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Note

Reversed-phase liquid chromatographic determination of cyclic adenosine 3′,5′-monophosphate in rat brain cortex

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There is now a considerable evidence that indicates that cyclic adenosine 3′,5′-monophosphate (cAMP) is a main biological effector [1,2] particularly prominent in the brain. Among the drugs acting on the cerebral adenylate cyclase system which produces cAMP, 5-hydroxytryptamine (5-HT) has been reported to induce the activation of the enzyme: a first activation corresponds to a low affinity constant ($K_m = 0.5–1\ \mu M$) [3–5] and a second activation has a high affinity constant ($K_m = 1\ nM$) [6]. An intermediate value has also been observed [7–9].

In order to clarify the mechanims of the interaction of 5-HT with the adenylate cyclase, which have important physiological implications, it was necessary to use a precise and sensitive method to determine cAMP. Different methods have been proposed for measuring cAMP concentrations in biological materials, e.g., competitive protein binding [10] or radioimmunoassay [11]; the latter technique is closely dependent on the specificity and sensitivity of the antibody used in the analysis. Various radiometric methods have also been employed, e.g., measuring the conversion of [^{32}P]ATP into [^{32}P]cAMP [12]. The major difficulty with these methods rests in the necessity to purify cAMP in order to eliminate the high background activity, which prevents a major proportion of [^{32}P]ATP from being converted into [^{32}P]cAMP. Multiple column chromatographic techniques are most commonly used, usually with Dowex 50-X4 and alumina [13]. In previous work it was pointed out that high-performance liquid chromatography (HPLC) is well suited to the rapid separation of adenine and guanine nucleotides [14–18].

In this work, a reversed-phase HPLC assay was developed for separating and

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determining cAMP in brain tissue, and thus to determine the adenylate cyclase activation induced by 5-HT.

EXPERIMENTAL

Chromatography

The chromatographic system consisted of a Pye Unicam 4011 solvent-delivery system, a Pye Unicam 4020 variable-wavelength detector (Philips, Bobigny, France) and an automatic recorder (Cole Parmer Instruments, Bioblock, Strasbourg, France). A pre-packed reversed-phase column (250 mm × 4.0 mm I.D.; 5-μm spherical particles) containing an octadecyl Si 100 polyol chemically bonded stationary phase obtained from Serva (Heidelberg, F.R.G.) was used. A guard column (50 mm × 4.0 mm I.D.) was packed with the same reversed-phase material.

Chemicals

Adenosine and guanosine derivatives from Sigma (St. Louis, MO, U.S.A.) were dissolved in the mobile phase, each at a concentration of 10^{-5} *M*. 5-HT, creatine sulphate, creatine phosphate, ethylene glycol tetraacetic acid (EGTA) and isobutylmethylxanthine (IBMX) were purchased from Serva (Heidelberg, F.R.G.), creatine phosphokinase from Sigma and [^{3}H]cAMP from New England Nuclear (Boston, MA, U.S.A.) (specific activity 33.5 Ci/mmol).

Chromatographic conditions

The eluent was 0.2 *M* NaH_2PO_4–0.1 *M* $NaClO_4$ (pH 5) (Merck, Darmstadt, F.R.G.) containing 10% (v/v) HPLC-grade methanol (Rathburn, Walkerburn, U.K.). It was prepared daily and filtered through a 0.45-μm Millipore membrane filter.

Adenylate cyclase activation

Adult male Sprague–Dawley rats (180–250 g) were killed by decapitation, their brains were rapidly removed and the cortex was dissected on ice. A crude mitochondrial fraction (P_2) was prepared according to Cotman and Matthews [19]. Shortly afterwards, the cortices were homogenized in an ice-cold buffer (30 mg/ml of tissue) containing Tris–HCl (5 m*M*) (pH 7.4), sucrose (0.32 *M*), EGTA (2 m*M*) and aprotinin (5 U/l), with a Potter glass–PTFE homogenizer. The homogenate was centrifuged at 4°C (1000 *g* for 5 min), the supernatant was discarded and the resulting pellet was resuspended and recentrifuged. Supernatants were added and centrifuged (20 000 *g* for 20 min). The resulting pellet was resuspended and lysed with magnetic stirring for 1 h in cold Tris–HCl buffer (5 m*M*) (pH 8.2) containing EGTA (2 m*M*) and protease inhibitor (aprotinin). Membranes were centrifuged (20 000 *g* for 10 min), resuspended in the same buffer and incubated for 10 min at 37°C to remove endogenous 5-HT. Aliquots (250 μl) of the tissue homogenate were added to a reaction mixture, kept at 0°C and containing ATP (1 m*M*) magnesium chloride (4 m*M*), EGTA (0.5 m*M*), IBMX (1 m*M*), GTP (0.1 m*M*), 5-HT (0.2 n*M* to 100 μ*M*), phosphocreatine (6.7 m*M*) and phosphocreatine kinase (0.2 mg/ml).

The reaction was initiated by placing the samples in a water-bath at 30°C and stopped after 10 min by transferring the tubes into a boiling water-bath and addition of ethanol (1 ml). The samples were centrifuged at 9000 *g* for 30 min at 4°C. The supernatants were collected and lyophilized with a Speed-Vac centrifuge (Bioblock, Strasbourg, France) coupled to a vacuum pump and kept at −80°C until used (less than one week later). They were then resuspended in the mobile phase (250 μl). The internal standards consisted of [^{3}H]cAMP (0.5 μCi) added to the reaction mixture; the radioactivity present in the cAMP fraction collected from the HPLC system was measured and used to identify the cAMP peak and provide a measure of the efficiency and reproducibility of the HPLC separation.

Tissue protein concentration was determined by the Folin reagent method as described by Lowry et al. [20].

RESULTS AND DISCUSSION

Mixtures of adenine and guanine derivatives were chromatographed isocratically under the conditions described under Experimental. Fig. 1 shows the chromatogram obtained for a mixture of adenine and guanine derivatives. The order of elution was ATP, 5′-AMP, 3′,5′-cAMP and adenosine. The separation of the guanine derivatives showed the same pattern as their adenine counterparts. All

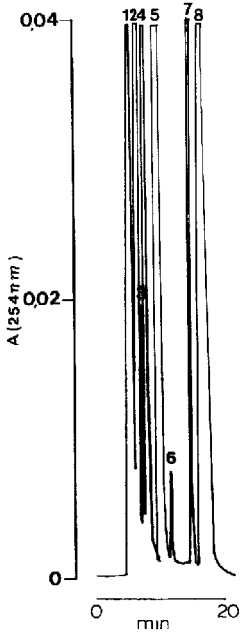

Fig. 1. Isocratic separation of eight adenine and guanine nucleotide reference samples on a reversed-phase octadecyl Si 100 polyol column (250 mm×4.0 mm I.D.; particle size 5 μm); 200 μl of the mixture of nucleotides (10^{-5} *M* for each compound) were injected. Other conditions as described under Experimental. Peaks: 1=5′-ATP (5.4 min); 2=5′-AMP (7.1 min); 3=5′-GTP (7.5 min); 4=5′-GMP (8.3 min); 5=3′,5′-cGMP (9.9 min); 6=guanosine (11.1 min); 7=3′,5′-cAMP (14.6 min); 8=adenosine (16.6 min).

TABLE I

EFFICIENCY OF THE SEPARATION METHOD

Rat cortex membranes were incubated for 10 min at 30°C in the presence of 5 pmol of [^{3}H]cAMP (specific activity 33.5 Ci/mmol) and IBMX (1 m*M*). After extraction (see Experimental), samples were lyophilized and resuspended in 250 μl of NaH_2PO_4 (0.2 *M*)-$NaClO_4$ (0.1 *M*) in methanol–water (10:90) and centrifuged for 5 min at 9000 *g*. The radioactivity content of the resulting supernatants was determined by liquid scintillation counting prior to the HPLC process. The cAMP peak eluted from the column was collected and the corresponding radioactivity was measured by liquid scintillation counting. The coefficient of variation of the percentage recovery was 5% ($n=10$).

	[^{3}H]cAMP recovered		Recovery (%)
	cpm	pmol	
Total radioactivity injected onto the HPLC column	116 900	3.92	78
Eluted cAMP fraction	99 372	3.34	67

the adenine and guanine derivatives were separated within 20 min. Under our experimental conditions, the retention time of cAMP was 14.6 min and it was reasonably separated from the other nucleotides. The sensitivity of the method permits the detection of amounts of this nucleotide in the range 80–2000 pmol. Good linearity of peak height versus concentration was obtained ($r=0.99$). The minimal amount of cAMP that can be detected is ca. 10 pmol. The recovery of the radioactivity used as an internal standard was 85% (Table I).

The chromatograms obtained were similar to those reported for other HPLC methods with regard to the separation patterns for the various adenine and guanine derivatives; however, the observed retention times were not identical.

cAMP in rat brain homogenate

In order to test the application of this system to a biological sample, the ethanol-soluble extract of brain homogenates was chromatographed as described under Experimental. Fig. 2 shows the nucleotide profiles. The cAMP peak was characterized by analysing a tritiated internal standard. It coincided with the radioactivity peak and corresponded to an identical retention time (14.6 min). The retention times for the non-radioactive or tritiated cAMP in the presence or absence of biological extract were very similar and corresponded to 14.6 min. cAMP was quantified by external calibration using a calibration graph obtained together with already known amounts of cAMP. A linear correlation between amount of cAMP and cAMP peak height was observed; the minimal amount of cAMP that could be measured with confidence was in the picomole range. The results for a typical assay are shown in Table II and Fig. 2. The apparent activation constant (K_m) and the velocity of the enzymatic reaction (V_{max}) were determined for the effect of 5-HT on the adenylate cyclase activity. In a separate experiment, the basal activity was 340 pmol/mg protein per min; in the presence of 5-HT the apparent activation constant was 0.8 n*M* with $V_{max}=120$ pmol/mg protein per

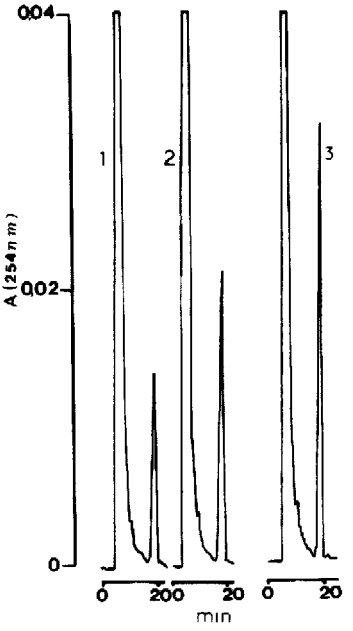

Fig. 2. Elution profile of total ethanol-soluble extract from rat brain cortex homogenates. (1) Profile for basal adenylate cyclase activity, (2) and (3) profiles for 5-HT- (5 nM) and 5-HT- (10 μM) activated adenylate cyclase, respectively. The assay conditions are give under Experimental. The cAMP peak was identified by its retention time (14.6 min).

min for the first activation and 0.3 μM with $V_{max} = 310$ pmol/mg protein per min for the second. The results show that serotonin increases the basal adenylate cyclase activity with two types of activation corresponding to 35 and 91%, respectively. These results are similar to those previously published by our laboratory and obtained by radioimmunoassay [5].

TABLE II

STIMULATION OF ADENYLATE CYCLASE ACTIVITY BY 5-HYDROXYTRYPTAMINE IN RAT CORTEX HOMOGENATES

The measurement of the adenylate cyclase activation was performed as described under Experimental. Values are means ± S.D. of fifteen determinations performed in duplicate. The coefficients of variation of basal and 5-HT-stimulated activities were less than 9% (intra-assay) and less than 4% (inter-assays).

Sample	cAMP fraction (pmol/mg protein per min)
Basal activity	340 ± 78
5-HT (2 nM)	460 ± 24
5-HT (10 μM)	650 ± 20

This method offers several clear advantages. It is reproducible, as the intra- and inter-assay variability are reduced, the coefficient of variation being below 9% for fifteen independent measurements. Moreover, the chromatographic separations give a precise determination of cAMP in the picomole range owing to an 85% recovery of the cAMP after the separation process, in addition to the clear identification of the cAMP peak in the nucleotide chromatogram. It is also possible to establish the presence of several compounds and to determine them: i.e. aden-osine which may result from the degradation of cAMP and indicate the degree of inhibition of the phosphodiesterase activity.

This isocratic method is easy to perform and sample manipulation before chromatography is minimal. Nevertheless, the number of samples that can be measured is limited to 20–50 per day, each determination requiring 20 min.

In conclusion, this method has proved reliable for analysing a serotoninergic physiological effector in a synaptosomal preparation. It can be considered useful for determining cAMP in various brain tissues and, therefore, for analysing the adenylate cyclase activity and its regulation by numerous potential activators or inhibitors in physiological or pathological situations.

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