cGMP levels of unstimulated cells were 0.3 ± 0.2 pmol cGMP/mg protein and 0.7 ± 0.3 pmol cGMP/mg protein, respectively. All data points are the mean ± SD of five determinations per treatment.

cyclase (GC) (Fig. 6). Stimulation of the particulate form of GC with 500 nM ANP, BNP or CNP resulted in significant increases in intracellular levels of cGMP compared to those observed under control conditions, with levels increasing by 130-, 400- and 200-fold, respectively. The levels of cGMP observed after stimulation with the bioactive peptides were comparable to those previously reported in cultured rat astroglia (39–41). Likewise, stimulation of the soluble form of GC with the nitric oxide donor SNP (500 μM) resulted in a 150-fold increase in intracellular cGMP levels over control levels, with the maximum intracellular levels measured being comparable to those previously reported (42,43). The increase in intracellular cGMP levels was reflected in the extracellular levels of cGMP. In the case of cells treated with ANP and CNP, the extracellular cGMP levels were approximately twice that observed intracellularly. In contrast, the extracellular level of cGMP observed following incubation with BNP was only 30% more than the intracellular level, whilst there were no significant changes between intracellular and extracellular levels of cGMP in astroglia that had been incubated for 15 min in the presence of SNP.

DISCUSSION

Many methods for the determination of cyclic nucleotides in biological samples have been described and several are now available from commercial suppliers. However, there are a number of limitations associated with the use of these techniques that can be of major significance, especially when considering their use for either large-scale experiments or use on a frequent basis. In need for a cheap, accurate and easy to perform method to assay cGMP levels in the CNS, we have developed our own ELISA-based method for the accurate detection and measurement of cGMP in a range of neural cell types. The method is a competitive ELISA based on the competition between immobilized cGMP and endogenous cGMP for a highly selective antiserum raised in the rabbit against cGMP. In the absence of endogenous cGMP, the antibody will readily bind to the immobilized antigen, which can then be readily visualized using a commercially available alkaline phosphatase-coupled secondary antibody. As the amount of endogenous cGMP in the sample increases, the amount of free antibody capable of binding to the immobilized antigen decreases and a subsequent reduction in absorbance is detected at 405 nm due to a reduced presence of alkaline phosphatase.

The assay for the measurement of cGMP in intracellular samples extracted from neural cell lines and primary cultures were fully validated with regards to antibody specificity, assay precision

and accuracy. The affinity and specificity of the antibody for antigen are the most important factors in determining the sensitivity and specificity of the assay for cGMP. The affinity of the antibody used in the present assay was previously reported to have a calculated K_D value of 23 nM (27). Since in most neural cell types, the level of cAMP considerably exceeds that of cGMP, a highly selective cGMP assays require cGMP antisera which do not bind cAMP at relatively high concentrations. Furthermore, as the phosphodiesterase inhibitor IBMX is routinely added to cells as part of the experimental protocol, cAMP levels will be increased as a function of incubation time due to strongly diminished hydrolysis. The antibody used in this ELISA failed to exhibit any significant cross reactivity when tested against a cohort of compounds such as bases, nucleosides and nucleotides that were considered to be the most likely candidates to interfere with the assay. Low levels of cross reactivity between the antibody and cAMP were detected, even at high concentrations of cAMP that would be considered to be unphysiological.

One further source of error that must be taken into consideration is that of matrix effects. The affinity of the antibody for the cyclic nucleotide and thus the final result may be altered by components of the extract milieu that could interfere with the antigen–antibody binding in a non-specific manner. Such errors were eliminated following high percentage recoveries from cellular samples containing varying concentrations of cGMP. This was consistent with a lack of non-specific inhibitory effect of the extract. Furthermore, the amount of measured cGMP was directly proportional to the amount of tissue extract included in the assay and the amount of exogenous cGMP added to the extract was correctly quantified. Consequently, the measured extract values determined are an accurate representation of the true cyclic nucleotide concentrations.

As with most assays, close attention to detail, appropriate pipetting techniques and the proper handling of reagents will help to minimize the likelihood of inaccuracies. In the event of problems, the following points should be considered.

Antibody. As repeated freezing and thawing can alter the binding characteristics of antibodies and thus assay performance, antisera should be stored frozen in small-undiluted aliquots. It is preferable if the dilutions of primary and secondary antibody are prepared immediately prior to use.

Acetylation. Samples and standards should be acetylated using freshly prepared acetylation mixture. The acetylation process should be carried out immediately prior to the point in the assay in which the samples and standards are added to the microtiter plate. Furthermore, the samples should be immediately mixed after the addition of the acetylation reagent to avoid incomplete and variable acetylation.

Standards and Blanks. Assay blanks should be treated identically to samples and standards. Consequently, the PBS designated for use to determine the minimum and maximum extinction values (E_o and E_m) should be acetylated in the same manner as the standards and samples. Diluted standard solutions may be frozen and used repeatedly, however it is suggested that freshly diluted solutions of standards are prepared on the day of the assay.

Plate Washing. Due care should be taken to ensure that all wells of the plate are satisfactorily washed and that all wash buffer has been removed from the wells prior to the start of the next step. Upon completion of the washing cycle, the plate should be blotted to remove any residual fluid. To facilitate the washing process, washing buffers may be prepared well in advance and stored at 4°C.

The ELISA described here was used to quantify changes in cGMP levels in astroglia-rich primary cultures following stimulation of the particulate and soluble forms of guanylate cyclase. The ability of ANP, BNP, CNP and SNP to significantly increase intracellular levels of cGMP compared to those observed under control conditions was consistent with the presence of at least two members of the guanylate cyclase-coupled natriuretic peptide receptors namely, NPR-A and NPR-B (44), and the soluble NO-sensitive form of guanylate cyclase. Astrocytes exhibited a distinct sensitivity in their response to stimulation, with a rank order of effect, with regards to the total amount of cGMP produced over a 20 min period, being BNP > CNP > ANP > SNP. This may suggest that the specific mode of stimulus not only influences the intracellular level of cGMP, but also dictates the degree in which astrocytes release cGMP into the intercellular space.

The method described here provides investigators with the opportunity to perform a cheap, reliable and reproducible assay for cGMP. The assay is simple to execute, sensitive and is performed using standard laboratory chemicals, and without the need for complicated preparatory steps of either

sample or antibody. The results of the assay reported here compare quite favorably to those obtained using radioimmunoassay techniques.

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