Biochimica et Biophysica Acta, 321 (1973) 165–170

BBA 66988

STIMULATION OF PURIFIED MUSCLE PROTEIN KINASE BY CYCLIC AMP AND ITS BUTYRATED DERIVATIVES

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(Received May 22nd, 1973)

SUMMARY

1. N^6,$O^{2'}$-Dibutyryl cyclic AMP, N^6-monobutyryl cyclic AMP and $O^{2'}$-monobutyryl cyclic AMP are able to activate to the same maximal velocity purified muscle cyclic AMP-dependent protein kinase. The concentrations required for this maximal stimulation, however, are widely different, the most effective being the N^6-monobutyryl derivative.

2. The relatively low K_a for N^6-monobutyryl cyclic AMP ($2 \cdot 10^{-7}$–$3 \cdot 10^{-7}$ M), (K_a for cyclic AMP $8 \cdot 10^{-8}$ M), the observed similarity with cyclic AMP in its ability to dissociate the protein kinase, and the reported formation of N^6-monobutyryl cyclic AMP from dibutyryl cyclic AMP in many cells strongly suggests a direct effect of the N^6 derivative on the protein kinase, not involving formation of free cyclic AMP or inhibition of phosphodiesterase.

INTRODUCTION

In studies of the effects of cyclic AMP on physiological processes[1] or cell behavior[2,3], the butyrated derivatives[4] are often utilized in place of the parent compound. The presumed reason is that cell membranes are more permeable to the butyrated compounds than to cyclic AMP and that the derivatives are less affected by cellular phosphodiesterases[1,4]. The experimental bases for these statements have not been extensively explored until very recently[5,6]. It has been reported that dibutyryl cyclic AMP is an inhibitor of cyclic AMP phosphodiesterase in some cells[7,8] but not in other cells[5,9], a difference which may lie with the type of phosphodiesterase[10]. Even where inhibition of phosphodiesterase is significant, however, the intracellular levels of dibutyryl cyclic AMP required are in the millimolar range[7]. Therefore, there is some question as to whether the biological effects of dibutyryl cyclic AMP can be ascribed entirely to the increase in intracellular cyclic AMP caused by inhibition of phosphodiesterase or a direct effect of dibutyryl cyclic AMP itself[11]. Furthermore, in some cells, cyclic AMP does not accumulate even though dibutyryl cyclic AMP is present in the cells[5]. Finally, some cells possess intracellular deacylases

which convert the dibutyryl cyclic AMP to the N^6-monobutyryl derivative[1,4,12–15], while others do not[7,16]. Thus, the mechanism of action of dibutyryl cyclic AMP will depend upon which derivatives are present in cells and how they affect the relevant enzymes.

If, in fact, the butyrated derivatives act like cyclic AMP in the cell, which is clearly suggested by their effects on phosphorylase activation[12] and glycogen metabolism[17], they should interact with and stimulate the protein kinases[1]. N^6-Butyryl cyclic AMP will bind the protein kinase in the assay of Gilman[18] for cyclic AMP, whereas $O^{2'}$-monobutyryl cyclic AMP and dibutyryl cyclic AMP will not[19], but the relationship of the binding to the enzymatic activity can only be interpreted by a direct study of the enzyme activity itself. To test enzymatic activity we have studied the effects of dibutyryl cyclic AMP and the two derivatives on the purified protein kinase of rabbit muscle. We will show that all three compounds stimulate protein kinase activity, but with very different efficiencies. N^6-Butyryl cyclic AMP is by far the most potent of the cyclic AMP derivatives and approaches cyclic AMP itself in its ability to stimulate protein kinase. In addition, it promotes dissociation of the kinase when analyzed on sucrose density gradients[20].

MATERIALS

[γ-^{32}P]ATP was prepared by the method of Post and Sen[21]. Specific activity was of the order of $2 \cdot 10^8$–$15 \cdot 10^8$ cpm/μmole after dilution with unlabelled ATP.

Disodium ATP, calf thymus arginine-rich histone (Type IV), cyclic AMP, dibutyryl cyclic AMP, N^6-butyryl cyclic AMP and $O^{2'}$-butyryl cyclic AMP were purchased from the Sigma Chemical Co. Beef heart phosphodiesterase was obtained from both Sigma Chemical Co., and Boehringer-Mannheim Corp.

METHODS

Rabbit muscle protein kinase in its cyclic AMP-dependent form was purified according to a method slightly modified from that of Schlender *et al.*[22]. The activity of the enzyme was 15–20-fold stimulated by added cyclic AMP. Protein kinase activity was measured by a modification of the method of Tao *et al.*[20]. The assay mixture contained 1 mM [^{32}P]ATP, 4 mg/ml histone, 18 mM $MgCl_2$, 50 mM Tris–HCl, pH 7.8, and cyclic AMP or cyclic AMP derivatives in a total volume of 50 μl to which 40 μl of enzyme in 50 mM Tris, 5 mM EDTA, pH 7.8 (spec. act. 6.8 nmoles ^{32}P incorporated/mg protein per 10 min) was added. After 10 min at 30 °C, samples of 75 μl were removed, spotted on filter paper squares (2 cm × 2 cm) and the protein precipitated on the paper by immersion in 10% trichloroacetic acid. After 5 min of washing (magnetic stirrer), the papers were further washed twice, 5 min each, with 5% trichloroacetic acid, 20 min with hot 5% trichloroacetic acid (90 °C), 5 min with cold 5% trichloroacetic acid, 5 min with 50% ethanol–50% ether and finally 5 min with ether. The papers were dried under a heat lamp and radioactivity incorporated in protein measured in a scintillation counter, using 0.5% PPO in toluene as the scintillation mixture.

The butyrated compounds were stored at −80 °C in solid form and in the initial experiments solutions were prepared without further purification immediately

prior to use. However, it was soon found that the monobutyrated derivatives were contaminated by cyclic AMP. In contrast, neither cyclic AMP nor monobutyrated derivatives were found to be present in our samples of dibutyryl cyclic AMP. Samples were analyzed on polyethyleneimine-cellulose thin-layer plates (Brinkmann Co.), using any one of three solvents: isobutanol–ammonia–water (7:2:1, by vol.), LiCl[5] or 0.85 M phosphate at pH 3.5. The latter two solvents allow a clear separation of N^6-butyryl cyclic AMP from $O^{2'}$-butyryl cyclic AMP as well as from dibutyryl cyclic AMP and cyclic AMP. By a suitable dilution series, it was estimated that N^6-butyryl cyclic AMP contained about 5% cyclic AMP and $O^{2'}$-butyryl cyclic AMP less than 1% cyclic AMP. Cyclic AMP was removed from the butyrated derivatives by short incubations with beef heart phosphodiesterase since the derivatives, but not cyclic AMP, have been reported to be resistant to enzymatic degradation[4,9,11,12]. The removal of cyclic AMP was verified by thin-layer chromatography. While N^6-butyryl cyclic AMP was not affected by phosphodiesterase during short incubations, some breakdown of the $O^{2'}$ derivative, presumably to monobutyryl 5'-AMP, was observed.

RESULTS

Activation of protein kinase

The effects of dibutyryl cyclic AMP, N^6-butyryl cyclic AMP, $O^{2'}$-butyryl cyclic AMP, and cyclic AMP on the activity of purified muscle protein kinase were compared. As shown in Fig. 1, the enzyme was activated by all analogues to the same maximum velocity but quite different concentrations were required. The N^6 derivative was the most potent and close to cyclic AMP, while the $O^{2'}$-butyryl and the dibutyryl forms required higher concentrations and were close to each other. The K_a of activation for the butyryl analogues was next determined using the butyryl derivatives directly without further purification as well as after phosphodiesterase pretreatment to

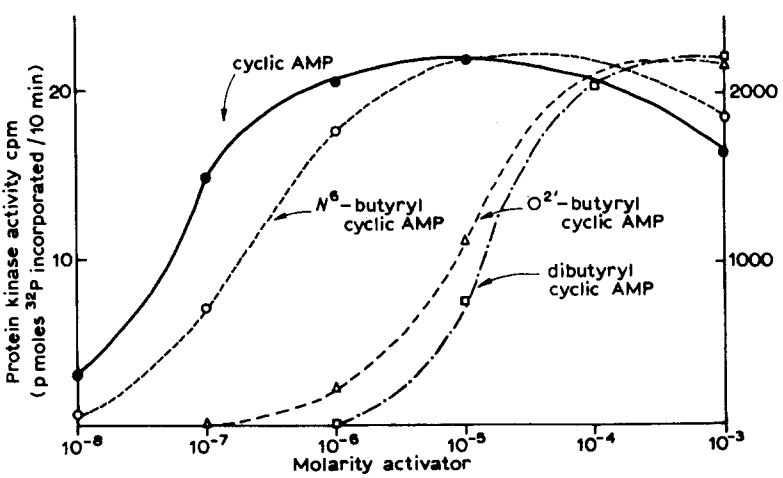

Fig. 1. Activation of purified rabbit skeletal muscle protein kinase by cyclic AMP and butyryl derivatives. Assay as described in Methods. In common with cyclic AMP and other cyclic nucleotides with low K_a for protein kinase, high concentrations of N^6-butyryl cyclic AMP appear to result in partial inhibition of the enzyme.

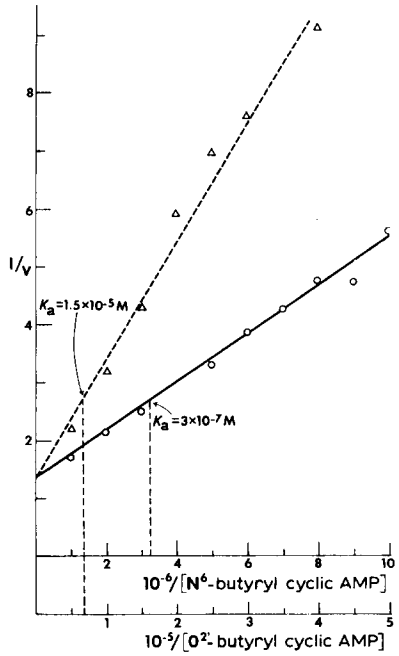

Fig. 2. Lineweaver–Burk representation of the activation of protein kinase by N^6-butyryl cyclic AMP and $O^{2'}$-butyryl cyclic AMP after purification as described by text.

remove contaminating cyclic AMP (Fig. 2). While enzymatic treatment had little effect on the K_a for N^6-butyryl cyclic AMP ($2 \cdot 10^{-7}$–$3 \cdot 10^{-7}$ M), it increased that of $O^{2'}$-butyryl cyclic AMP from $5 \cdot 10^{-6}$ M to $1.5 \cdot 10^{-5}$ M. Since no cyclic AMP was detected in the phosphodiesterase-treated $O^{2'}$-butyryl cyclic AMP solutions the stimulation obtained must be due to the compound itself. However, as some breakdown of $O^{2'}$-butyryl cyclic AMP probably occurred during treatment with phosphodiesterase, the exact concentration is not known and the correct K_a value is probably lower than the value reported. No further work was attempted with the $O^{2'}$ derivative since N^6-butyryl cyclic AMP appeared to be the more potent of the two monobutyryl derivatives and since it appears to be the compound that accumulates in cells possessing an intracellular esterase[5,13–15,19]. The activation by dibutyryl cyclic AMP required higher concentrations (K_a approx. 10^{-5} M).

Dissociation of the protein kinase

In order to determine whether the mode of action of the butyryl derivatives is the same as that of cyclic AMP, that is by dissociation of the enzyme into subunits[20], the following experiments were performed. The protein kinase was incubated in the presence of 1 μM cyclic AMP or 1 μM N^6-butyryl AMP and applied to sucrose density gradients (10–25%) containing the same concentrations of these activators. Controls were run without addition of activators. The samples were analyzed after centrifugation for kinase activity. Fig. 3 shows that both cyclic AMP and N^6-butyryl cyclic AMP caused dissociation of the enzyme into smaller subunits[20].

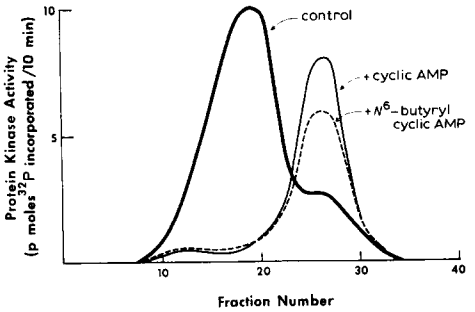

Fig. 3. Sucrose density gradient centrifugation of protein kinase in the presence of buffer (50 mM Tris–HCl, 5 mM EDTA, pH 7.8) alone, or with the addition of 10^{-6} M cyclic AMP or 10^{-6} M N^6-butyryl cyclic AMP. Centrifugation at 100 000 × *g* for 18 h. Activity assayed as described in Methods in the presence of cyclic AMP.

DISCUSSION

The mechanism of action of the butyrated derivatives of cyclic AMP has come under discussion recently and, as will appear, may be different depending upon the tissue investigated. In bone[7] and in intact adipocytes[16], conversion of dibutyryl cyclic AMP to monobutyryl cyclic AMP or cyclic AMP itself has not been detected and it is assumed that the effects of dibutyryl cyclic AMP are due to the compound itself and not to an intracellular metabolic product. The mode of action of the compound suggested by Heersche *et al.*[7] is solely through its ability to inhibit the phosphodiesterase and therefore to cause an increase in intracellular levels of cyclic AMP. While the data reported[7] could support this hypothesis, they are not sufficient to rule out a direct contribution due to stimulation of protein kinase by dibutyryl cyclic AMP itself, a result suggested by prior work indicating such a stimulation of phosphorylase kinase[1], likely mediated by protein kinase. The present work supports this possibility since samples of dibutyryl cyclic AMP without detectable cyclic AMP or mono butyryl derivatives show stimulation of muscle protein kinase. However, as in the case of the phosphorylase kinase system[1], nearly three orders of magnitude higher concentrations of the dibutyryl AMP are required as compared with cyclic AMP itself. In some cells, therefore, in which dibutyryl cyclic AMP is not converted to its monobutyryl form, the compound may act either by direct stimulation of protein kinase or in addition, by inhibition of phosphodiesterase activity. In other cells, *e.g.* thyroid, where dibutyryl cyclic AMP is not an inhibitor of phosphodiesterase[5], some effect must be due to direct stimulation of the protein kinase by dibutyryl cyclic AMP, although the major effect is probably due to N^6-butyryl cyclic AMP which is formed within the cell[5,14].

Other cells, such as HeLa[12,19] and sea urchin eggs (Nath, J. and Rebhun, L. I., unpublished), however, possess such potent intracellular esterases (deacylases) that only N^6-butyryl cyclic AMP increases intracellularly in the presence of external dibutyryl cyclic AMP. The present work indicates that in such cells activity of dibutyryl cyclic AMP is most likely through stimulation of protein kinase by separation of regulatory and catalytic subunits.

The mode of action of externally applied dibutyryl cyclic AMP is, therefore,

likely to be complex depending upon several factors: (a) the presence of deacylases and their activity relative to the amount of dibutyryl cyclic AMP applied, (b) whether the phosphodiesterase in the cells is inhibited by dibutyryl cyclic AMP or N^6-butyryl cyclic AMP, (c) the rate of production of cyclic AMP by adenyl cyclase and (d) the degree to which the protein kinase may vary in its ability to react with dibutyryl cyclic AMP or N^6-butyryl cyclic AMP. In addition, we cannot infer that all the effects of dibutyryl cyclic AMP, or the monobutyryl cyclic AMP derivatives are through the cyclic AMP regulatory system. It is possible that each compound has other unique actions which may account for the opposite effects of cyclic AMP and dibutyryl cyclic AMP, *e.g.* as reported in adipocytes[24], or HeLa cells[25], or thyroid[5]. A comparative study of esterases for the $O^{2'}$-acyl group and of the relative effects of cyclic AMP and its derivatives on phosphodiesterase and protein kinase would be of considerable interest.

ACKNOWLEDGEMENTS

This work was supported by a grant to L. I. Rebhun from the Damon Runyon Cancer Fund and to Dr C. Villar-Palasi from the U.S. Public Health Service. We wish to thank Dr Jayasree Nath for running the phosphodiesterase and thin-layer techniques and Dr L. Huang for a critical reading of the manuscript.

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