A SPECIFIC BINDING SITE FOR 3', 5' CYCLIC AMP IN RAT PAROTID MICROSOMES

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SUMMARY

Binding of 3', 5' cyclic AMP (cAMP) was investigated in view of the role of this nucleotide in the induction of enzyme secretion in the rat parotid gland. The experiments were carried out with the microsomal fraction which showed a high affinity for cAMP. Half maximal binding was obtained at 2×10^{-7} M cAMP. N^6-monobutyryl cAMP which is an efficient inducer of enzyme secretion in the parotid gland inhibited the binding of ^3H labeled cAMP more efficiently than any other nucleotide tested. Pre-incubation of the microsomes with either phospholipase C or trypsin caused a marked decrease in the amount of ^3HcAMP bound/mg protein. Partial separation between smooth and rough microsomes indicates that the binding site for cAMP is localized preferentially in the smooth membranes.

It seems by now well established that cAMP is an intermediate in the induction of enzyme secretion in the rat parotid gland (1, 2). However, the reaction in which cAMP participates to bring about enzyme secretion has not been identified. In order to try and locate such a reaction it was decided to look for a subcellular component of the gland cell which would specifically bind cAMP. Since the process of secretion involves extensive membrane interactions (3) the binding of cAMP was studied at first in subcellular membrane fractions. The present communication reports on high affinity binding of cAMP by components of the microsomal

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Abbreviations: cAMP, 3', 5' cyclic AMP; dibutyryl cAMP, N^6-2-O-dibutyryl 3', 5' cyclic AMP; monobutyryl cAMP, N^6-monobutyryl 3', 5' cyclic AMP; PMB, p-hydroxymercuribenzoate.

fraction. A communication by Gill and Garren on cAMP binding protein from adrenal glands has recently appeared (4).

EXPERIMENTAL

Rat parotid homogenate was prepared in 0.3 M sucrose pH 7.5 (Na_2CO_3) containing also 0.2 μg/ml of diphenyl-p-phenylene diamine (see also ref. 2). The supernatant obtained by centrifugation for 10 min at 10,000 g served for isolation of the microsomal fraction by further sedimentation for 60 min at 10^5xg. This fraction contained more than 50% of the cAMP binding capacity of the particulate fractions.

^{3}HcAMP obtained from Schwartz Bioresearch was purified by thin layer chromatography on cellulose powder using the solvent mixture isopropyl alcohol, ammonia, water (7:1:2). Binding of the labeled nucleotide was measured in the following standard reaction mixture. ^{3}HcAMP, 50-1000 $\mu\mu$moles; $MgCl_2$, 5 μmoles; glycylglycine pH 7.5, 75 μmoles and aminophylline, 5 μmoles. The microsomal fraction, 0.3-3 mg protein, was added as the last component. The final volume was 1 ml.

In earlier phases of this work, incubation was for 30 min at 0-4°. Subsequently, experiments were performed at 30° for 5 min during which time almost maximal binding was obtained. No significant differences were found by this change of conditions. The reaction was terminated by centrifugation at 15,000xg for 5 min. Protein and the amount of radioactivity measured by scintillation counting, were assayed in the pellet. The amount of cAMP actually bound was obtained by subtracting the amount of unbound radioactivity retained in the pellet. The latter was determined on a control test system which contained the same amount of radioactivity as the experimental system and a huge excess of unlabeled cAMP (1 μmole) which

reduced the specific radioactivity at least a thousand fold. Thus, essentially only unlabeled cAMP would be bound and the radioactivity found in the control pellet would be due solely to the amount of fluid retained.

RESULTS AND DISCUSSION

The binding of ^{3}HcAMP to microsomes could be described by a typical saturation curve when tested in the concentration range $5x10^{-8}$ - 10^{-6} M. As calculated from a Lineweaver-Burk plot (5), maximal binding at saturating concentration was 2-3 $\mu\mu$moles cAMP/mg protein. Half maximal binding was reached at $2x10^{-7}$ M cAMP. Binding increased linearly with protein concentration in the range 0.3-3 mg. Of the bound radioactivity at least 93% were recovered as cAMP when the microsomal pellet was eluted by heating for 5 min at 100° and the material was analysed by thin layer chromatography. The exchange at 30° of bound ^{3}HcAMP by addition of a tenfold excess of unlabeled cAMP was rather slow. About 75% of the label was exchanged in 15 min although maximal binding is achieved within 5 min.

The specificity of the binding site for cAMP was studied by measuring the inhibition of ^{3}HcAMP binding caused by adding other nucleotides to the test system (Table I).

It is demonstrated that the binding is rather specific for cAMP. Furthermore, the cyclic nucleotides show a markedly higher affinity for the site than the other nucleotides tested. It is interesting to note that the most effective inhibition was obtained with monobutyryl cAMP which was much more effective than the dibutyryl derivative. Posternak and Sutherland who first synthesized the butyryl cAMP derivatives reported that monobutyryl cAMP was much more potent than dibutyryl cAMP when tested in the phosphorylase activating system (6). Our own observations indicate that the monobutyryl

TABLE I

INHIBITION OF THE BINDING OF cAMP TO PAROTID MICROSOMES BY VARIOUS NUCLEOTIDES

Inhibitor	Inhibitor/cAMP Molar ratio	Per cent inhibition of cAMP binding
6-monobutyryl cAMP	8	50
3', 5'-cyclic IMP	50	50
3',5'-cyclic GMP	400	50
2', 6'-dibutyryl cAMP	600	50
3', 5'-cyclic UMP	4,000	50
2', 3'-cyclic AMP	20,000	16
2', 3'-cyclic GMP	20,000	6
5'-AMP	2,000	20
3'-AMP	2,000	10
ATP	20,000	20
GTP	20,000	0
Pyrophosphate	20,000	0

All systems contained 50 pmoles of ^{3}HcAMP (about 50,000 cpm). The system without added inhibitor contained a total of 1,710 cpm in the pellet. The amount of unbound cAMP retained in the control pellet was 590 cpm. Thus the net amount actually bound was 1,710-590=1,120 cpm. The decrease in the net amount of bound radioactivity caused by the addition of an inhibitor compound was expressed as % inhibition.

All compounds were tested at least at 4 different concentrations.

derivative is also more effective in the induction of amylase secretion by parotid slices (unpublished). Thus the present direct measurements of affinity for the binding site are in good agreement with the observations on the biological activity of the cyclic nucleotide derivatives.

Several experiments were performed to test the possible involvement of various components of the microsomal membrane in the binding of cAMP. It is shown in Table II that PMB had only a slight effect and it is

TABLE II

EFFECT OF VARIOUS AGENTS ON BINDING OF cAMP TO MICROSOMES

Exp.	Compound added		cAMP bound pmoles/mg protein of pellet	Per cent inhibition of binding
1	None		0.71	0
	PMB	0.1 mM	0.55	22
2	None		0.36	0
	Phospholipase C	75 μg/ml	0.32	11
		150 μg/ml	0.23	36
		300 μg/ml	0.18	50
3	None		0.26	0
	Trypsin	2 μg/ml	0.09	65

Microsomes, 1.5 mg/ml, were preincubated at 30° in the presence of sucrose, 150 mM; glycylglycine pH 7.5, 50 mM and the reagents shown above in a final volume of 2 ml. The systems with phospholipase C and trypsin also contained 0.5 mM calcium. Preincubation times were: Exp.1, 5 min; Exp.2, 30 min; Exp.3, 10 min. At the end of preincubation aliquots were added to the standard assay system at 30°. The concentration of ^{3}HcAMP was 5×10^{-8} M.

therefore tentatively concluded that sulfhydryl groups are not an essential part of the binding site.

It is further demonstrated that degradation of phospholipd and of protein in the microsomal membrane both lead to reduced binding. Only in the case of trypsin treatment a decrease of about one third in the amount of protein in the pellet was observed. Since the amount of cAMP bound per mg protein remaining in the pellet was strongly diminished by trypsin it is evident that the binding site is selectively affected by this enzyme.

In order to define more closely the membrane involved in the binding of cAMP the microsomal fraction was partially resolved into smooth

and rough microsomes by flotation in 1.6 M sucrose (7). The floating band enriched in smooth microsomes bound 5 times more cAMP/mg protein than the precipitate of rough microsomes. Thus the binding site seems to be localized preferentially in smooth membranes, which in the rat parotid gland are derived from the Golgi vesicles and the cell membrane. The dynamic modification of the cell membrane during the secretion process mediated via cAMP has recently been described (3). Observations by Amsterdam, Ohad and Schramm (manuscript in preparation) point to formation of vacuoles in the Golgi area of the cell during the first minutes after onset of secretion. Experiments are in progress to test whether these changes in the smooth membranes are related to their ability to bind cAMP.

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