ADENYL CYCLASE ACTIVITY IN A PLASMA MEMBRANE FRACTION PURIFIED FROM "GHOSTS" OF RAT FAT CELLS

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1. Introduction

In several animal species, the metabolism of adipose tissue is altered by a great variety of hormones having a disparate molecular structure [1–5]. Isolated fat cells are even more sensitive to these agents than the parent tissue [6].

Plasma membrane sacs ("ghosts") of rat fat cells possess a single unit adenyl cyclase system activated by the lipolytic hormones [7] through distinctive selective sites (receptors or discriminators). However, it has not been proved that antilipolytic hormones (insulin, prostaglandins) reduce adenyl cyclase activity in ghosts.

Purification of a ghost fraction from rat adipose cells was performed on a linear sucrose gradient. The result was a 3-fold increase in nucleotidase activity and respectively 5- and 10-fold increase in basal and epinephrine-stimulated adenyl cyclase activities. Optimum pH was determined to be 8.0 and a linear relationship between adenyl cyclase activity and amount of enzyme was established in the range 1 to 10 μg protein. Insulin had no effect on adenyl cyclase activity.

2. Materials and methods

Groups of 10 male rats of the Sprague Dawley strain (Sodelabo, France) weighing 150–180 g were used. The rats were fed ad libitum on standard diet and had free access to tap water.

Collagenase was purchased from Worthington Biochemical Corp. (Freehold, N.J., USA). Fatty acid-poor BSA Fraction V was obtained from Pentex (Miles Laboratories Inc. Kankake, Ill., USA).

α-AT^{32}P (250–880 mCi/mmole) was obtained from C.E.A. (Saclay, France). Adenosine-2-^{3}H-5′-monophosphate (4.6 Ci/mmole) and adenosine 8-^{3}H-3′, 5′-cyclic monophosphate (5 Ci/mmole) were supplied by the Radiochemical Centre (Amersham, England).

Porcine insulin (Novo S 8563) was a gift from Dr. Rosselin.

Isolated fat cells and ghosts were prepared with the methods described by Rodbell [6, 8]. The yield of fat cell ghosts from 10 g epididymal fat pads was about 8.0 mg protein.

24 ml linear sucrose gradients were prepared after a modification of the method described by McKeel et al. [9] and contained 1 mM EDTA, 5 mM Tris-HCl with or without 1 mM ATP; final pH was 7.4. Centrifugation at 24,000 rpm for 60 min in a Beckman L50 ultracentrifuge SW 25.1 rotor resulted in a sharp band of membranes in the upper part of the tube. This band was removed and diluted 4/1 (v/v) with 25 mM Tris pH 7.4; after centrifugation 15 min at 13,000 rpm, membranes were suspended again in 25 mM Tris or 5 mM Tris–1 mM EDTA, pH 7.4. Total preparation time was about 3.5 hr after sacrificing the rats. The yield of membranes was 1.2–1.5 mg protein from 8.0 mg of ghosts. Membranes were kept in liquid nitrogen for 1 or 2 wk without noticeable loss of activity.

Adenyl cyclase activity was determined according to Rodbell [7] except that the ATP-regenerating system consisted of 5 mM phosphocreatine and 0.1 mg/ml of creatine kinase.

5′-Nucleotidase was determined by the radiotest of Avruch et al. [10] and protein by the procedure of

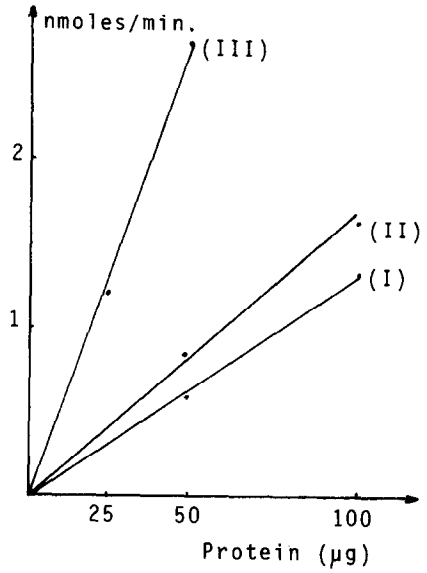

Fig. 1. Influence of pH and enzyme concentration on nucleotidase activity in ghosts (I:pH 7.4; II:pH 8.5) and membranes obtained by purification of ghosts on a linear sucrose gradient (III, pH 8.5).

Lowry et al. [11] with crystalline bovine serum albumine as a standard.

3. Results

Nucleotidase activity measured on a suspension of ghosts in 1 mM $KHCO_3$ showed a linear relationship between 50–100 μg protein. Activity increased when pH reached 8.5 and was then 16.8 nmoles AMP/mg prot./min. Nucleotidase activity was increased about 3 times after linear sucrose gradient (fig. 1).

The average basal adenyl cyclase activity measured on ghosts was 280 pmoles/mg prot./10 min. In previous experiments performed with different batches of collagenase (158 units/mg, instead of 200) and albumin (Calbiochem, instead of Pentex), basal activity was 436 pmoles/mg prot./10 min. A simultaneous drop in the lipolytic activity of a cell suspension was observed.

The comparison of adenyl cyclase activity in ghosts and membranes was performed with the same lot of products. As can be seen in fig. 2, adenyl cyclase activity of plasma membranes obtained through a linear

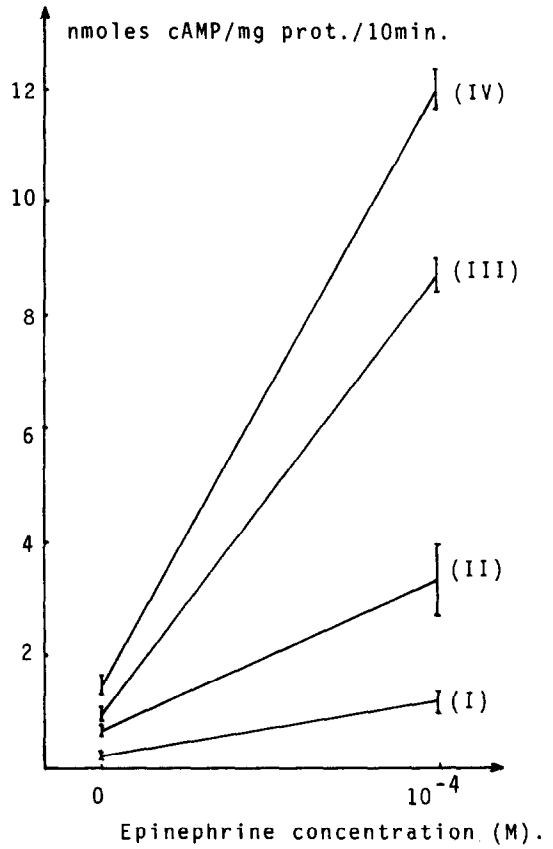

Fig. 2. Comparison of basal and epinephrine-stimulated activities in ghosts (I) and membranes obtained through different conditions, at pH 7.4. I: 50 μg ghost protein in 1 mM $KHCO_3$. II:30 μg membrane protein in 25 mM Tris. Gradient without ATP. III:30 μg membrane protein in 25 mM Tris. Gradient with 1 mM ATP. IV:20 μg membrane protein in 5 mM Tris–1 mM EDTA. 1 mM ATP.

sucrose gradient without ATP (II) was increased by a factor of 3, compared with ghosts (I). The presence of 1 mM ATP in the gradient (III) preserved the deterioration of enzymatic activity. Adenyl cyclase activity is very labile: the keeping of "ghosts" in 1 mM $KHCO_3$ at 4° for 2 hr (time needed for purification on sucrose gradient) resulted in a drop of 60% of their enzymatic activity. On the other hand, the activity of a ghost suspension incubated for 2 min at 30° just before the test of adenyl cyclase decreased by 30%; after 5 min at 30°, the activity drop was 70%. EDTA in the incubation medium increased the adenyl

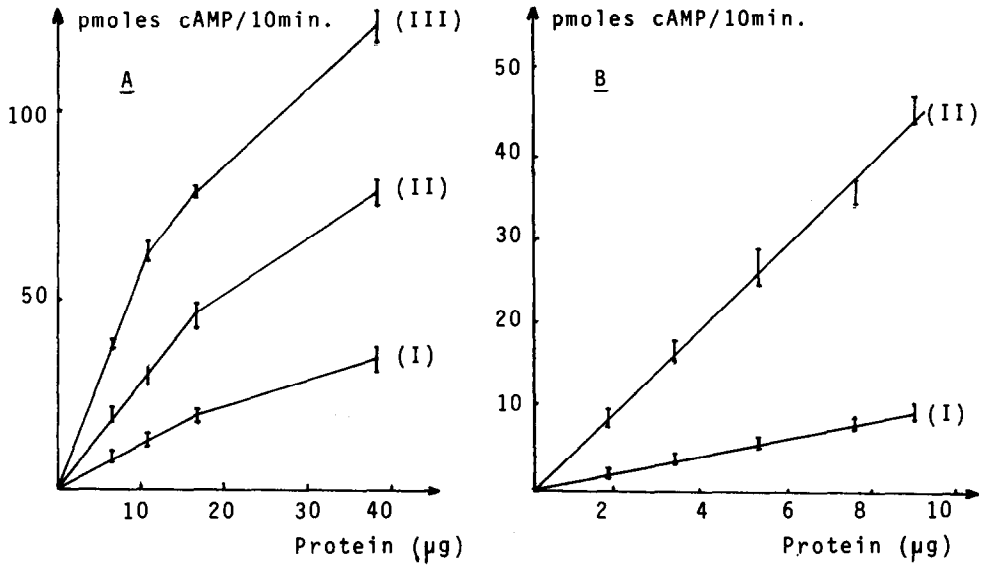

Fig. 3 A and B. Relationship between amount of membrane protein and basal (I), 10^{-4} M epinephrine (II) and 10 mM fluoride (III) stimulated adenyl cyclase activities at pH 7.8.

cyclase activity (IV). Moreover, it was found that 1 mM EDTA preserves inactivation of membranes kept in liquid nitrogen.

According to the observations described in the last paragraph, optimum conditions were adopted, i.e. gradients were performed with 1 mM ATP and membranes stored in 5 mM Tris–1 mM EDTA, pH 7.4.

Adenyl cyclase activities (basal, epinephrine- and fluoride-stimulated), estimated at pH 7.8, were proportional to amounts of membranes lower than 15 μg (fig. 3 A). From 1.8 to 9.0 μg protein, a linear relationship was confirmed (fig. 3 B).

Basal and epinephrine-stimulated adenyl cyclase activities were maximum at pH 8.0 (fig. 4). A small drop in stimulation percentage (760 to 720%) of adenyl cyclase activity was observed with increasing pH from 7.4 to 7.8; the stimulation decreased to 550% at pH 8.0 and even more at pH 8.2.

Insulin in concentrations ranging from 10^{-6}–10^{-8} M did not influence basal cyclase activity when the amount of membrane protein added varied from 9–20 μg and time of incubation was prolonged from 10 to 20 min. In these conditions, insulin did not prevent stimulation of adenyl cyclase activity by 10^{-5} M epinephrine.

Preincubation of membranes in presence of insulin 10^{-6} M for 2–5 min prior to determination of basal or epinephrine-stimulated adenyl cyclase activities was again unsuccessful in showing an inhibitory effect of insulin.

4. Discussion

Purification of a ghost fraction from rat fat cells on a linear sucrose gradient resulted in a marked increase in basal adenyl cyclase activity, 3–5-fold, depending upon experimental conditions. Similarly, epinephrine-stimulated activity reached a 3–10-fold increase.

The smaller increase in nucleotidase activity (3 times) may be due to contamination of ghosts by nuclei, mitochondria and microsomes containing nucleotidase activity [10], while adenyl cyclase activity is located only in the plasma membrane [12, 13].

Following the 85% protein loss after purification, one would expect a maximal 7-fold increase in adenyl cyclase activity. Maximal increase of basal activity is less important, probably due to a loss of enzymatic activity during the purification procedure. The 10-fold

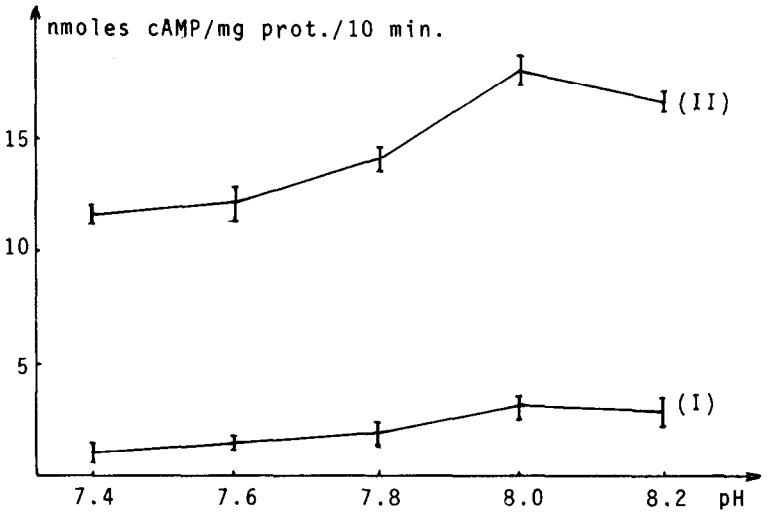

Fig. 4. Effect of pH on basal (I) and 10^{-4} M epinephrine-stimulated adenyl cyclase activities in adipocyte membranes.

increase of epinephrine-stimulated activity seems discordant; nevertheless, it is likely that contaminants in ghosts disturb processes occurring at the cell surface (e.g. binding of epinephrine). Purification of a ghost fraction could therefore have a double effect: increase of specific adenyl cyclase activity through elimination of non-membranous proteins, and reduction of the side effects of contaminants on the reactions occurring at the cell surface.

Jungas [14] showed a 30% decrease of adenyl cyclase activity in homogenates from epididymal fat pads preincubated with insulin. Moreover, Butcher et al. [15] found that addition of insulin caused a striking decrease in intracellular cyclic AMP levels in fat pads incubated with epinephrine and caffeine. On the other hand, Rodbell [16] and Cryer et al. [17] on fat cell ghosts and Vaughan [18] on homogenates of adipose tissue did not show any influence of insulin on basal or hormone-stimulated adenyl cyclase activities; our experiments with a membrane preparation containing a high adenyl cyclase activity confirm these results.

The decrease in cyclic AMP content of epididymal fat pad incubated with insulin may then be due to a phosphodiesterase stimulation. Hepp [19] and Blecher [20] could not observe any influence of insulin on this enzymatic activity but more recently Loten [21] demonstrated, with adequate substrate concentrations, an inhibitory effect of insulin on phosphodiesterase activity.

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