Bull. Fac. Agr., Saga Univ. (佐賀大農彙) No. 64 : 21～26 (1988)

Effects of the Cytosol Fraction and ATP on cAMP Binding to Erythrocyte Membranes : Association and Dissociation Kinetics

Takuji TSUKAMOTO and Yutaka YAMAKAWA
(Laboratory of Biological Chemistry)
Received September 22, 1987

Summary

Regulation of cAMP binding to human erythrocyte membranes by ATP and cytosol was characterized through measurements of the kinetic association and dissociation constants (k_1 and k_{-1}).

The cytosol fraction of human erythrocytes as well as 1 mM ATP decreased the rate constant (k_1) of association of [^{3}H]cAMP with human erythrocyte membranes. However, the effect of 1 mM ATP on the association kinetics was much greater than that of the cytosol fraction.

On the other hand, the cytosol fraction and 1 mM ATP increased the rate constant (k_{-1}) of dissociation of bound [^{3}H]cAMP from the human erythrocyte membranes. However, the cytosol fraction affected the dissociation kinetics to a far greater extent than 1 mM ATP. Thus, although both cytosol and 1 mM ATP decreased the affinity of [^{3}H]-cAMP for binding sites on human erythrocyte membranes at equilibrium, the detailed mode of their action on the membrane [^{3}H]cAMP binding sites appears to be different.

Introduction

Binding of adenosine cyclic 3',5'-monophosphate (cAMP) to membrane receptors is important in regulation of cell functions. With respect to membrane cAMP binding sites in human erythrocytes, intracellular binding of cAMP to the inner surface of membranes was shown to be much less than its binding to isolated membranes where most of the cytosol constituents were removed[6]. Further, equilibrium binding studies revealed that intracellular cAMP binding to membrane appeared to be mainly regulated by adenosine 5'-triphosphate (ATP)[7]. However, it is apparent that ATP regulation (inhibition) of cAMP binding to membranes observed through equilibrium binding experiments does not accurately reflect the effect of ATP on the kinetics of the cAMP binding process. Therefore, ATP as well as cytosol regulation of cAMP binding to human erythrocyte membranes was characterized through measurements of the kinetic association and dissociation constants (k_1 and k_{-1}).

Materials and Methods

Materials. cAMP, ATP and bovine serum albumin were obtained from Schwartz-Mann Co. and Sigma Chemical Co. [^{3}H]cAMP was purchased from Amersham Co. Other chemicals were of reagent grade.

Preparation of erythrocytes and erythrocyte membranes. Blood from young healthy male donors (19-31 years old) was collected directly into heparinized vacutainer tubes. The blood was centrifuged at 4°C and washed five times with 10 mM Tris buffer containing 150 mM NaCl (pH 7.8). Each erythrocyte preparation was monitored with a cell count of erythrocytes, reticulocytes and leukocytes including a leukocyte differential count.

The residual leukocyte count in the erythrocyte preparations was $0.01\pm0.002\%$, with $0.066\pm0.021\%$ reticulocytes. Membranes were prepared from washed erythrocytes essentially by a modification[2] of the method of Dodge *et al.*[1].

[^{3}H]cAMP binding assay. The [^{3}H]cAMP binding assay using isolated human erythrocyte membranes was performed essentially by the method of Rubin *et al.*[4]. The assay mixture (0.1 ml) contained 40 mM potassium phosphate buffer (pH 7.0), 8 mM $MgSO_4$, 50 nM [^{3}H]cAMP (10,000-13,000 cpm/pmol), and 33-40 μg of membrane protein. Association kinetics; after incubation for various times at 30°C, 2.0 ml of ice-cold 20 mM potassium phosphate buffer (pH 6.0) was added to the reaction mixture. The diluted reaction mixture was filtered through a 24-mm cellulose ester (Millipore) filter with a 0.45-μm pore size. The filters were washed with 10 ml of ice-cold 20 mM potassium phosphate buffer (pH 6.0) and dried in scintillation vials. The membrane bound [^{3}H]cAMP in the filters was measured as previously described[7]. Nonspecific binding was defined as those counts not displaced by excess amounts of nonradioactive cAMP (10 μM) and was subtracted from the total [^{3}H]cAMP binding to obtain the specific binding. Dissociation kinetics; after the binding of [^{3}H]cAMP to human erythrocyte membranes reached equilibrium through 50 min incubation at 30°C, the reaction mixture was diluted 100-fold into 40 mM potassium phosphate buffer containing 8 mM $MgSO_4$ (pH 7.0) and subsequently incubated for various times at 30°C. The residual membrane bound [^{3}H]cAMP was determined as described for association kinetics. Although the speed of the agitation of the water bath used for the previous [^{3}H]cAMP binding studies[7] was set at 120 cycles per minute, it was raised to 150 cycles per minute in this study to ensure sufficient mixing of the reaction mixture during the rapid phase of association and dissociation. The kinetic parameters (k_1 and k_{-1}) were estimated according to a previously described method[5].

Chemical analysis. Protein concentration was determined by the method of Lowry *et al.*[3]. The non-hemoglobin protein was determined as described previously[7].

Results and Discussion

The rates of association and dissociation of [^{3}H]cAMP binding to isolated human erythrocyte membranes are shown in Figs. 1 and 2. The kinetic parameters (k_1 and k_{-1}) obtained from the experimental data in Figs. 1 and 2 are shown in Table I.

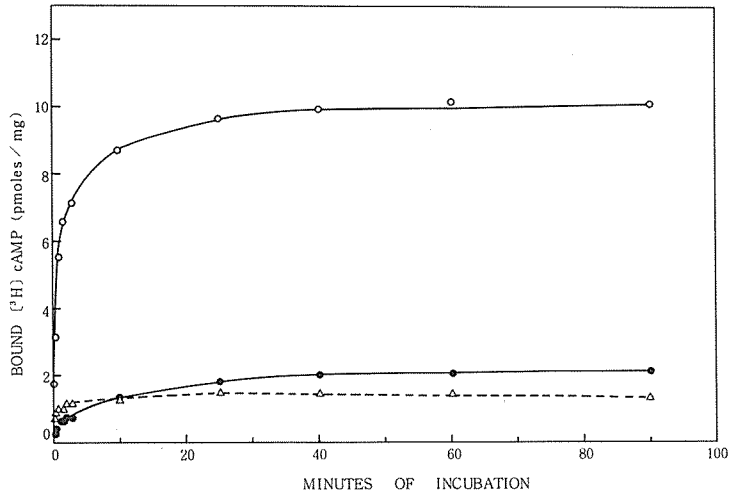

Fig. 1. Rate of association of [^{3}H]cAMP with human erythrocyte membranes. ○, No additives (control); ●, plus 1 mM ATP; △, plus cytosol fraction (4 mg protein/0.1 ml reaction mixture).

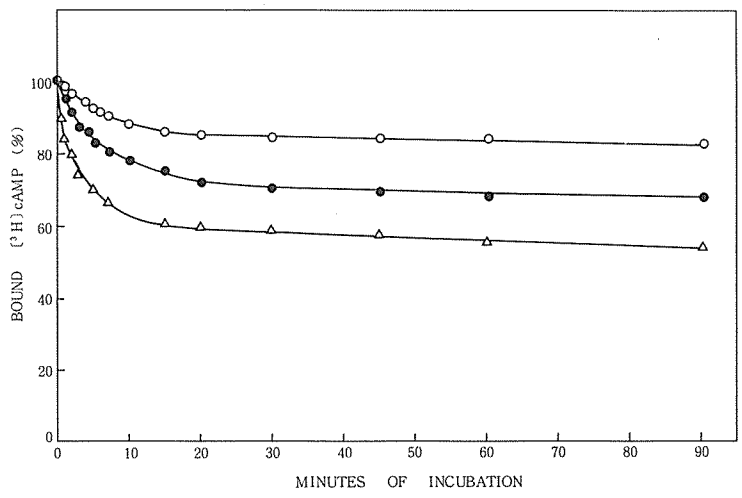

Fig. 2. Rate of dissociation of [^{3}H]cAMP bound to human erythrocyte membranes.
○, no additives (control); ●, plus 1 mM ATP; △, plus cytosol fraction (4 mg protein/0.1 ml reaction mixture).

Table I. Kinetic Parameters Derived from Data in Figs. 1 and 2.

Assay conditions	k_1 (M^{-1}, min^{-1})	k_{-1} (min^{-1})
No additives	1.78×10^{7}	1.82×10^{-2}
+Erythrocyte hemolyzates (cytosol fraction)	6.45×10^{6}	1.16×10^{-1}
+1 mM ATP	2.91×10^{6}	3.85×10^{-2}

Although the dissociation curve (Fig. 2) suggests the presence of multiple classes of [^{3}H]cAMP binding sites (rapidly and slowly dissociating binding sites) in human erythrocyte membranes, only k_{-1} was determined because the rate of dissociation of [^{3}H]cAMP from the slowly dissociating binding sites was too slow to analyze without computer programs. In addition, neither ATP nor cytosol appeared to have a significant influence on the rate of dissociation of [^{3}H]cAMP from the slowly dissociating binding sites (Fig. 2).

As shown in Fig. 1, at equilibrium of [^{3}H]cAMP binding, in the presence of the cytosol fraction or 1 mM ATP, the [^{3}H]cAMP binding to human erythrocyte membranes was only 14.2% or 19% of the control binding.

As shown in Table I, the cytosol fraction as well as 1 mM ATP decreased the rate of association of [^{3}H]cAMP with membranes and increased the rate of dissociation of bound [^{3}H]cAMP from the membranes. However, the effect of 1 mM ATP on the association kinetics was much greater than that of the cytosol fraction. On the other hand, the cytosol fraction affected the dissociation kinetics to a far greater extent compared to 1 mM ATP.

The apparent kd for high affinity [^{3}H]cAMP binding sites calculated from k_1 and k_{-1} values for the control binding, binding in the presence of the cytosol fraction and binding in the presence of 1 mM ATP were 1.02 nM, 18 nM, and 13.2 nM, respectively. Therefore, both cytosol and 1 mM ATP decreased the affinity of [^{3}H]cAMP for high affinity binding sites on human erythrocyte membranes, though the detailed mode of their action on the membrane [^{3}H]cAMP binding sites appears to be quite different. The results obtained in this study suggest the existence of other factors which contribute to the regulation of intracellular membrane cAMP binding in human erythrocytes.

Acknowledgements

The authors gratefully acknowledge the kind cooperation of Dr. Yoshifumi Harada, Ms. Miyako Shimaya and Ms. Hisako Nagabuchi of the Health Care Center for drawing blood from donors. We are grateful to Ms. Tae Kan of the National Hospital of Saga for her valuable suggestions in leukocyte and reticulocyte countings.

We are very grateful to Dr. Hidetoshi Kishikawa and Dr. Yutaka Takagi for their kind cooperation in our study which was performed in the Laboratory of Radioactive Isotopes.

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan and the Mishima Kaiun Memorial Foundation of Japan.

References

1) Dodge, J.T., C.D. Mitchell and D.J. Hanahan (1963). *Arch. Biochem. Biophys.*, **100**, 119.
2) Hanahan, D.J. and J.E. Ekholm (1974). *Methods in Enzymol.*, **31**, 168.
3) Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall (1951). *J. Biol. Chem.*, **193**, 265.
4) Rubin, C.S., J. Erlichman and O.M. Rosen (1972). *J. Biol. Chem.*, **247**, 6135.
5) Salvaterra, P.M. and H.R. Mahler (1976). *J. Biol. Chem.*, **251**, 6327.
6) Tsukamoto, T., K. Suyama, P. Germann and M. Sonenberg (1980). *Biochemistry*, **19**, 918.
7) Tsukamoto, T., Y. Yamakawa and T. Inaba (1987). *Agric. Biol. Chem.*, **51**, 1033.

赤血球膜へのcAMP結合に及ぼす細胞質画分およびATPの影響：会合解離の反応速度論

塚本　卓治・山川　　裕
（生物化学研究室）
昭和62年9月22日受理

摘　　要

ヒト赤血球膜と[^{3}H]cAMPの会合解離過程に及ぼすヒト赤血球内容物（細胞質画分）とATPの影響を調べた．細胞質画分またはATP（1 mM）の存在下，[^{3}H]cAMPと膜の会合の速度定数（k_1）の値は低下したが，ATPの方がより効果的な会合過程の阻害剤であった．一方，細胞質画分またはATPの存在下，[^{3}H]cAMP･膜複合体の解離速度定数（k_{-1}）の値は上昇したが，細胞質画分の方が複合体の解離をより著しく促進した．

すなわち，細胞質画分およびATPは共に，平衡状態における膜の[^{3}H]cAMPに対する親和性を低下させるが，その結合部位への作用の様式は異なることが推察される．