EFFECT OF ADENOSINE NUCLEOLIPIDS ON THE ACTIVITY OF RABBIT SKELETAL MUSCLE PROTEIN KINASE

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Received April 3rd, 1980

The inhibitory effects of adenosine 5'-phosphate esters with lipoid hydroxy compounds on rabbit skeletal muscle protein kinase are in comparison with adenosine or adenosine 5'-monophosphate relatively weak. The inhibitory effect is in some cases preceded by the enzyme stimulation which can reach up to 70—80% of the stimulation by cyclic AMP. While the inhibitory effect seems to be caused by the adenosine moiety of the compound, the nature of the stimulatory effect is not yet elucidated.

In our studies following the effects of adenosine 5'-phosphate esters with hydroxy compounds (adenosine nucleolipids) on the activity of enzymes of cyclic AMP system we have found several new features of these compounds[1-3]. The most pronounced effect of adenosine nucleolipids is the inhibition of the activity of adenylate cyclase[1,2,4]. The activity of cyclic AMP phosphodiesterase was inhibited only moderately[1,2]. The effects of adenosine 5'-phosphate esters with hydroxy compounds on the activity of protein kinase were quite inconsistent, depending on the alcohol moiety[1,2,5].

The activity of protein kinase from rat liver and adipose tissue, basal as well as stimulated by cyclic AMP, was inhibited by ethyl ester of 5'-AMP while dihydroxypropyl ester of 5'-AMP revealed stimulation of basal enzyme activity which reached[5] about one half of the stimulatory effect of cyclic AMP. Some of the adenosine nucleolipids also revealed the stimulation of protein kinase from rat heart and adipose tissue and the inhibition of the enzyme by high drug concentrations[1,2].

EXPERIMENTAL

Materials

Adenosine 5'-[γ^{32}P]triphosphate, triethylammonium salt (50—100 Ci/mmol) was prepared in our laboratory[6] from carrier free $^{32}PO_4^{3-}$ without Cl^- (22[P 032]003) supplied by Isocommerz, Berlin, G.D.R. Sephadex G—100 was a product of Pharmacia Fine Chemicals AB. Sweden, and umbelliferone a product of Sigma, U.S.A. Cyclic AMP and other chemicals used were

supplied by Lachema, Czechoslovakia. Histone for the phosphorylation of protein kinase was prepared from the calf thymus[7,8].

Adenosine 5'-phosphate ethyl ester (E-AMP), 2,3-dihydroxypropyl ester (G-AMP), 2-palmitamidoethyl ester (PEA-AMP), 2-oleamidoethyl ester (OEA-AMP) and octadec-1-yl ester (C_{18}-AMP) (Table I) were prepared as described earlier[1,2,4,5].

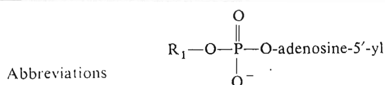
Protein Kinase Assay

Protein kinase preparation was isolated from the fresh rabbit skeletal muscle through the ammonium sulphate precipitation step[9]. Fraction 1 (0—25% ammonium sulphate saturation) contained the enzyme sensitive to cyclic AMP stimulation, fraction 2 (25—45% ammonium sulphate saturation) contained mostly the catalytic unit of protein kinase. To remove the rest of regulatory subunit from protein kinase, the fraction 2 was further purified[10] in the presence of 0·3 μM cyclic AMP on a Sephadex G—100 column (0·9 × 10 cm; the second peak eluted by 10 mM phosphate buffer with 10 mM EDTA pH 6·0) was not stimulated by cyclic AMP and was considered as a catalytic unit of protein kinase (fraction 3).

The activity of protein kinase was determined according to Miyamoto and coworkers[9] with slight modifications[5]. The standard incubation medium of total 100 μl contained: 50 mM phosphate buffer (pH 6·0), 6 mM Mg^{2+}, 0·5 mM EDTA, 5 mM sodium fluoride, 5 mM theophylline, 100 μg of histone, enzyme preparation corresponding to 20—30 μg of protein[11], 10 μM ATP (in kinetic studies increasing concentrations 10—100 μM ATP), and about 500 000 cpm of ^{32}P-γ-ATP. The stimulation of enzyme was carried out by 1 μM cyclic AMP. The reaction was started by the addition of ATP to the ice-cold mixtures and the samples were incubated at 30°C for 20 min. The reaction was stopped, and the phosphorylated histone precipitated by the addition of 3 ml of ice-cold 7·5% trichloracetic acid; 0·2 ml of 1% human albumin were added as histone carrier. In experiments in which the effects of adenosine nucleolipids were tested in the presence of albumin, the amount of histone carrier was lowered to obtain the same final

TABLE I

Chemical Structure and Abbreviations of Adenosine 5'-Phosphate Esters with Various Hydroxy-Compounds



Abbreviations	R_1
E-AMP	ethyl
G-AMP	2,3-dihydroxypropyl
PEA-AMP	2-palmitamidoethyl
OEA-AMP	2-oleamidoethyl
C_{18}-AMP	1-octadecyl

albumin concentration in all samples. After centrigugation, the precipitate was dissolved in 0·3 ml of 1M-NaOH. Precipitation and centrifugation (5 min at 2400 g) were carried out four times. The last precipitate was dissolved in 0·2 ml of 1M-NaOH and the radioactivity was transferred quantitatively from the centrifugation tubes into the scintillation vials. ^{32}P was measured by the Cerenkov's radiation in solution containing 30 mg of umbelliferone in 1 l of water.

All results are representative experiments carried out in triplicate or duplicate. They are expressed in absolute amount of ^{32}P incorporated into histone/mg protein/20 min incubation period. In kinetic studies the results are presented in a double-reciprocal way according to Lineweaver and Burk.

RESULTS

The effects of adenosine 5'-phosphate esters with various hydroxy-compounds were tested on the activity of partially purified rabbit skeletal muscle protein kinase. The effects of drugs were tested either on the holoenzyme (fraction 2), unstimulated or stimulated by 1 μM cyclic AMP, or on the catalytic unit of protein kinase (fraction 3). The aim of the work was to find the explanation for the inconsistent effects of various esters of AMP on the activity of protein kinase.

Data presented in Fig. 1 compare the inhibitory effects of E-AMP and G-AMP, with the inhibitory effects of adenosine and 5'-AMP on the fraction 2 of muscle

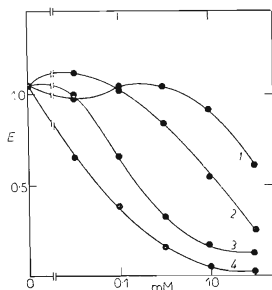


FIG. 1

Effect of G-AMP (1), E-AMP (2), Adenosine (3) and AMP (4) on Rabbit Skeletal Muscle Protein Kinase

The enzyme activity (E) of the fraction 2 (see Methods) was estimated in the presence of 1 μM cyclic AMP and is expressed as nmol for mg protein.

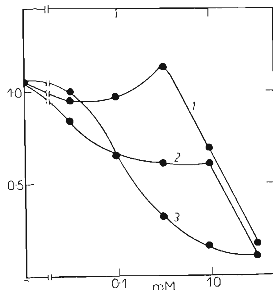


FIG. 2

Comparison of the Inhibitory Effects of PEA-AMP (1) and OEA-AMP (2) with the Effect of Adenosine (3) on Rabbit Skeletal Muscle Protein Kinase

Experimental conditions were identical as described in Fig. 1.

protein kinase stimulated by cyclic AMP. The enzyme inhibition caused by E-AMP and G-AMP were much weaker than the inhibitory effects of adenosine and its 5'-monophosphate. G-AMP was inhibitory only at concentrations above 1 mM. Fig. 2 shows inhibitory effects of two adenosine nucleolipids on the same preparation of protein kinase as described in Fig. 1. In comparison with adenosine the inhibitory effects of PEA-AMP were observed only when the concentrations above 0·3 mM were used. The inhibitory effects of OEA-AMP, which contain the unsaturated lipoid moiety, show quite unusual type of inhibition which was similar to that of PEA-AMP only at the highest drug concentration.

PEA-AMP and C_{18}-AMP revealed the inhibition of the catalytic unit of protein kinase. This inhibitory effect was preceded by the slight activation of the enzyme by lower drug concentrations (Fig. 3). This stimulatory effect of PEA-AMP was very pronounced on the unstimulated holoenzyme. While PEA-AMP stimulation reached 70–80% of the stimulation of 1 μM cyclic AMP, no stimulation was observed when OEA-AMP was tested (Fig. 4).

Data shown in Figs 5 and 6 quantitatively analyse the inhibitory effects of adenosine and PEA-AMP on the catalytic unit of muscle protein kinase. The inhibitory effects of adenosine in the range of 0·1 to 3·2 mM concentrations were purely competitive

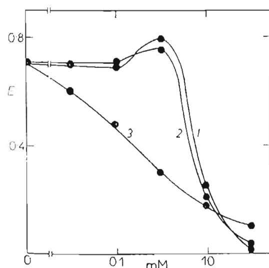


FIG. 3

Comparison of the Inhibitory Effects of C_{18}--AMP (1) and PEA-AMP (2) with the Effects of Adenosine (3) on the Rabbit Skeletal Muscle Protein Kinase

The effects of drugs (mM) were estimated on the activity of the catalytic unit of protein kinase, isolated on Sephadex G—100 (fraction 3).

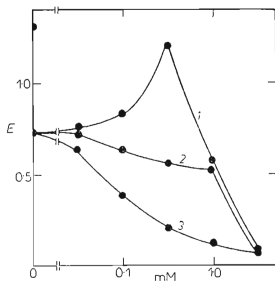


FIG. 4

Comparison of the Effects of PEA-AMP (1) and OEA-AMP (2) with the Effects of Adenosine (3) and 1 μM cyclic AMP (an upper circle) on the Rabbit Skeletal Muscle Protein Kinase

The effects of drugs (mM) were estimated on the fraction 2 of protein kinase unstimulated by cyclic AMP.

with respect to ATP (Fig. 5). Similar competitive type of inhibition was observed when PEA-AMP was tested in the narrow inhibitory concentrations from 0·59 to 1·30 mM (Fig. 6).

In the last experiment (Fig. 7) the inhibitory effects of PEA-AMP on the holoenzyme, stimulated by cyclic AMP, were tested without and in the presence of 0·3 and 1·0% of human albumin. The addition of albumin to the assay system shifted the inhibitory dose-response curve of PEA-AMP to the right, however, the steep shape of the inhibition by this nucleolipid remained unchanged by albumin addition.

DISCUSSION

In order to explain inconsistent effects of various esters of 5'-AMP on the activity of protein kinase[1,2,5] we have compared these effects with the well known inhibitory effects[9,12-14] of adenosine and 5'-AMP on the partially purified enzyme from the

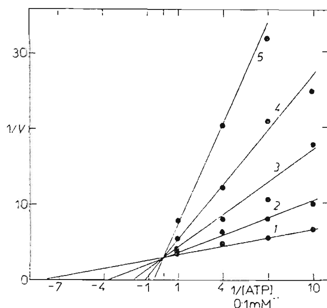


Fig. 5

Inhibitory Effect of Adenosine on the Activity of Rabbit Skeletal Muscle Protein Kinase Expressed in Double Reciprocal Plots

The effects of increasing concentrations of adenosine, 0 mM (*1*), 0·1 mM (*2*), 0·3 mM (*3*), 1·0 mM (*4*) and 3 mM (*5*), were estimated on the activity of the catalytic unit of muscle protein kinase (fraction 3). V, ^{32}P incorporated from ^{32}P-γ-ATP into histone expressed as μmol/mg protein/20 min of incubation.

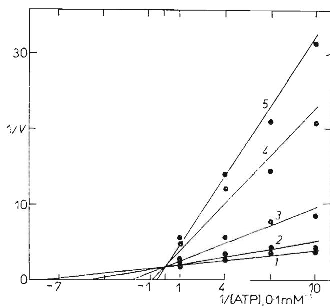


Fig. 6

Inhibitory Effect of PEA-AMP on the Activity of Rabbit Skeletal Muscle Protein Kinase Expressed in Double Reciprocal Plots

The effects of increasing concentrations of PEA-AMP, 0 mM (*1*), 0·59 mM (*2*), 0·77 mM (*3*), 1·0 mM (*4*), and 1·3 mM (*5*) were estimated on the activity of the catalytic unit of muscle protein kinase (fraction 3). V, ^{32}P incorporated into histone mol/mg protein/ /20 min of incubation.

rabbit skeletal muscle. Protein kinase activity, stimulated by cyclic AMP, was inhibited by esters of AMP much weaker than by adenosine and 5'-AMP and appeared only when higher concentrations (above 0·3 mM) of these drugs were used (Figs 1–4). The inhibition of the enzyme by adenosine nucleolipids was, however, nearly complete within one order of the drug concentration (Figs 2–4).

In the experiment using partially purified catalytic unit of protein kinase (fraction 3), the strong inhibitory effects of adenosine nucleolipids PEA-AMP and C_{18}-AMP followed after the slight enzyme activation by the lower drug concentrations (Fig. 3). This effect was much more pronounced in the experiment using the holoenzyme unstimulated by cyclic AMP. Here, the stimulatory effect of PEA-AMP reached 70–80% of the stimulatory effect of the natural protein kinase activator (Fig. 4). Also in this experiment, similarly to the experiment shown in Fig. 2, OEA-AMP (with the unsaturated lipoid moiety) did not show any stimulatory effect.

Thus, it can be summarized that adenosine 5'-phosphate esters revealed the inhibitory effects on protein kinase which depended on the alkyl moiety of the drug used and could be demonstrated only in a very narrow range of drug concentrations. The inhibitory effects of most nucleolipids follow after very inconsistent effects of these drugs and depend on the drug used and the condition of enzyme studied.

The nature of the inhibitory effects of adenosine nucleolipids could be estimated from the quantitative analysis of the inhibitory effects of adenosine nucleolipids (Fig. 6). High concentrations of these drugs showed similar competitive type of inhibition with respect to ATP on the catalytic unit of protein kinase as adenosine. These results suggest that the basis of the inhibitory effects of adenosine nucleolipids is the presence of adenosine moiety in their structure.

Data presented in Fig. 7 offer the explanation for the delayed start of the inhibitory effects on protein kinase at very high concentrations of adenosine nucleolipids. The shift of the dose-response curve to the right of the inhibitory concentrations

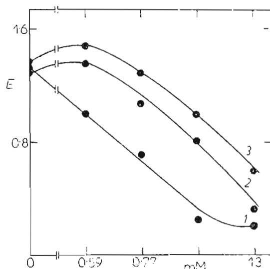


FIG. 7

Effect of Albumin on the Inhibitory Effect of PEA-AMP on Rabbit Skeletal Muscle Protein Kinase

The inhibitory effect of PEA-AMP were tested using enzyme fraction 2 stimulated by 1 μM cyclic AMP in the presence of 0 (*1*), 0·3 (*2*) or 1·0% (*3*) of human albumin added to the assay system. E, enzyme activity expressed as nmol per mg protein, mM, concentration of PEA-AMP.

of adenosine nucleolipids in the presence of human albumin added to the assay system, together with the preservation of the steep inhibitory curve, suggest that the binding of the lipoid moiety of adenosine nucleolipids to the enzyme proteins is responsible for the delayed inhibitory effect of these drugs. It seems that the inhibitory effect of adenosine nucleolipids is the manifestation of the unbound portion of the drugs.

The stimulatory effects of some adenosine nucleolipids on protein kinase as shown previously[1,2] and in this paper are interesting from the theoretical point of view. The nature of this effect is not yet known and therefore it seems desirable to find whether this stimulation is similar or different from the mechanism of the stimulation of protein kinase by cyclic AMP.

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Translated by the authors.