EFFECT OF N^6, 2'-O-DIBUTYRYL CYCLIC AMP UPON THE INTERCONVERTIBLE FORMS OF CYCLIC AMP PHOSPHODIESTERASE FROM HUMAN PLATELETS

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SUMMARY - The influence of dibutyryl cyclic AMP upon the equilibrium existing between three interconvertible forms - 2.5 S, 4.8 S and 7 S - of cyclic AMP phosphodiesterase from human platelets was investigated. It shifted the equilibrium towards the lighter form. It also exerted a protective effect against the thermo-inactivation of the enzyme. It is suggested that the analogue-induced equilibrium shift towards the dissociated high Km form of the phosphodiesterase might reflect a regulatory mechanism occurring also with natural cyclic nucleotides.

INTRODUCTION - Human platelets possess a high 3':5'-cyclic AMP (cAMP) phosphodiesterase activity, and some of its characteristics have already been defined (1,2). In a previous report (3), we have shown that after sucrose density gradient sedimentation of a 33,000 g supernatant, three peaks - respectively 2.5 S, 4.8 S and 7 S - with cAMP phosphodiesterase activity were obtained. They correspond to interconvertible molecular species forming an association-dissociation equilibrium depending on the concentration of enzyme (3). *In vitro* the dissociated form, which is predominant at low enzyme concentration, possesses a lower "affinity" for cAMP (Km : 3-5. 10^{-4} M) and a higher specific activity. At high enzyme concentration, the dissociated form is in equilibrium with associated forms whose "affinity" for cAMP is greater (Km : 3-5. 10^{-5}M) and whose specific activity is lower. In this latter condition both Km's are observed (3).

It was tempting to assume that such a transconformational equilibrium also takes place *in vivo* and might represent a mechanism of regulation. Any ligand modifying the equilibrium would

induce modifications of the phosphodiesterase kinetics. On the other hand it would be extremely useful to block the interconversion, or even to completely shift the equilibrium towards the fully associated or dissociated form so as to deal only with one single species for subsequent studies.

We therefore investigated the influence of N^6, 2'-O-dibutyryl cyclic AMP (dibutyryl cAMP), a non metabolised analogue of cAMP, as well as the effect of heat and ionic strength.

EXPERIMENTAL - Human platelets were prepared by combining several outdated platelet enriched plasma units from blood donors (Centre National de Transfusion Sanguine, Paris). Contaminating erythrocytes and leukocytes were removed by sequential centrifugation steps, ending with a pure pellet of platelets which was stored at -20°C. As previously described (2), platelet extracts were prepared in 3 vol. of cold 50 mM Tris-Hcl buffer, pH 7.4, containing 2 mM Mg SO4, and the 33,000 g supernatant was used for all subsequent studies. The cAMP phosphodiesterase activity was measured by the method of Thompson and Appleman (4) at a saturating level of 2mM cAMP. Sedimentation in sucrose density gradient was carried out as described in the legend to fig.2.

RESULTS AND DISCUSSION - When the enzyme activity was plotted against the enzyme concentration in the assay, we obtained, as previously reported (3), a triphasic curve which is due to a concentration dependent aggregation equilibrium (fig.1). If dibutyryl cAMP, an analogue of cAMP which is not degraded by phosphodiesterase (5), is added to the platelet extract at a final concentration of 1mM, the curve is shifted to the right, and the plateau is prolonged to 600 µg protein per ml instead of 260 µg protein per ml in the absence of the analogue. This suggests that dibutyryl cAMP prevents to some extent the aggregation of the enzyme.

This was further demonstrated by sucrose density gradient sedimentation. In standard conditions (fig. 2), as already reported (3), three peaks of cAMP phosphodiesterase were obtained, with S values compatible with mol. wt ratios of 1 : 2 : 4 . Any of these peaks generated the others after reconcentration followed by resedimentation in sucrose gradient (3). When 1 mM dibutyryl cAMP was incorporated in the sucrose density gradient, the distribution pattern of phosphodiesterase was shifted towards the lighter peak

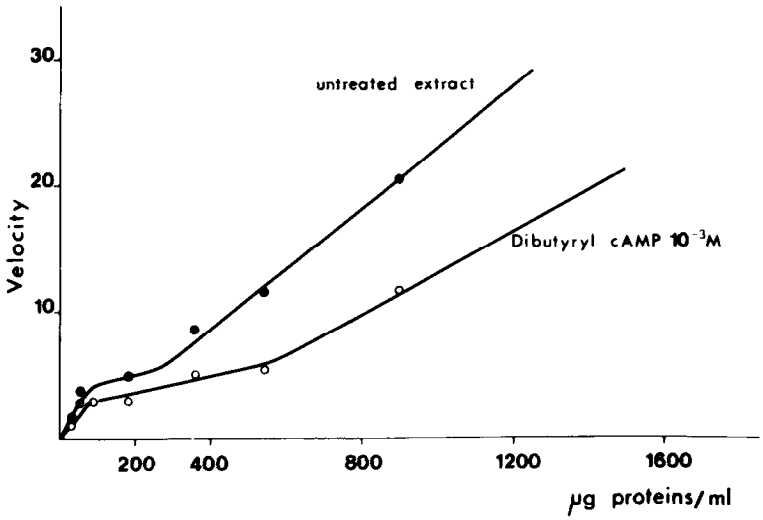

Fig. 1 Influence of dibutyryl cAMP upon the enzyme concentration dependance of cAMP phosphodiesterase activity (assayed with 2 m M cAMP, in a final volume of 0.5 ml).

●——●——● : no dibutyryl cAMP added

o——o——o : 1 mM dibutyryl cAMP in the extract and in the assay.

The inhibitory effect of dibutyryl cAMP was more pronounced at high enzyme concentration (50 %) than at low enzyme concentration (25 %)

(fig. 2). This finding confirms our foregoing conclusion that dibutyryl cAMP did influence the interconversion equilibrium by favoring the dissociated form of the enzyme.

The dibutyryl derivative also had a protective effect on the thermo-inactivation of the phosphodiesterase. When a platelet extract was heated at 51°C in the presence of 1 mM dibutyryl cAMP, no loss of phosphodiesterase activity occurred for the first 20 min, although in the absence of the analogue there was a 50 % decrease of enzyme activity (fig. 3). This finding, combined with the dibutyryl cAMP induced shift of the equilibrium towards the dissociated form (fig. 2), suggests that this latter form might be more thermostable.

CONCLUSION - The effects of dibutyryl cAMP are of great significance because this compound had been found to be a non-competitive

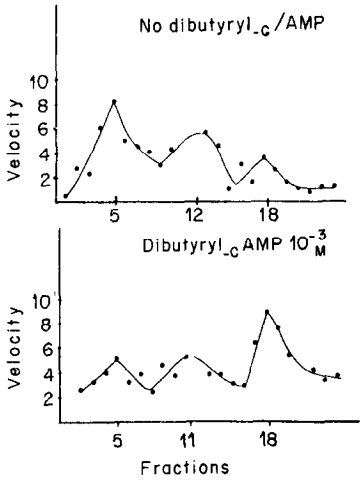

Fig. 2 Effect of dibutyryl cAMP upon the sedimentation pattern of platelet cAMP phosphodiesterase in a sucrose density gradient.
0.2 ml of platelet extract containing 1 mg protein was layered onto 5 ml tubes containing a linear (5 to 20 %) sucrose density gradient made in the extraction medium. After centrifugation at 38,000 rpm in a Spinco S W 39 rotor, the bottom of the tubes was punctured, and 0.2 ml fractions were collected and assayed for cAMP phosphodiesterase (4) with 2 mM unlabelled cAMP and 20 nM (8 -^{3}H) cAMP (ca. 200,000 cpm in 0.5 ml). S values were calculated according to the method of Martin and Ames (6).
When present, dibutyryl cAMP was 1 mmolar in the sucrose gradient.

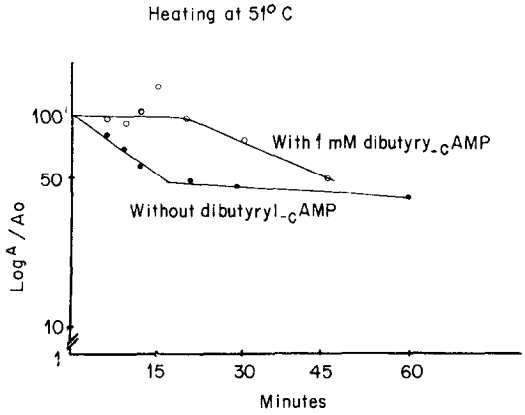

Fig. 3 Effect of heating at 51° C a platelet extract containing 12 mg prot/ml.

●—●—● : in the absence of dibutyryl cAMP

o—o—o : in the presence of 1 mM dibutyryl cAMP

No dibutyryl cAMP was added in the test tube during the assay.

inhibitor of phosphodiesterase at high substrate concentration, and to be uncompetitive at low substrate concentration (personal unpublished data). This suggests the existence on the enzyme of a binding, and possibly regulatory site distinct from the catalytic site.

We demonstrate here that the binding of this substrate analogue induces drastic changes in the enzyme transconformational equilibrium leading to the predominance of a dissociated form of the enzyme (2.5 S) with lower "affinity" for its substrate. That this analogue induced equilibrium shift represents the molecular basis for the apparent "negative cooperativity" kinetics of phosphodiesterase (7) is a likely possibility. Artificial ligands, such as pharmacological effectors, might also exert a regulatory effect by modifying the equilibrium between the interconvertible forms.

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