An assessment of the effect of hormone and neurotransmitters on adenine and guanine derivatives simultaneously in rat brain cortical slices

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Summary. A 2-dimensional thin-layer method has been developed for the separation on cellulose of adenine and guanine derivatives. Using incubated rat cerebral cortex slices it was shown that noradrenaline and acetylcholine stimulated cAMP and cGMP production respectively but glutamate and γ-aminobutyric acid stimulated production of both cyclic nucleotides.

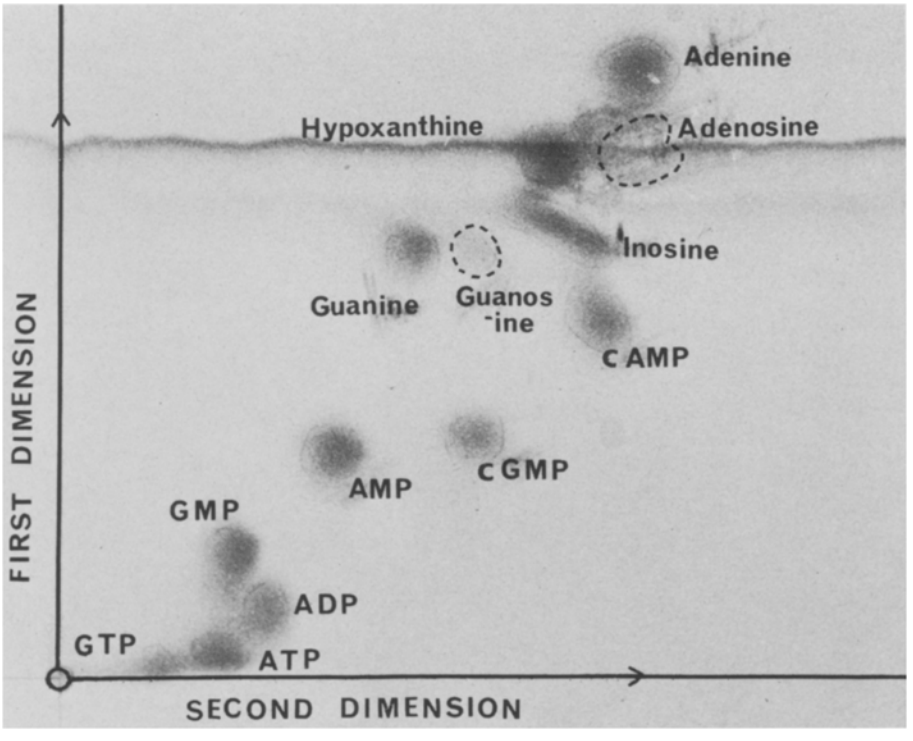
The aim of the present study was to develop a sensitive method for the simultaneous determination of cyclic 3′,5′-adenosine monophosphate (cAMP) and cyclic 3′,5′-guanosine monophosphate (cGMP)[2]. The method was used to test whether the excitatory or inhibitory postsynaptic action of a transmitter correlates with the increased production of either cyclic nucleotide[3].

Materials and methods. Samples (5–10 µl) were spotted in 1 corner of thin-layer cellulose sheets 20 × 20 cm with fluorescent indicator (Eastman Kodak limited, England) at a point 2.5 cm from 2 adjacent sides. Plates were developed at room temperature (23–25 °C) using Shandon (England) thin-layer ascending chromatography developing jars. The plates were developed 1st in 1 dimension using solvent A,n-butanol:acetone:glacial acetic acid: ammonium hydroxide (5%):water (10:5:2:3:2), until the solvent front had travelled approximately 16 cm. The plates were thoroughly dried and developed again in the same direction using the same solvent.

After drying the plates were developed in the 2nd direction with solvent B,n-propanol:methanol:ammonium hydroxide:water (10:1:5:2). The component spots were visualized under UV-light and removed for counting by cutting away the marked area. The separated spots were placed in scintillation vials, mixed with water and blended with Brays scintillation fluid[4]. Spots of similar size were counted as control with each experiment. Final dpm was used for calculation of the results after quench correction due to the different amount of thin-layer plates and radioactivity.

Tissue slices (60–80 mg wet wt) were cut from the cerebral cortex of Sprague-Dawley female rats[5], were incubated at 37 °C in Krebs-bicarbonate medium (5 ml) as previously described[6]. The slices were incubated in the presence of [U-^{14}C]adenine or a mixture of [8-^{3}H] adenine and [8-^{3}H] guanine (Radio-chemical Centre, England) for 30 min. The slices were then lifted out of the incubation fluid, drained and rinsed in fresh Krebs-bicarbonate medium containing the test substance and incubation was carried out for a further 5 min. At the end of incubation the brain slices were each extracted with trichloroacetic acid (5%) containing 1 µmole each of adenine, adenosine, cAMP, guanine, guanosine and cGMP. After removal of trichloroacetic acid the sample freeze dried and dissolved in water before use.

Results and discussion. In the rat cerebral cortex extracts, radioactivity migrated with authentic cAMP and cGMP. cAMP and cGMP spots were removed, extracted with water, concentrated by evaporation to dryness, dissolved in 40 mM Tris buffer, 2 mM $MgSO_4$ (pH 7.5) and treated



Chromatography of a standard mixture of adenine and guanine nucleotides and nucleosides. Standard mixture (5 µl) containing 10 nmoles of each compound, developed 2-dimensionally in solvent A(n-butanol:acetone:glacial acetic acid: 5% ammonium hydroxide:water 10:5:2:3:2) and solvent B (n-propanol:methanol:ammonium hydroxide:water 10:1:5:2) as described in the text. Abbreviations: only 5′-nucleotides were used in the study. AMP, ADP, ATP = the mono-, di- and triphosphates of adenosine. GMP, GDP, GTP = the mono-, di- and triphosphates of guanosine.

The effect of noradrenaline, acetylcholine, glutamate and GABA on the levels of adenine, adenosine, cAMP, cGMP, guanine and guanosine formed in rat cerebral cortex slices from radioactive adennine and guanine

Radioactive labelling of tissue nucleotides as percent incorporated per 100 mg wet wt of tissue from [^{14}C] adenine

	Number of rats used	Adenine	Adenosine	cAMP	cGMP	Guanine	Guanosine	Total
Control	4	2.69 ± 0.25	0.34 ± 0.04	0.22 ± 0.03	0.017 ± 0.008			3.267
Noradrenaline 0.1 mM	6	2.19 ± 0.64	0.37 ± 0.17	0.52* ± 0.08	0.010 ± 0.003			3.090

Radioactive labelling of tissue nucleotides as percent incorporated per g wet wt of tissue from [8-^{3}H] adenine and [8-^{3}H] guanine

Control	8	1.76 ± 0.35	0.58 ± 0.08	0.46 ± 0.10	0.07 ± 0.01	0.83 ± 0.29	0.25 ± 0.07	3.95
Noradrenaline 0.1 mM	4	2.66 ± 1.42	0.54 ± 0.04	5.03* ± 1.84	0.07 ± 0.01		0.18 ± 0.01	8.48
Acetylcholine 0.2 mM (neostigmine 0.33 mM)	6	2.10 ± 0.38	0.55 ± 0.08	0.43 ± 0.05	2.53* ± 0.09		0.32 ± 0.08	5.93
Glutamate 10 mM	5	1.35 ± 0.04	0.36 ± 0.03	4.01* ± 1.20	0.25* ± 0.07	1.33 ± 0.03	0.23 ± 0.07	7.53
GABA 5 mM	3	1.23 ± 0.30	0.33 ± 0.05	2.03* ± 1.21	0.42* ± 0.05	1.47 ± 0.02	0.28 ± 0.06	5.76

Rat cerebral cortex slices were incubated for 30 min at 37 °C in Krebs-bicarbonate medium containing 10 mM glucose and 2 μCi [U-^{14}C] adenine (281 mCi/mmol) for the top experiment. Everything was the same in the bottom experiment except 0.1 μM adenine and 0.1 μM guanine, 5 μCi [8-^{3}H] adenine (27,000 mCi/mmole) and 5 μCi [8-^{3}H] guanine (1000 mCi/mmole) was used instead of [^{14}C] adenine. The slices were rinsed in saline and transferred to fresh medium containing no radioisotope and incubation under test condition was carried out for 5 min. The values are means ± SEM calculated as a percentage of the total radioactivity in the incubation medium. * denotes that the value is significantly greater than control $p \leqslant 0.05$.

with beef heart cyclic nucleotide phosphodiesterase (Boehringer Co., London). The extract was then redeveloped 2-dimensionally and 95–98% of the UV absorption and radioactivity due to both cAMP and cGMP was lost following this treatment.

After 2-dimensional chromatography of a standard mixture, using solvent A for the 1st development and solvent B for the 2nd development, there was complete separation of cAMP, cGMP, adenine, adenosine, inosine, hypoxanthine, guanine, guanosine, AMP, GMP, ATP, GTP and ADP from each other (figure). cCMP and cIMP ran between cAMP and cGMP but were not routinely included in the standard carrier as they were not present in detectable amounts in the rat brain extract.

Rat cerebral cortex slices incubated with [^{14}C]adenine incorporated radioactive label into adenosine, cAMP and cGMP. The incorporation increased linearly over a 30–80-min period. The addition of theophylline (0.5 mM) to the incorporation medium did not affect the incorporation of radioactivity into cAMP. Using a mixture of [8-^{3}H]-adenine and [8-^{3}H]guanine suitable incorporation of tritium into cGMP was obtained. Noradrenaline produced a significant increase in the cAMP (table) both with [^{14}C]adenine or tritiated mixture of adenine and guanine and no significant change in cGMP. In contrast, acetylcholine significantly increased cGMP production without affecting cAMP. Glutamate (10 mM), used at a concentration capable of depolarising cerebral cortex slices, significantly increased both the levels of cAMP and cGMP but the effect on cAMP was greater than on cGMP. γ-aminobutyric acid (GABA) also significantly raised the levels of both cAMP and cGMP, but in this case the effect on cGMP was greater. No significant effect on adenosine or guanosine was detected in response to any of the compounds.

The effects with glutamate and GABA may be more complex than those obtained with noradrenaline and acetylcholine, since the amino acids cause widespread depolarization or hyperpolarization which could lead to secondary transmitter release and activation of cyclic nucleotide formation. However, the proportion of cells responsive to acetylcholine or noradrenaline in the cortex is much smaller. Noradrenaline and acetylcholine are more exclusive in their action whereas the amino acids appear to have multiple effect.

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2 I. Das and J. S. de Belleroche, in: 10th International Congress of Biochemistry, Hamburg, July 1976, abstracts, p. 457.
3 P. Greengard, Nature *260*, 101 (1976).
4 G. A. Bray, Analyt. Biochem. *1*, 279 (1960).
5 H. McIlwain and R. Rodnight, in: Practical Neurochemistry. Churchill, London 1962.
6 J. S. de Belleroche, I. Das and H. F. Bradford, Biochem. Pharmac. *23*, 835 (1974).