EFFECT OF ETHYL- AND GLYCEROL-1 ESTER OF ADENOSINE-5'-PHOSPHATE ON THE ACTIVITY OF ADENYLATE CYCLASE, SPECIFIC PHOSPHODIESTERASE AND PROTEIN KINASE FROM RAT LIVER AND ADIPOSE TISSUE

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Adenosine-5'-phosphate ethyl ester and glycerol-1 ester affect both basal activity and the activity stimulated by cyclic AMP in protein kinase from the liver and adipose tissue of rats. Neither the activity of adenylate cyclase, nor that of specific phosphodiesterase of the same tissues is affected by the compounds mentioned.

The preparations of adenylate cyclase from various tissues synthesize in the presence of alcohols in addition to cyclic AMP also new nucleotides[1-3]. In our preceding paper[1] we have identified these nucleotides as esters of adenosine-5'-phosphate (5'-AMP). All the above -mentioned papers stress the possibility that the presence of alcohols might lead to the formation of nucleotides which are separated together with cyclic AMP by the method of Krishna and coworkers[4], and may thus lead to falsely high values for adenylate cyclase activity. However, it is not excluded that the esters of 5'-AMP may possess a feed-back regulatory role in living tissues. The effect of the glycerol ester of 5'-AMP (glycerol-5'-AMP) in adipose tissue (which sets glycerol free during lipolysis) is considered in the first place, and in the second place also that of ethyl ester of 5'-AMP (ethyl-5'-AMP) in liver, after ingestion of ethanol. One of the possibilities would be to influence the synthesizing and degrading enzymes or enzymes mediating the effects of cyclic AMP. For the elucidation of these questions we synthesized both mentioned esters of 5'-AMP and also tested their effects on the activity of adenylate cyclase, specific phosphodiesterase and protein kinase in the liver and adipose tissue of rats.

EXPERIMENTAL

Adenosine 5'-[α^{32}P]triphosphate, trisodium salt (500–3000 mCi/mmol), and adenosine 5'-[(γ^{32}P]triphosphate, trisodium salt (500–3000 mCi/mmol), were the products of the Radio-

chemical Centre, Amersham, England, and [^{3}H—G]-3′,5′-cyclic adenosine phosphate (24 Ci/mmol) was supplied by New England Nuclear, Boston, Mass., U.S.A. 2-Phosphoenol-pyruvate (tricyclohexylammonium salt), pyruvate kinase from rabbit muscle (300 I.U. per mg of protein) and snake venom of Ophiophagus hannah (King cobra) were the products of Calbiochem, San Diego, U.S.A. Cyclic AMP was purchased from Lachema, Brno, Czechoslovakia. Neutral aluminum oxide for chromatographic adsorption analysis according to Brockmann (act. II) was a product of Reanal, Hungary. The naphthalene-dioxane scintillation cocktail SLD-31 was supplied by Spolana, Neratovice, Czechoslovakia. The histone $F_{2\alpha}$, F_3 was prepared from calf thymus[5].

Adenosine-5′-phosphate Ethyl Ester

Pyridinium salt of N,O$^{2'}$,O$^{3'}$-triacetyladenosine-5′-phosphate (55 mg; 0·1 mmol) was evaporated twice with pyridine (3 ml each time), and 2,3,5-triisopropylbenzenesulfonyl chloride (60 mg), pyridine (1 ml) and ethanol (5 µl) were added. After 6 hours' standing under exclusion of moisture water was added (20 µl), followed by concentrated aqueous ammonia (3 ml) after an additional 30 minutes standing. After 20 hours the mixture was evaporated, the residue dissolved in 20% aqueous ethanol (2 ml) and chromatographed on one sheet of Whatman No 3MM paper in 2-propanol–conc. ammonia–water (7 : 1 : 2). The UV-absorbing zone of R_{Up} 6·7 was eluted with water. $800A_{260}$ (56%) of ethyl adenosine-5′-phosphate was thus obtained. UV spectrum (water): λ_{max} $26_{min}0$ nm, λ 231 nm. Electrophoretic mobility at pH 7·5 was $0{\cdot}44_{Up}$.

Adenosine-5′-phosphate Glycerol Ester

The substance was prepared analogously as ethyl ester, starting with 0·1 mmol of protected adenosine-5′-phosphate and 50 µl of glycerol. Preparative paper chromatography gave $305A_{260}$ (22%) of a compound of R_{Up} 4·3. Electrophoretic mobility (at pH 7·55) was 0·41. With excess glycerol phosphorylation in the position 2 is unlikely.

Preparation of Tissue Homogenates

For the experiments male rats of the Wistar strain, weighing 150—200 g were used and fed ad libitum; they were supplied by Velaz, Prague. After killing by cervical dislocation the liver was withdrawn together with the epididymal adipose tissue. For activity determination of adenylate cyclase crude liver homogenates were used (75 mg per 5 ml of ice-cold 75 mM Tris-HCl buffer of pH 7·5 and 15 mM $MgCl_2$ and 2·5 mM EDTA). The adipose tissue was homogenized in the same homogenization mixture at a ratio of 1 g tissue and 3 ml solution. After a short centrifugation at 1000 g the separation of the fat component took place, and for the activity determination of adenylate cyclase the infranatant was used. For the determination of activity of phosphodiesterase and protein kinase the above-mentioned preparations were further centrifuged for 10 minutes at 3000 *g* then further diluted with a suitable medium (see appropriate methods) to the required protein concentration[6].

Activity Determination

The activity of adenylate cyclase was determined under the conditions mentioned earlier[7]. To 30 µl of the incubation mixture, containing 1 µCi of ATP-[α^{32}P] 20 µl of the enzyme preparation was added (corresponding to 50—70 µg of protein) and the mixture was incubated at 37°C for 10 minutes. The final composition of the incubation mixture was: ATP, 0·1 mM;

Tris-HCl buffer 30 mM (pH 7·5); cyclic AMP, 0·1 mM; Mg^{2+}, 6 mM; K^{+}, 5 mM; EDTA, 1 mM; phosphoenol pyruvate, 5 mM; phosphoenol pyruvate kinase, 40 μg/ml; myokinase, 20 μg/ml. The values for blanks were determined for each experiment separately in the absence of the enzyme preparation. The reaction was stopped by addition of one ml of 0·05M-HCl containing 50 μg of cyclic AMP for the calculation of the recovery value. Immediately after the end of the reaction the samples were boiled in a water bath for 5 minutes. The test tube content was chromatographed on alumina[8], applying 1 ml of the solution onto a column of alumina (1 g; 4 cm × 0·6 cm). Cyclic AMP was eluted with 2·5 ml of 0·1M Tris-HCl buffer, pH 7·5. The recovery was measured by optical densitometry at 260 nm.

The phosphodiesterase activity was determined by this modification of Brooker's method[9]: The activity determination was carried out in the total volume of 200 μl in a scintillation vial. The final composition of the incubation medium was: Tris-HCl buffer, (pH 7·5), 60 mM; $MgCl_2$, 10 mM; EDTA, 0·4 mM; ^{3}H-cyclic AMP (about 25000 c.p.m.), cyclic AMP, 10^{-6} and 10^{-4}M and tissue homogenate corresponding to 60—80 μg of protein. After addition of the enzyme preparation the samples were first incubated at 30°C for 10 minutes (concentration of cyclic AMP 10^{-6}M) or 20 min (concentration of cyclic AMP 10^{-4}M). Snake venom (20 μl) was then added, having 5'-nucleotidase activity (2 mg of venom of king cobra per one ml), and the samples were incubated for another 10 minutes. The reaction was stopped by addition of 1 ml of a mixture of Dowex 1 (×2) with ethanol (1 : 1, v/v) and the radioactivity which remained unretained on the ion exchanger (unquenched) determined the amount of the ^{3}H adenosine formed and hence the decomposition of cyclic AMP as well. The unspecific binding of ^{3}H adenosine was not taken into consideration. After the subtracting of the values of unquenched radioactivity in substrate (less than 10% of the added c.p.m.) the results were expressed in % of the decomposed cyclic AMP, and the absolute value was calculated from the concentration of the substrate added.

The activity of protein kinase was determined according to Miyamoto and coworkers[10], with slight modifications. The standard incubation medium (total 100 μl) contained: Tris-HCl buffer (pH 6·5), 50 mM; Mg^{2+}, 6 mM; EDTA, 0·5 mM; NaF, 5 mM; theophylline, 5 mM; 100 μg histone $F_{2\alpha}$, F_3, enzyme preparation corresponding to 30 μg of protein; $^{32}P\gamma$ATP about 250000 c.p.m.; and ATP $2 . 10^{-5}$M. The stimulation of the enzyme was carried out with cyclic AMP at a 10^{-5}M concentration. The reaction was started by addition of ATP to the ice-cold mixture and the samples were incubated at 30°C for 15 minutes. The reaction was stopped and the phosphorylated histone precipitated on addition of 3 ml of 7·5% trichloroacetic acid; 0·2 ml of 1% human albumin were added as histone carrier. The precipitate was dissolved in 0·3 ml of 1M-NaOH. The precipitation and the centrifugation were carried out four times. Then the radioactivity was transferred quantitatively from the test tubes into the scintillation vials. The results are expressed in absolute amount of ^{32}P transferred to histone.

All the results are a representative experiment which was carried out in triplicate or duplicate. ^{3}H was measured in SLD-31 and ^{32}P by Cerenkov's radiation[11].

RESULTS

In the first series of experiments the effects of synthetic ethyl-5'-AMP and glycerol-5'-AMP were tested on the activity of stimulated and unstimulated adenylate cyclase activity. The esters were tested within the dosage range from 10^{-6} to 10^{-3}M, *i.e.* at concentrations which possibly could occur in tissues. Fig. 1 (on the left) shows that ethyl-5'-AMP (which is intensively formed by the preparations of liver adenylate cyclase[3] in the presence of ethanol) does not affect either the basal activity of this

enzyme or the activity stimulated by glucagon and sodium fluoride. Virtually identical results were found with the highest concentrations of glycerol-5′-AMP used (data not shown). In the adipose tissue, where a physiological formation of glycerol-5′-AMP from the liberated glycerol during lipolysis can be supposed, this substance did not lead to the influencing either of the basal activity or the activity stimulated by isoproterenol and sodium fluoride (Fig. 1, on the right) in the above mentioned dosage range. The highest concentration of ethyl-5′-AMP (10^{-3}M) did not affect the adenylate cyclase activity in the adipose tissue either (data not shown).

In the second series of experiments the effect of alcoholic esters of 5′-AMP were tested on phosphodiesterase activity. For a comparison of the effects we simultaneously tested papaverine which is a strong inhibitor of this system. As the majority of tissues contain two phosphodiesterases with differing K_m, we followed the inhibitory effect of these substances at two different substrate concentrations, *i.e.* at 10^{-6} and 10^{-4}M concentrations. Fig. 2*a* shows the results with liver, where either ethyl-5′-AMP or the highest concentrations of glycerol-5′-AMP did not affect the phosphodiesterase activity substantially, while papaverine led to a 90% inhibition of the enzyme. A similar situation can also be observed in Fig. 2*b* where the effects of glycerol-5′-AMP and the highest concentrations of ethyl-5′-AMP on the phosphodiesterase activity were observed in preparations of adipose tissue. In comparison

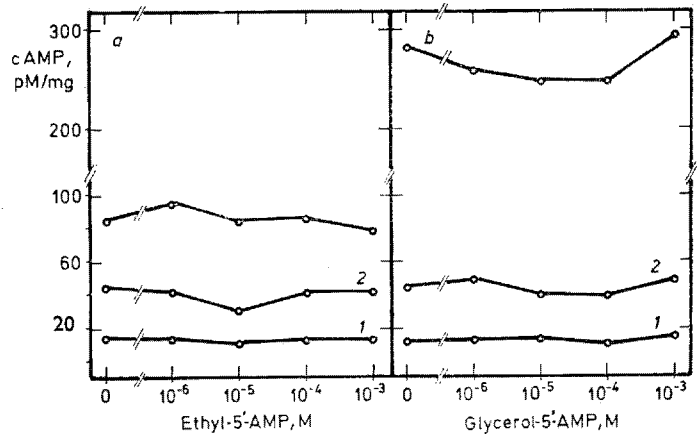

FIG. 1

Effects of Ethyl-5′-AMP and Glycerol-5′-AMP on Adenylate Cyclase Activity in homogenates of Rat Liver (*a*) and Adipose Tissue (*b*)

The enzyme activity was determined after 10 minutes' incubation (see Methods). On the left: *1* basal values, *2* glucagon 10^{-6}M, unnumbered curve sodium fluoride 10^{-2}M. On the right: *1* basal values, *2* isoproterenol 10^{-4}M, unnumbered curve sodium fluoride 10^{-2}M.

with the inhibitory effects of papaverine no substantial influencing of phosphodiesterase activity took place here either. Only at a higher substrate concentration a certain drop in enzyme activity was observed, which, however, seems to be independent of the concentration of 5'-AMP esters.

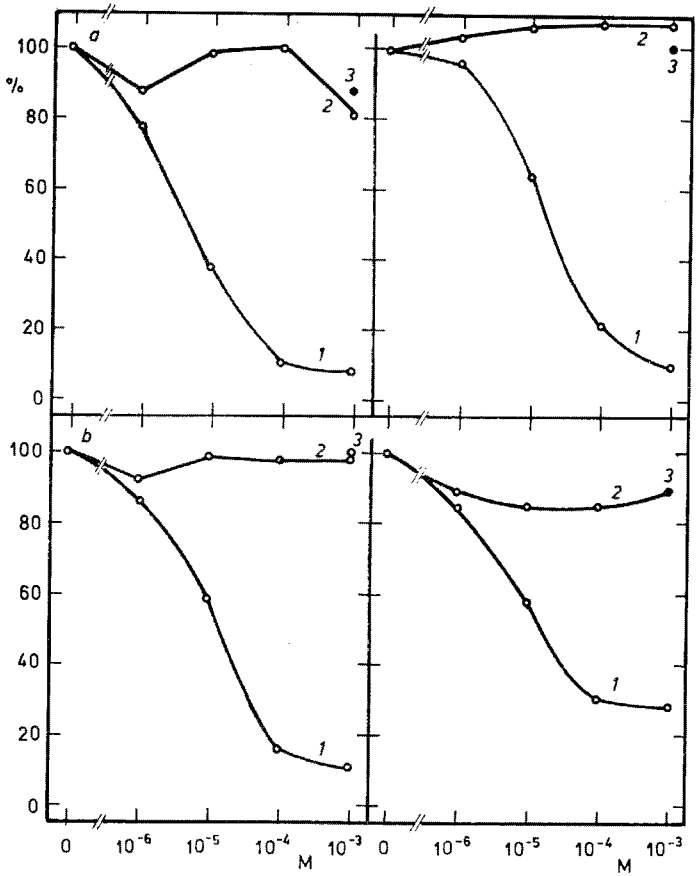

FIG. 2

Affecting of Phosphodiesterase Activity from Liver (*a*) and Adipose Tissue (*b*) with Ethyl-5'-AMP and Glycerol-5'-AMP, in Comparison with the Effect of Papaverine

a The activity of enzyme in liver was determined in the presence of cyclic AMP (10^{-6}M) for 20 minutes (on the left), and (10^{-4}M) for 30 minutes (on the right). The basal activities were 0·78 nM/1 mg, or 28·37 nM/1 mg, resp. The results are given in % of these values. *1* Papaverine, *2* ethyl-5'-AMP, *3* glycerol-5'-AMP.

b The activity of enzyme in adipose tissue was determined in the presence of cyclic AMP (10^{-6}M) for 20 minutes (on the left), and (10^{-4}M) for 30 minutes (on the right). The basal values were 0·86 nM/1 mg, or 28·54 nM/1 mg, resp. The results are given in % of these values. *1* Papaverine, *2* glycerol-5'-AMP, *3* ethyl-5'-AMP.

In the third series of experiments we investigated the effect of ethyl-5′-AMP and glycerol-5′-AMP on the activity of protein kinase, *i.e.* an enzyme mediating the effect of cyclic AMP. On Fig. 3*a* the basal activity of protein kinase in liver is shown, as well as its activity in the presence of cyclic AMP at a 10^{-5}M concentration. Ethyl-5′-AMP led to a considerable decrease both of the basal activity and of the activity stimulated by cyclic AMP. The concentration of glycerol-5′-AMP from 10^{-5}M up displayed, on the contrary, a stimulative effect which attained about half of the cyclic AMP effect. This effect is far more distinct in adipose tissue where the stimulatory effect of cyclic AMP was more pronounced (Fig. 3*b*). However the in-

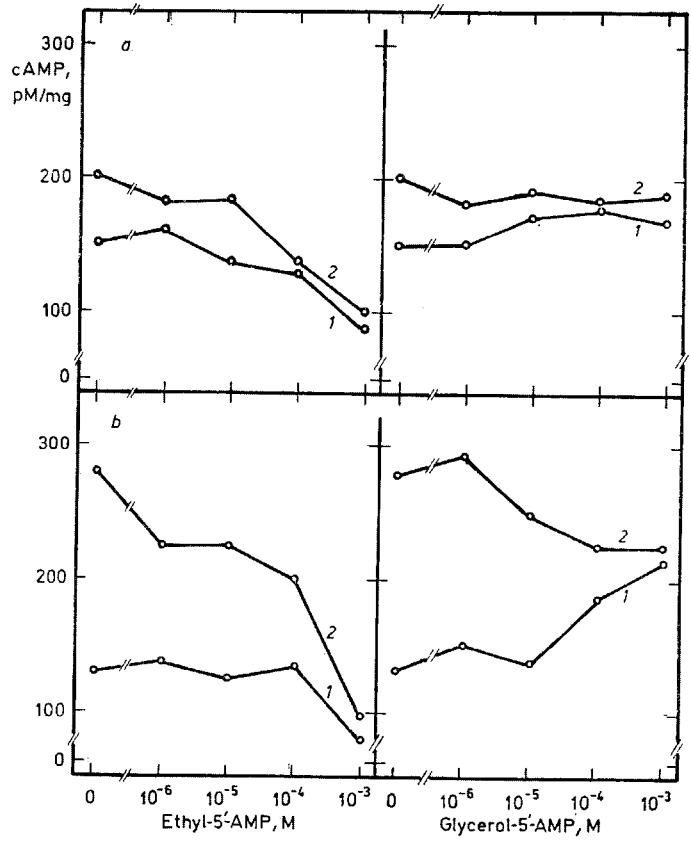

FIG. 3

Effects of Ethyl-5′-AMP and Glycerol-5′-AMP on Protein Kinase Activity from Livers (*a*) and Adipose Tissue (*b*) of Rats

For the activity determination see Methods. On the left: Effects of ethyl-5′-AMP; *1* basal values, *2* values after cyclic AMP (10^{-5}M). On the right: Effects of glycerol-5′-AMP; *1* basal values, *2* values after cyclic AMP (10^{-5}M).

creasing concentrations of glycerol-5'-AMP partly antagonized the effect of cyclic AMP. Similarly as in the liver, ethyl-5'-AMP had an antagonistic effect in the preparations of protein kinase from adipose tissue as well. While the basal activity was affected only by the highest concentration only, the activity stimulated by cyclic AMP was strongly inhibited and the highest concentrations led to its inhibition below the basal values.

DISCUSSION

The formation of unknown nucleotides from ATP and various alcohols using adenylate cyclase preparations has been described by several authors. In addition to indirect evidence that they are esters of AMP we also have proved it directly by comparison with authentic esters[1] of AMP. Although it is still unknown whether the formation of these esters takes place under *in vivo* conditions, a number of authors[2,3] envisage their possible physiological importance. Hypothetically the condensation products of ethanol with 5'-AMP may be most likely formed when ethanol is ingested, and that of glycerol with 5'-AMP when this polyhydric alcohol is liberated during the lipolysis in the adipose tissue.

The affecting of enzymes of the cyclic AMP system represents one possibility of a regulative interference of alcohol esters of 5'-AMP. Therefore in this study we followed the effect of ethyl-5'-AMP and glycerol-5'-AMP on the activity of adenylate cyclase, phosphodiesterase and protein kinase in preparations of rat liver and epididymal adipose tissue. The activity of adenylate cyclase was not affected by these substances within a broad dosage range and therefore it seems that these substances will probably not function as a feed-back control at this level. As regards the phosphodiesterase activity a substantial inhibition of the enzyme did not take place in any of the tissues investigated. However, the possible inhibition of the enzyme cannot be regarded as a regulatory mechanism, because the inhibition would lead rather to the strengthening of the effect under study. The results do not indicate that an increase in phosphodiesterase activity could take place, which in turn could weaken the effect mediated by cyclic AMP.

Most important changes were found in the protein kinase activity. Ethyl-5'-AMP has inhibitory effects not only in liver, but in adipose tissue as well. The stimulative effect of cyclic AMP on the activity of protein kinase is completely antagonized. In contrast to this, one can observe in the case of glycerol-5'-AMP that a certain stimulation does occur, which attains about one half of the effect of cyclic AMP. The stimulation of protein kinase by a cyclic nucleotide is, however, partly inhibited by glycerol-5'-AMP, so that this ester could act as a competitive dualist. It is out of question that the described effects could be caused by a contamination by cyclic AMP.

The results mentioned indicate that the AMP esters could affect protein kinase only among the enzymes of the cyclic AMP system. Whether a similar regulatory mechanism can also apply *in vivo* is not yet known. The changes in the activity of this enzyme are remarkable especially because of the fact that relatively very few substances are known which change protein kinase activity. A direct influencing of some intracellular enzymes, which are not stimulated by cyclic AMP would represent the second possibility of the regulatory interference of 5′-AMP esters; the possibility also exists that the formation of these esters could decrease the intracellular level of ATP that is necessary as an energy source for intracellular reactions In connection with this the possibility of the formation of alcoholic liver steatose[3] is considered in the first place. However, the possibility of participation of these regulatory mechanisms requires further study in which the level of ethyl-5′-AMP in intact tissue after administration of ethanol would be investigated, as well as a direct study of the effect of this ester on enzymes responsible for liver steatose.

REFERENCES

1. Hynie S., Ledvina M., Smrt J.: This Journal *41*, 1448 (1976).
2. Petrack B., Ma D., Sheppy F.: J. Biol. Chem. *249*, 3661 (1974).
3. Gold D. I., Volicer L.: Private communication.
4. Krishna G., Weiss B., Brodie B. B.: J. Pharmacol. Exp. Ther. *163*, 379 (1968).
5. Johns E. W., Buttler J. A.: Biochem. J. *82*, 15 (1962).
6. Lowry O. H., Rosenbrough N. J., Farr A. L., Randall R. J.: J. Biol. Chem. *193*, 265 (1961).
7. Hynie S., Wenke M.: Eur. J. Pharmacol. *30*, 230 (1975).
8. Ramachandran J.: Anal. Biochem. *43*, 227 (1971).
9. Brooker G., Thomas L. J. jr, Appelman M. M.: Biochemistry *7*, 4177 (1968).
10. Miyamoto E., Kuo J. F., Greengard P.: J. Biol. Chem. *244*, 6395 (1969).
11. Hynie S.: This Journal *39*, 2325 (1974).

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