Some Biochemical Properties of Alkyl Phosphotriesters of cyclic AMP

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SUMMARY

The biochemical properties of several alkyl phosphotriesters of cyclic AMP were studied with respect to their interactions with beef heart protein kinase and cyclic nucleotide phosphodiesterase. Ethyl and propyl triesters did not enhance the phosphorylation of histone by protein kinase and methyl, ethyl, propyl and butyl triesters were poor competitors for the cyclic AMP binding site of the enzyme. However, these alkyl phosphotriesters were effective inhibitors of cyclic nucleotide phosphodiesterase with the Ki's arrayed in the following order: methyl > ethyl > propyl > butyl > cetyl triester. Metabolic studies with mice indicated that intraperitoneal injection of low doses of propyl triester for one week significantly increased cyclic AMP concentration.

INTRODUCTION

Neutral alkyl phosphotriesters of cyclic AMP are potent inhibitors of tumor growth and may undergo degradation to cyclic AMP in biological systems (1, 2).

The chemical properties of these alkyl phosphotriesters indicate that they may be converted to cyclic AMP *in vivo*. Alkaline hydrolysis results in ring opening whereas nucleophilic attack yields cyclic AMP as the principal product (3). The biological activity of these triesters could be based on their interactions with enzymes associated with cyclic AMP metabolism directly as well as on the *in vivo* conversion to cyclic AMP itself. Here we present a study of the inhibition of cyclic nucleotide phosphodiesterase, the activation of protein kinase and the displacement of tritiated cyclic AMP from protein kinase with various triesters. The elevation of cyclic AMP *in vivo* was effected by administering triesters to outbred Swiss mice. These various experiments should be helpful in understanding and evaluating the biological effects of these new cyclic AMP derivatives.

MATERIALS AND METHODS

Methyl, ethyl, propyl, butyl and cetyl cyclic AMP alkyl phosphotriesters were synthesized and purified as previously described (3). Concentrations of the various triesters were determined spectrophotometrically using a molar extinction coefficient of 14,000 at 260 nm.

Cyclic nucleotide phosphodiesterase was purchased from Sigma Chemical Co. and activity was assayed by the method of Butcher and Sutherland (4)

using Bartlett's (5) method for the determination of inorganic phosphate following treatment with bacterial alkaline phosphatase (Sigma). Assays with cetyl triester were filtered through HA millipore filters after phosphatase treatment to remove insoluble cetyl triester which interfered with subsequent phosphate assays. Cetyl alcohol was used as a control and had no influence on either phosphodiesterase or phosphatase activity. Triesters had no effect on alkaline phosphatase activity. Enzyme activity was expressed as μmoles cyclic AMP hydrolyzed/min.

Protein kinase from beef heart was purified by the method of Kuo and Greengard (6). Enzyme activity was assayed by following the incorporation of ^{32}P from $\gamma[^{32}P]$ ATP (New England Nuclear, approximately 2 Ci/mmole) into mixed histone (Sigma) using the method of Kuo et al. (7).

Displacement of [^{3}H] cyclic AMP (Schwartz-Mann, 21 Ci/mmole) was done by Gilman's (8) method in a volume of 0.2 ml and saturating cyclic AMP concentrations of 40 nM.

The effects of ethyl and propyl triester on cyclic AMP metabolism in vivo were studied using outbred Swiss mice (average weight, 20 grams). Four groups of six mice each were injected intraperitoneally with saline, 1 mg cyclic AMP, 1 mg ethyl triester and 1 mg propyl triester per mouse respectively for six consecutive days and the mice were sacrificed 24 hours after the final injection. The liver was removed and a 250 mg sample frozen for cyclic AMP determination.

The frozen samples were homogenized in 4 ml of a solution of 0.03 M triethylammonium bicarbonate buffer pH 7.0 and ethanol 3:7, v/v, centrifuged at 17,000 g, and the supernatant collected and dried in a nitrogen stream. The residue was suspended in 4 ml of water, filtered through glass wool, and applied to a column (4 mm x 4 cm) of AG 1-X8 resin (formate). The column was washed with 5 ml of water followed by 5 ml of 0.3 M formic acid. Cyclic AMP was eluted with 10 ml of 1.0 M formic acid and lyophilized. The samples were dissolved in water and assayed using Gilman's method (8) as outlined above. Use of tritiated cyclic AMP in the extraction indicated a recovery of approximately 80%. The values reported are uncorrected. Measurement of cyclic AMP was confirmed by digestion of sample aliquots with cyclic nucleotide phosphodiesterase.

RESULTS AND DISCUSSION

Ethyl and propyl triesters did not enhance the phosphorylation of histone by beef heart protein kinase (Fig. 1), and methyl and butyl as well as ethyl and propyl triesters had low affinities for the enzyme. Preliminary experiments indicated no real pattern of affinity among the four triesters and the binding data were averaged and plotted as 1/pmoles [^{3}H] cyclic AMP bound as a function of added nucleotide. Comparison of the slopes of these plots (Fig. 1 and ref. 9) for the triesters and cyclic AMP indicates that beef heart protein kinase has a fifty-fold higher affinity for cyclic AMP than for the triesters. The linearity of the triester plot and the intersection at the ordinate indicates that triesters and cyclic AMP are competing for the same binding site (9).

Beef heart cyclic nucleotide phosphodiesterase showed heterogeneous behavior indicating the presence of a high and low Km enzyme (Fig. 2). The estimated Km for the low Km enzyme is approximately 4×10^{-5} M in agreement with values of others (Km = $3.6 - 3.9 \times 10^{-5}$ M, ref. 10) for this enzyme. All triesters inhibited this enzyme to differing degrees.

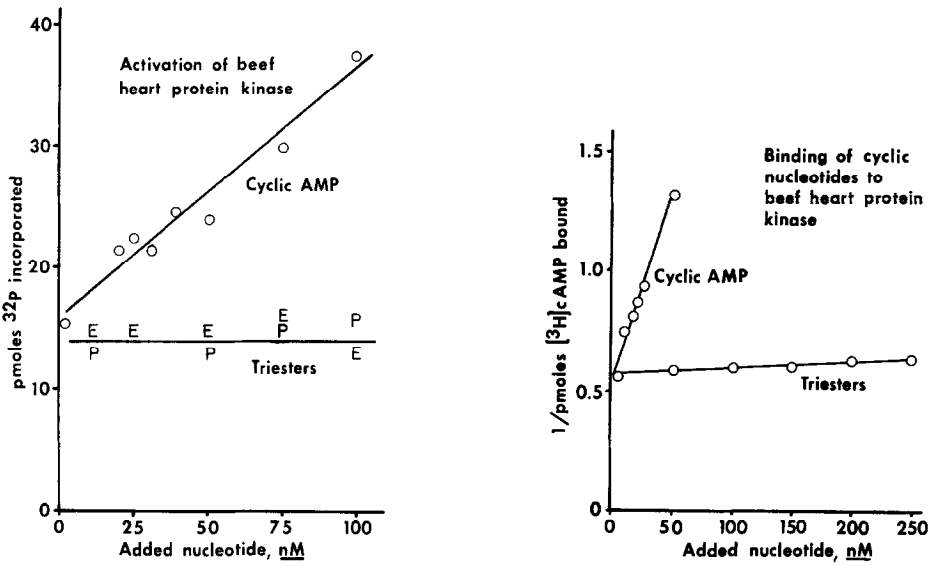

Figure 1. (Left) The phosphorylation of histone by protein kinase in the presence of cyclic AMP, ethyl (E) and propyl triester (P). (Right) The reciprocal of the amount of [^{3}H] cyclic AMP bound to protein kinase as a function of added nucleotide concentration. Values for methyl, ethyl, propyl and butyl triesters were averaged for the triester line. The ratio of the slopes: 0.015 (cyclic AMP) / 0.0003 (triester) = 50 is a measure of the relative affinity of protein kinase for cyclic AMP as compared to triester (9).

TABLE 1

The Inhibition of Cyclic Nucleotide Phosphodiesterase

With Alkyl Phosphotriesters of Cyclic AMP

Triester	Ki, M	Triester Concentration, M
Methyl	2.6×10^{-4}	5.3×10^{-4}
Ethyl	2.1×10^{-4}	7.7×10^{-4}
Propyl	1.5×10^{-4}	4.6×10^{-4}
Butyl	1.5×10^{-4}	4.8×10^{-4}
Cetyl	1.0×10^{-4}	1.5×10^{-4}

In general, the shorter aliphatic groups such as methyl and ethyl conferred a lesser inhibitory effect than the longer chain derivatives such as propyl, butyl and cetyl triesters. Theophylline yielded a Ki = 8.0×10^{-4} M. Like theophylline, the triesters were all competitive inhibitors of cyclic nucleotide phosphodiesterase. Ethyl triester was used in a high enough concentration to inhibit the high Km enzyme. This enzyme had a Km = 4.8×10^{-4} M and the measured Ki = 3.3×10^{-4} M for ethyl triester.

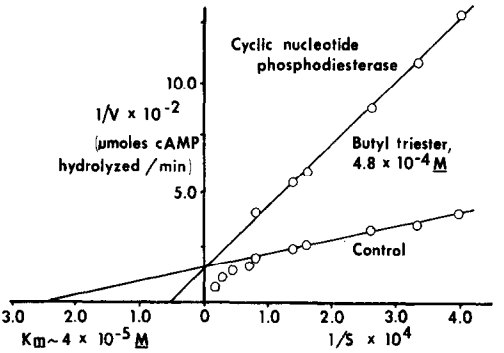

Figure 2. The inhibition of cyclic nucleotide phosphodiesterase with butyl phosphotriester.

TABLE 2

Cyclic AMP in Outbred Swiss Mouse Liver

Treated with Ethyl and Propyl Triester

Treatment	Cyclic AMP pmoles/mg wet weight
Saline	0.51 ± 0.21
Cyclic AMP	0.38 ± 0.13
Ethyl Triester	0.52 ± 0.24
Propyl Triester	1.12 ± 0.13

A comparison of the ability of two triesters to elevate cyclic AMP levels *in vivo* was performed using outbred Swiss mice. Treatment with propyl triester clearly elevated liver cyclic AMP concentration (Table 2). The variation in the data indicates that saline, cyclic AMP and ethyl triester treatment may all be equivalent.

Alkyl phosphotriesters of cyclic AMP have been shown to be more effective growth inhibitors than cyclic AMP (1, 2). It is unlikely that this biological activity is attributed to the direct interaction of these triesters with protein kinase, since they have a low affinity for binding and fail to enhance protein kinase activity. We have already proposed that the biological activity could be attributed to enhanced penetration of these neutral molecules followed by breakdown to cyclic AMP (3). The present results show that these triesters inhibit cyclic nucleotide phosphodiesterase activity and could increase tissue cyclic AMP concentrations via this inhibition. This latter effect may be responsible for the prolonged elevation of cyclic AMP by the propyl ester *in vivo*. The ethyl triester inhibits phosphodiesterase to a lesser extent and is more vulnerable to degradation to cyclic AMP. Short term rapid increases are obsèrved

with ethyl triester (2) and longer lasting effects are found with propyl triester. These observations further establish the utility of cyclic AMP triesters as superior exogenous sources of cyclic AMP which can be used with advantage for numerous *in vivo* studies.

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