DIFFERENCES IN MACROMOLECULAR BINDING BETWEEN CYCLIC AMP AND ITS DIBUTYRYL DERIVATIVE IN VITRO*

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SUMMARY

Rat liver cytosol binds ^{3}H-cAMP and ^{3}H-DBcAMP in vitro. Fractionation of bound radioactivity by DEAE-Sephadex chromatography shows that ^{3}H-cAMP is associated with a different cytosolic protein than is ^{3}H-DBcAMP. The pI's of the cAMP-protein and the ^{3}H-DBcAMP-protein complexes are 6.7 and 3.9, respectively. Competition studies between ^{3}H-cAMP and its structural analogues have shown the following order of effectiveness in competing for binding sites in rat liver cytosol: cAMP > N^{6}-MBcAMP > $O^{2'}$-MBcAMP. No inhibition of ^{3}H-cAMP binding was observed with 5'-AMP, adenosine, cGMP or DBcAMP. In vitro binding experiments with rat serum has shown that only ^{3}H-DBcAMP binds to any significant extent.

Differences in the effectiveness of cAMP and DBcAMP have been observed in the stimulation of glucose production from glycogen in rat liver perfusion experiments (1) and in the activation of phosphorylase in dog liver slices (2). It has been suggested (3,4) that the differences in potency of cAMP and its dibutyryl derivative may be due to either increased permeability into the cell or a decrease in sensitivity to enzyme degradation. Menahan and co-workers (5) have shown that in rat liver cAMP is most sensitive to the hydrolytic activity of phosphodiesterase. The dibutyryl derivative

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The abbreviations used are: cAMP, adenosine 3',5'-monophosphate; DBcAMP, N^{6},$O^{2'}$-dibutyryl cAMP; 5'-AMP, adenosine-5'-monophosphate; cGMP, guanosine 3',5'-monophosphate; 3'-AMP, adenosine-3'-monophosphate, ATP, adenosine-5'-triphosphate; GTP, guanosine-5'-triphosphate.

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is neither degraded by this enzyme nor is it an inhibitor of phosphodiesterase. Experiments with HeLa cells (6) have shown that the greater intracellular accumulation of DBcAMP as compared to cAMP is due to resistance to enzyme degradation rather than enhanced permeability. Different results were recently obtained for the intracellular accumulation of cAMP and DBcAMP with Strain L and WI-38 cells in monolayer culture (7); these cells transported cAMP better than DBcAMP. No additional explanations have been offered for the initial biological fate of these cyclic nucleotides once they enter the cell. This communication describes *in vitro* experiments with rat liver cytosol which shows that cAMP and DBcAMP bind to separate and distinct cytosolic protein(s).

MATERIALS AND METHODS

All cyclic nucleotides and derivatives were obtained from Sigma Chemical Company. Adenosine was obtained from Calbiochem; adenosine-5'-monophosphate from Boehringer, Mannheim. ^{3}H-cAMP (Specific Activity 24.1 Ci/mmole) and ^{3}H-DBcAMP (Specific Activity 7.05 Ci/mmole) were purchased from the New England Nuclear Corporation.

The purity of all compounds was established by descending paper chromatography using Whatmann No. 3MM paper with ethanol-0.5 M ammonium acetate (5:2, v/v) as solvent (4) and by high voltage paper electrophoresis using pyridine-acetate buffer, pH 3.5, containing 0.01 M EDTA (8).

Male adrenalectomized rats (Charles River Breeding Laboratories) weighing 100-130 g were killed by decapitation. Livers were perfused *in situ* with cold 0.9% saline. The livers were sliced, weighed, minced and homogenized with 1 volume of cold 0.05 M tris-HCl, pH 7.5, containing 0.25 M sucrose. The homogenate was centrifuged 27,000xg for 20 minutes. The post-mitochondrial supernatant was centrifuged at 100,000xg for 1 hour. The 100,000xg supernatant was used for the *in vitro* binding and competition studies. An equivalent amount of cytosolic protein was used for all experiments.

For the competition experiments an aliquot of ^{3}H-cAMP or ^{3}H-DBcAMP

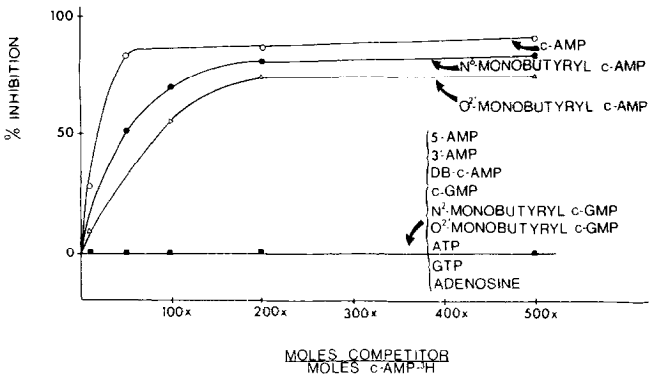


Fig. 1. cAMP competition studies. % binding and inhibition of cytosolic reaction mixtures with ^{3}H-cAMP at final concentration of 1.4 x 10^{-7} M in the presence or absence of non-radioactive competitors were determined as described in Materials and Methods. 0% and 90.2% inhibition are equivalent to 49.0% and 4.8% binding, respectively. Incubations were done in presence of 5 mM theophylline.

was dried under a stream of N_2 at 18°C. At zero time, both cytosol and competitor were added to the reaction vial. The final concentration of labeled nucleotide was 1.4 x 10^{-7} M. Incubation was carried out at 4°C for 90 minutes with constant stirring. At the end of the incubation period, the cytosol was fractionated on a 2 x 53 cm Sephadex G-25 column into protein bound and unbound radioactivity. The columns were eluted with 1 mM tris, pH 7.5. Determination of the % binding was calculated from the following relationship:

$$\% \text{ Binding} = \frac{\text{DPM bound pool}}{\text{DPM in total recovered radioactivity}} \times 100$$

% inhibition was calculated as the difference in the percent binding in control as compared to reaction mixtures containing inhibitor.

Further fractionation of the excluded protein bound radioactivity was carried out on DEAE Sephadex A-50 as previously described (9). The cyclic nucleotide binding fraction was electrofocused using the 110 ml column of LKB Instrument, Inc. (10) with either a 3-10 or 3-6 pH gradient. The pH was measured at 4°C.

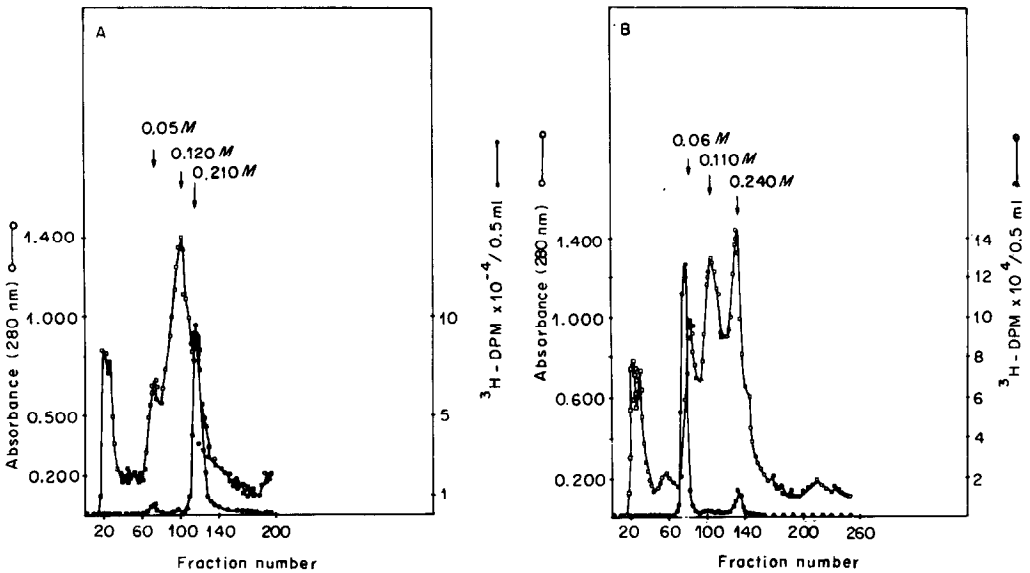
Rat serum, prepared from the blood of the vena cava, was allowed to stand for 15 minutes and was centrifuged in a clinical centrifuge for 10

minutes. The supernatant was removed and recentrifuged. This final supernatant was used for binding experiments.

RESULTS AND DISCUSSION

Figure 1 illustrates the specificity of binding of ^{3}H-cAMP in cytosol. Maximal inhibition occurs with a 500-fold excess of non-radioactive cAMP (90.2% inhibition). The monobutyryl derivatives at maximal concentration levels are less effective inhibitors of ^{3}H-cAMP binding (85% and 75% inhibitions, respectively). No inhibition of ^{3}H-cAMP binding was observed with 5'-AMP, 3'-AMP, adenosine, dibutyryl cAMP or cGMP. When the competition experiments were done using ^{3}H-DBcAMP and increasing levels of competitor, no inhibition of labeled nucleotide binding was observed with cAMP (data not shown). Since the monobutyryl derivatives of cAMP were effective competitors of ^{3}H-cAMP and ^{3}H-DBcAMP binding to rat liver cytosol it seemed at first strange that these two nucleotides were not mutual inhibitors. The possibility existed that these two structurally related cyclic nucleotides were binding to different cytosolic fractions.

Figure 2 shows the chromatographic patterns of the G-25 excluded protein labeled with either ^{3}H-DBcAMP or ^{3}H-cAMP. In Figure 2A, 96% of the radioactivity (8.40 pmoles ^{3}H-DBcAMP/mg protein) is found in the region corresponding to 0.21 M KCl and 4% of the radioactivity is associated with protein eluting in the 0.05 M KCl region. ^{3}H-cAMP shows a reversed binding pattern (Fig. 2B). Ninety-two percent of the recovered radioactivity (17.1 pmoles ^{3}H-cAMP/mg protein) is associated with the cytosol fraction eluting at 0.06 M KCl. The remaining 8% of the radioactivity is found in the fractionation region corresponding to 0.24 M KCl. Preliminary *in vivo* experiments in our laboratory have shown that at short intervals after injection, the liver cytosol binding pattern is similar to those *in vitro* (unpublished observations). Addition of 5 mM theophylline and/or 1 mM EDTA did not effect the binding of ^{3}H-DBcAMP to rat liver cytosol. However, ^{3}H-cAMP binding was reduced by 50% when incubated with cytosol in the absence of theophylline



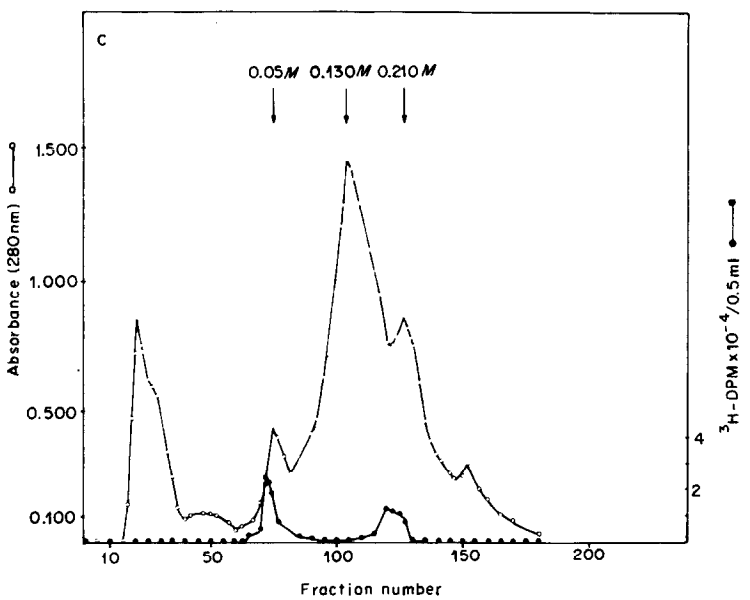


Fig. 2A. DEAE-Sephadex A-50 chromatography of G-25 excluded protein bound ^{3}H-DBcAMP. Fractionation of the cytosol bound pools was done on a column of DEAE Sephadex A-50 that was 3 x 75 cm using a linear 0-1.0 M KCl gradient in 0.05 Tris-HCl, pH 7.5. 6.0 ml fractions were collected. The positions of the various protein fractions are determined by their elution in the KCl gradient. 75% of the bound radioactivity was recovered.

Fig. 2B. DEAE-Sephadex A-50 chromatography of G-25 excluded protein bound ^{3}H-cAMP. Conditions are identical to those described in 2A. 100% of the bound radioactivity was recovered.

Fig. 2C. DEAE-Sephadex A-50 chromatography of G-25 excluded protein bound ^{3}H-cAMP that was initially incubated in the presence of 500-fold excess of N^6-monobutyryl cAMP. Fractionation conditions identical to those described in Fig. 2. 93% of the bound radioactivity was recovered.

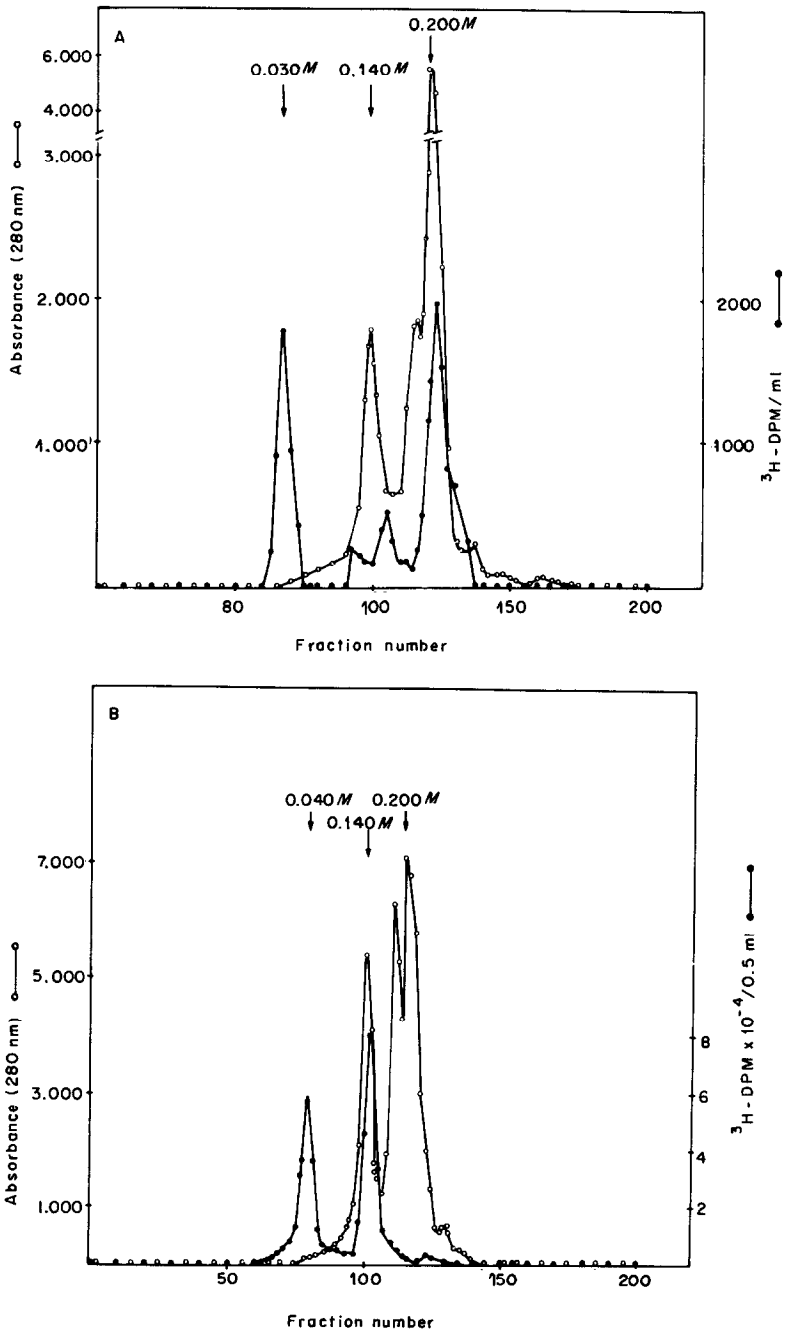


Fig. 3A. DEAE-Sephadex A-50 chromatography of G-25 excluded serum protein bound ^{3}H-cAMP. 72% recovery of bound radioactivity. Chromatographic conditions identical to those described in the legend to Fig. 2.

Fig. 3B. DEAE-Sephadex A-50 chromatography of G-25 excluded serum protein bound to ^{3}H-DBcAMP. 96% recovery of radioactivity. Conditions same as those described in the legend to Fig. 2.

(unpublished data). Also both cyclic nucleotide binding regions were not associated with any protein kinase activity.

Isoelectrofocusing of the major nucleotide binding proteins gave a pI of 6.70 for ^{3}H-cAMP-protein complex and 3.90 for ^{3}H-DBcAMP (data not shown).

Direct demonstration that the competition of ^{3}H-cAMP binding with the various effective competitors to a specific cytosol fraction is shown in Figure 2C. Cytosol was incubated with a 500-fold excess of N^{6}-monobutyryl cAMP in the presence of ^{3}H-cAMP and theophylline. Incubation conditions and fractionation procedure are described in Materials and Methods. The radioactivity associated with the 0.05 M KCl region is dramatically reduced when compared to the binding of ^{3}H-cAMP in the absence of inhibition (Fig. 2B). However, the radioactivity associated with the 0.21 M KCl region shows no apparent change in labeling.

To determine whether any of the rat liver cytosol proteins that bound labeled nucleotides was a serum protein, rat serum was prepared. Binding experiments showed that 1.3% of the ^{3}H-cAMP was bound as compared to 49.0% in rat liver cytosol. Figure 3A shows the DEAE-Sephadex A-50 chromatography of the serum-^{3}H-cAMP G-25 protein bound radioactivity (excluded pool). The major binding fraction of rat liver cytosol is not present in the serum fractionation chromatogram. However, when serum was incubated with ^{3}H-DBcAMP, 45% binding was observed as compared to 62.2% binding in rat liver cytosol. Figure 3B shows that the major nucleotide binding occurs in the 0.14 M KCl region and that the 0.20 M region is unlabeled. Therefore, ^{3}H-cAMP and ^{3}H-DBcAMP appear to bind to different protein fractions in rat liver cytosol and serum.

The data presented show that at physiological concentrations, cAMP and its dibutyryl derivative bind to different cytosolic proteins. This is the first time that it has been demonstrated that DBcAMP binds to an intracellular protein fraction. Little is known about the butyryl derivatives. It has been generally accepted that DBcAMP mimics the effects of cAMP by its greater permeability into intact cells and its greater resistance to phos-

phodiesterase (2,3). Blecher et al. (11) have recently presented data to show the presence of N^6-acyl amidohydrolase and $O^{2'}$-acyl esterase in a variety of tissues. This deacylase activity is predominantly located in the cytosol compartment of the cell. Preliminary in vivo experiments in our laboratory with ^{3}H-DBcAMP have shown that at increasing times after injection the radioactivity associated with protein in the 0.21 M KCl region of the DEAE-Sephadex A-50 chromatogram decreases while there is a concomitant increase in the cAMP labeled region (unpublished data). This suggests that as the acyl derivative of cAMP is deacylated there is an increased affinity for the cAMP binding protein. The results from the competition experiments and preliminary in vivo time study of ^{3}H-DBcAMP binding support this transfer hypothesis. Sutherland has suggested that the resistance of the butyryl derivative to phosphodiesterase may allow the cyclic nucleotide to be transported to a particular site of action (cytosol or nuclear?) and once there acts as a "slow feed" of cAMP (12). Thus, a more effective level of cAMP could be supplied than if cAMP were given. It is tempting, therefore, to speculate that the intracellular DBcAMP binding protein may be involved in the regulation of cAMP levels. Current work in our laboratory is directed at the purification and characterization of these nucleotide binding proteins and clarification of the biological activation of cAMP and DBcAMP in view of this difference.

REFERENCES

1. Levine, R.A. and Lewis, S.E., Biochem. Pharmacol., 18, 15 (1969).
2. Henion, W.F., Sutherland, W.E. and Pasternak, T., Biochim. Biophys. Acta, 148, 106 (1967).
3. Robison, G.A., Butcher, R.W. and Sutherland, E.W., Ann. Rev. Biochem., 37, 149 (1968).
4. Pasternak, T., Sutherland, E.W. and Henion, W.F., Biochim. Biophys. Acta, 65, 558 (1962).
5. Menahan, L.A., Hepp, K.D. and Wieland, O., Eur. J. of Biochem., 8, 435 (1969).
6. Kaukel, E. and Hilz, H., Biochem. Biophys. Res. Commun., 46, 1011 (1972).
7. Ryan, W. and Durick, M.A., Science, 177, 1002 (1972).
8. Sebring, E. and Salzman, N., Anal. Biochem., 8 , 126 (1964).

9. Singer, S. and Litwack, G., Endocrinology, 88, 1448 (1971).
10. Singer, S. and Litwack, G., Cancer Res., 31, 1364 (1971).
11. Blecher, M. and Hunt, N., J. Biol. Chem., 247, 7479 (1972).
12. Robison, G.A., Butcher, R.W. and Sutherland, E.W., Cyclic AMP, Academic Press, New York, 1971, p. 101.