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OBSERVATIONS ON THE BINDING OF ADENOSINE 3′:5′-MONOPHOSPHATE TO CELL MEMBRANE FRAGMENTS FROM OX CEREBRAL CORTEX

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Summary

Microsomal or synaptosome membrane fragments from ox brain bind cyclic AMP with a pH optimum of 7.0. Scatchard analysis shows the presence of at least two binding sites. Cyclic GMP and cyclic IMP only inhibit binding at concentrations 5000 times that of cyclic AMP and even higher concentration ratios of ATP and AMP have no effect. Membrane fragments saturated with cyclic [^{3}H]AMP lost less than 7 % of bound nucleotide on incubation at 0°C for 45 min but lost 25 % in the same period in the presence of 10 μM non-radioactive cyclic AMP.

Cyclic AMP is bound by membrane fragments from a variety of tissues, including brain [1], liver [2,3], kidney [4] and adrenal cortex [5]. Purified proteins prepared from the cytoplasm of the cell also bind cyclic AMP [4,6,7] and binding to the regulatory unit of protein kinase enzymes has been demonstrated [8,9]. Membrane fragments from brain are rich in bound protein kinase activity stimulated by cyclic AMP, which catalyses the phosphorylation of endogenous membrane-bound proteins [10—15]. Our interest in this enzyme led us to investigate the binding of cyclic AMP to membrane fragments from brain.

Membrane fragments from frozen ox brain were prepared as previously described [16,17] and binding measured in a final volume of 0.2 ml by incubation at 0°C for 1 h in the presence of 50 mM Tris·HCl (pH 7.0) unless otherwise stated. Reactions were terminated by the addition of 1 ml of 20 mM potassium phosphate buffer (pH 6.0). Reaction mixtures were

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filtered through cellulose acetate discs (Oxoid division of Oxo Ltd., London, S.E.1., U.K.) and the discs washed with 8 ml of the same buffer in small lots, dried at 80°C, placed in scintillation vials and counted [18]. Binding of cyclic AMP to the filters in the absence of protein was negligible and the filters retained over 90% of the protein applied.

Both synaptosome membrane and microsomal fragments bound cyclic AMP with a similar time-course taking about 1 h to reach saturation. The pH optima was found to be 7.0 with a smaller peak at 5.0 (Fig. 1). Cheung [1] found the pH optima for a soluble binding protein from brain to be 6.5.

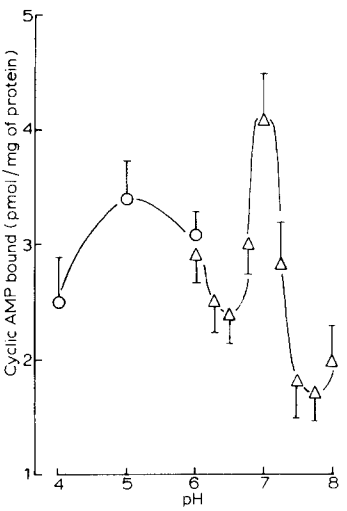

Fig. 1. The effect of pH on the binding of cyclic AMP by synaptosomal membrane fragments. Binding was measured at 0°C in 50 mM sodium acetate (○) or 50 mM sodium phosphate buffer (△) with 16 pmol of cyclic [^{3}H] AMP and 0.1 mg of membrane protein in a volume of 0.2 ml as described in the text. Incubation time was 60 min. Results are means of 6 observations, with vertical bars representing one standard deviation.

Membrane fragments from brain contain bound phosphodiesterase activity which could result in some loss of cyclic AMP during the binding process. Although the reaction was carried out at 0°C to minimise this effect, it was found that after incubation with 0.5 μM cyclic AMP for 1 h under the conditions used to measure binding, 50—60% of the cyclic AMP was broken down to 5′-AMP (as detected by paper chromatography of extracts made by heating the incubation mixture for 2 min at 100°C). It was found, however, that binding in the concentration range 0.1—500 μM cyclic AMP was not affected by the inhibitors of cyclic nucleotide phosphodiesterase theophylline (10 mM) or papaverine (1 mM) or by EDTA (50 mM), although the latter substance, by chelating Mg^{2+}, would also be expected to reduce phosphodiesterase activity. It thus appears that any breakdown of cyclic AMP that occurs during the course of the binding reaction does not greatly affect the amount of binding. Further, since 5′-AMP does not compete with cyclic AMP for binding sites (Table I) it appears that the bound radioactivity is not due to 5′-[^{3}H]AMP resulting from the hydrolysis of cyclic AMP.

TABLE I

INHIBITION OF THE BINDING OF CYCLIC AMP BY VARIOUS COMPOUNDS

Binding was measured with 0.1 mg of membrane protein in 0.2 ml of 50 mM Tris·HCl at 0°C for 1 h as described in the text. Results quoted are means ± standard deviation with the number of observations in parentheses except where less than 4 observations were made.

Compound (mM)	Concentration of cyclic AMP (nM)	Percent Cyclic AMP bound (control=100)	
$MgCl_2$ (0.1)	2	37, 40	
$MgCl_2$ (0.1)	500	70 ± 5.6	(6)
$MgCl_2$ (1.0)	2	35 ± 3.6	(6)
$MgCl_2$ (1.0)	500	70 ± 5.6	(6)
Acetylcholine (1.0)	2	80 ± 14	(9)
Acetylcholine (1.0)	500	88 ± 9	(8)
Cyclic GMP (0.01)	2	10, 12, 16	
Cyclic GMP (0.01)	500	100, 105, 110	
Cyclic IMP (0.01)	2	5 ± 4	(4)
Cyclic IMP (0.01)	500	83 ± 20	(4)
Dibutyryl cyclic AMP (0.01)	2	54 ± 7	(4)
Dibutyryl cyclic AMP (0.01)	500	97 ± 10	(4)

Bound cyclic AMP does not appear to be lost from the membrane fragments at 0°C though the bound nucleotide does exchange with non-radioactive cyclic AMP (Fig. 2). At 25°C considerable loss occurs, perhaps due to phosphodiesterase activity.

The effect of cyclic nucleotide concentration on the binding was determined in the range 0—500 nM (Fig. 3). Very little difference was seen between synaptosome membrane and microsomal fragments and Scatchard analysis [19] indicated the presence of at least two binding sites. For microsomal fragments the capacities of the high and low affinity sites were 4 and 1000 pmol/mg of protein with affinity constants of $2.2 \cdot 10^8$ M^{-1} and $7 \cdot 10^3$ M^{-1} respectively.

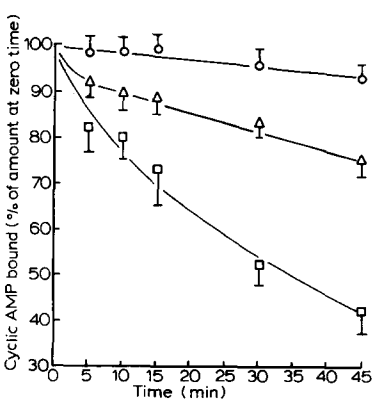

Fig. 2. Stability of bound cyclic AMP. Synaptosome membrane fragments were incubated at 0°C at a concentration of 0.6 mg/ml with 500 nM cyclic [^{3}H] AMP and 50 mM Tris·HCl at pH 7.0. After 60 min the protein was spun out at 12500 g for 10 min in a Sorvall RC2B centrifuge. The pellet was washed once with 12 ml of water and resuspended at a protein concentration of 0.6 mg/ml in 50 mM Tris·HCl (pH 7.0). ○——○, experiments at 0°C in the absence of 10 μM cyclic AMP; △——△, experiments at 0°C in the presence of 10 μM cyclic AMP; □——□, experiments at 25°C in the absence of cyclic AMP. Samples (0.2 ml) were taken at the stated times, filtered, and the protein residues washed and radioactivity determined as described in the text. The results are means of 6 observations with the vertical bars representing one standard deviation.

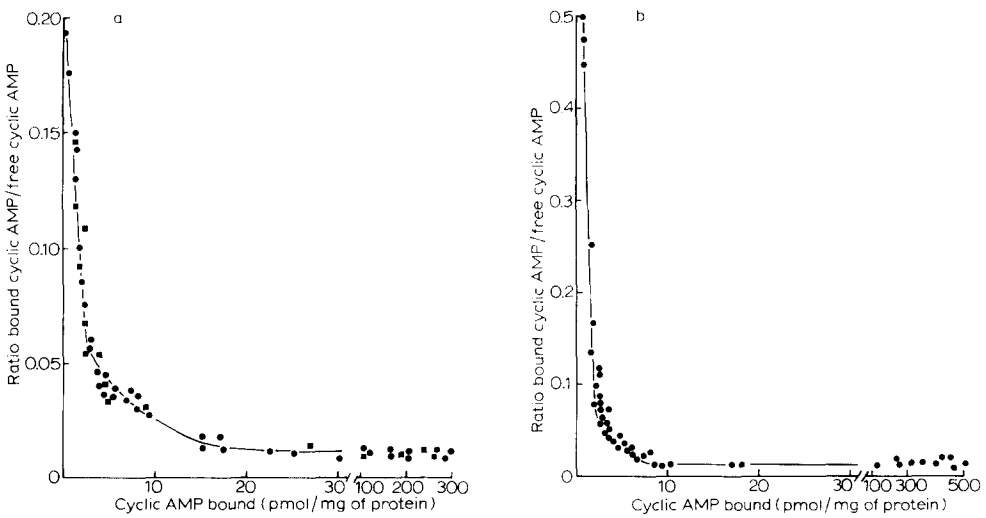

Fig. 3. Scatchard plots of the binding of cyclic AMP to membrane fragments. Binding was measured in 0.2 ml of 50 mM Tris·HCl (pH 7.0) with the stated concentration of cyclic [^{3}H] AMP and 0.1 mg of membrane protein as described in the text. In the presence (■) or absence (●) of 1 mm papaverine (a) microsomal fragments (b) synaptosome membrane fragments.

For synaptosomal fragments the respective figures were 3 and 2000 pmol/mg of protein and $5 \cdot 10^7$ M^{-1} and $2 \cdot 10^4$ M^{-1}. Multiple binding sites for cyclic AMP have also been observed with plasma membranes from liver [4].

The effect of a number of compounds on binding was examined. Neither NaCl (100 mM), KCl (100 mM) or $CaCl_2$ (10 mM) had any effect at either high (500 nM) or low (2 nM) concentrations of cyclic AMP. Binding was, however, decreased by $MgCl_2$ (0.1—1.0 mM) particularly at low concentrations of cyclic AMP, indicating the effect to be on high rather than low affinity sites (Table I). Of the putative neurotransmitters examined noradrenaline (1 mM) and dopamine (1 mM) had no significant effect but acetylcholine gave a small but significant ($P < 0.01$) decrease in binding (Table I). Of the various nucleotides tested, neither adenosine (1 mM), ATP (1 mM) or 5′-AMP (1 mM) had any significant effect ($P > 0.1$), but cyclic GMP, cyclic IMP and, to a lesser extent, dibutyryl cyclic AMP (all at 10 μM concentration) inhibited the binding (Table I) of low but not high concentrations of cyclic AMP. Strychnine (1 mM), cysteine (1 mM) and ethanol (1%) were all without significant effect ($P > 0.2$).

These results indicate that cerebral membrane fragments have a high capacity for binding cyclic AMP. The affinity of the nucleotide for its binding sites is also high, since 5′-AMP did not interfere with the reaction and since cyclic GMP and cyclic IMP only inhibited the binding at concentrations several thousand times higher than that of cyclic AMP.

The content of cyclic AMP in the cerebral cortex is 10—25 pmol/mg of protein, rising to 100 pmol/mg on stimulation for 10 min with electrical pulses [20], The mean concentration of the nucleotide in cerebral tissue has been calculated to be 1—2.5 μM, rising to 10 μM on stimulation [2]. Our results show that changes of cyclic AMP concentration in this range may be accom-

panied by substantial changes in the amount of the nucleotide bound to cell membranes.

The nature of the proteins in the membrane fragments to which cyclic AMP binds is unknown. Some of the binding is presumably occurring to the phosphodiesterase and cyclic AMP-stimulated protein kinase enzymes in the membrane. However, adenosine and K^+ which inhibit the latter enzyme [11, 23] have no effect on binding.

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