Separation and Some Characteristics of Two Factors from Rat Liver Particulate Fraction Which Stimulate and Inhibit the Low-K_m Adenosine 3′,5′ Cyclic Monophosphate (cAMP) Phosphodiesterase of Rat Fat Cells

Hiroshi KUMAGAI,* Shi-jie ZHANG and Seiyu HIROSE

Faculty of Pharmaceutical Sciences, Chiba University, 1–33 Yayoi-cho, Inage-ku, Chiba 263, Japan. *Received September 24, 1991*

Two factors were separated from rat liver particulate fraction treated with insulin, one of them having a stimulating effect on low-K_m adenosine 3′,5′ cyclic monophosphate (cAMP) phosphodiesterase activity of crude microsomal fraction (P-2 fraction) and the other having an inhibiting effect on the activity of low-K_m cAMP phosphodiesterase solubilized with 0.3% Brij 58 from P-2 fraction. Trypsin and heat treatments had essentially no effect on these two factors.

The stimulating factor did not significantly change the apparent K_m value of enzyme in P-2 fraction but increased the maximal velocity of the reaction. The inhibiting factor raised the K_m value of solubilized enzyme without affecting the maximal velocity of the reaction.

The stimulating factor level in diabetic rat was larger than that in normal rat while the inhibiting factor level in diabetic rat was smaller than that in normal rat. Possible participation of both factors in insulin action is discussed.

Keywords stimulating factor; inhibiting factor; cAMP phosphodiesterase; insulin; diabetes; rat liver

Introduction

Insulin has been shown to lower the adenosine 3′,5′ cyclic monophosphate (cAMP) concentration raised by epinephrine in rat adipose tissue.[1,2] This lowering of cAMP concentration is due to the decrease in the rate of cAMP formation or to the increase in the rate of its degradation or both. Loten and Sneyd[3] first reported that the increase in cAMP degradation in the homogenate of rat adipose tissue in response to insulin was caused by the elevation of the activity of low-K_m cAMP phosphodiesterase.

The low-K_m cAMP phosphodiesterase activity was found in the microsomal fraction of rat adipose tissue.[4] It was reported that the cAMP phosphodiesterase in the plasma membrane fraction of rat liver was activated by insulin through phosphorylation of the enzyme, while the enzyme in the microsomal fraction was activated by insulin without being phosphorylated.[5] Thus, the mechanism of the cAMP phosphodiesterase activation in the microsomal fraction of adipose tissue and liver by insulin appears to be different from that in the plasma membrane fraction of liver.

Larner *et al.*[6] found a factor that inhibited cAMP-dependent protein kinase in deproteinized extracts of insulin-treated and control rat muscle. The level of this factor was higher in insulin-treated muscle than in control muscle. This factor stimulated the glycogen synthase phosphoprotein phosphatase in rat muscle.[6] A stimulating factor for pyruvate dehydrogenase was also found in extracts of insulin-treated rabbit and rat adipose tissues.[7–9] Saltiel and Cuatrecasas[10] partially purified two substances that stimulated the high-affinity cAMP phosphodiesterase from insulin-treated bovine hepatic plasma membranes and reported their apparent molecular weights of approximately 1400. Thus, the stimulating factors found in various tissues treated with or without insulin mimicked the action of insulin on glycogen synthase D phosphatase, pyruvate dehydrogenase or low-K_m cAMP phosphodiesterase. Although these factors are thought to be involved in the activation of cAMP phosphodiesterase by insulin, the exact chemical nature and mode of action of them remain unclear.

We found two factors in rat liver particulate fraction; one stimulated the activity of low-K_m cAMP phosphodiesterase in microsomal fraction from rat fat cells and the other inhibited the activity of the enzyme solubilized from this fraction with 0.3% Brij 58. In this paper, we describe some characteristics of both factors obtained from rat liver particulate fraction. In addition, we examined the differences in levels of both factor in the livers of normal and diabetic rats.

Materials and Methods

Materials Bovine serum albumin (fraction V) and insulin (bovine pancreas) were purchased from Sigma Chemical Co. Snake venom (*Crotalus adamateus*) and collagenase S-1 were obtained from Funakoshi Pharmaceutical Co. and Nitta Gelatin Co., respectively. cAMP, adenosine 5′ monophosphate (AMP) and adenosine were purchased from Yamasa Shoyu Co. Streptozotocin was obtained from Wako Pure Chemical. [2,8-^{3}H]Adenosine 3′,5′ cyclic phosphate ammonium salt ([^{3}H]cAMP, 1.34 TBq/mmol) was supplied by New England Nuclear Corp. Sephadex G-25 and AG1-X2 were obtained from Pharmacia LKB Biotechnology Inc. and Bio-Rad Laboratories, respectively. Laboratory chow (MF) was purchased from Oriental Yeast Co., Ltd.

Animals and Their Treatments Male Wistar rats (150—160 g body weight) were purchased from Takasugi Experimental Animal Co., Ltd. They were maintained in a temperature-controlled (22±2 °C) room with a 12 h light–12 h dark cycle (light on at 7.00 a.m.), and received laboratory chow and tap water *ad libitum*. The rats were kept under the above conditions for 4 d after purchase and healthy rats with normal body weight gains were used in the experiment. Diabetic rats were prepared by injecting the tail vein with streptozotocin (65 mg/kg body weight) dissolved in 50 mM citrate buffer (pH 4.5) after the rats were fasted for 16 h.[11] Blood samples were collected from the tail vein every 24 h and immediately centrifuged for 10 min at 7000 × *g* to obtain plasma. Plasma glucose was measured with the *o*-toluidine method.[12] Streptozotocin-diabetic rats had a blood sugar of 494±10.8 mg/dl as compared to the control of 150±13.0 mg/dl on day 3.

Preparation of Rat Liver Particulate Fraction This was performed by the method of Saltiel and Cuatrecasas[10] with a slight modification. Rats (about 200 g body weight) were exsanguinated from the common carotid artery without anesthesia. Livers (60 g) were removed quickly and rinsed in an ice-cold 50 mM NH_4HCO_3 solution (pH 7.5). The rinsed livers were homogenized in 5 volumes of the above solution using a Teflon glass homogenizer. The homogenate was centrifuged for 10 min at 1000 × *g*. The supernatant was centrifuged further for 25 min at 30000 × *g* to obtain pellet. The pellet was suspended in 5 volumes of 50 mM NH_4HCO_3 solution (pH 7.5) and centrifuged again for 25 min at 30000 × *g*. The resulting pellet was resuspended in 300 ml of the same solution at a final concentration of 2 mg protein per ml.

Preparation of Crude Extract Containing Stimulating and Inhibiting Factors This was performed by the method of Saltiel and Cuatrecasas.[10] The suspension (300 ml) of rat liver particulate fraction prepared as

described in the preceding section was divided into 150-ml portions. The 150-ml portion was incubated with 10 nM insulin for 10 min at 37 °C. The pH of the mixture was then adjusted to 4.0 with 1 N formic acid. After centrifugation for 15 min at 30000 × *g*, the supernatant was removed and mixed with an equal volume of $CHCl_3$–CH_3OH (2 : 1, v/v) with shaking. The mixture was centrifuged for 2 min at 1000 × *g*. The water layer was separated from the chloroform layer and evaporated to dryness *in vacuo* at 40 °C. The resulting powder was resuspended in 3 ml of 50 mM NH_4HCO_3 solution (pH 7.5) and centrifuged for 10 min at 10000 × *g* to obtain a crude extract containing stimulating and inhibiting factors. As a control, the 150-ml portion to which no insulin was added was treated similarly. Thereafter, crude extract, stimulating and inhibiting factors, unless stated otherwise, means the crude extract, the stimulating and inhibiting factors from the insulin-treated liver particulate fraction of rats, respectively.

Preparation of Isolated Rat Fat Cells Isolated fat cells were prepared from rat epididymal adipose tissue by the method of Rodbell[13] with a slight modification. Rats (about 200 g body weight) were exsanguinated without anesthesia. The epididymal adipose tissues were removed quickly and rinsed in 0.154 M KCl. Each pad (about 2 g tissue) was cut into four pieces and then transferred to a 100 ml polyethylene flask containing 2 ml of KRBA buffer (118 mM NaCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 4.7 mM KCl, 25 mM $NaHCO_3$ and 20 mg/ml bovine serum albumin) supplemented with 3 mg/ml collagenase. KRBA buffer was bubbled before use with 95% O_2–5% CO_2 at 37 °C for about 10 min to make the pH of the solution 7.4. After the addition of fat tissue, the polyethylene flask was bubbled with 95% O_2–5% CO_2 for 15 s and then incubated at 37 °C for 40 min with gentle shaking. During incubation, the tissue was dispersed into small fragments and fat cells were liberated from the tissue fragments. After incubation, the contents of the flask were filtered through the nylon filter to obtain fat cell suspension which was transferred into a 50 ml polyethylene tube. The tissue fragments remaining on the nylon filter were washed four times with 2 ml of KRHA buffer (118 mM NaCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 4.7 mM KCl, 32 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid–NaOH (pH 7.4) and 20 mg/ml bovine serum albumin). The washings were added to the fat cell suspension. The tube was centrifuged at 400 × *g* for 1 min. The precipitate debris and KRHA buffer were removed carefully by aspiration to obtain the fat cell preparation that had been on the top layer after centrifugation. The fat cells thus obtained were washed four times with 10 volumes of KRHA buffer.

Treatment of Isolated Fat Cells with Insulin This was performed by the method of Makino *et al.*[14] The isolated fat cells (about 500 mg) from the preceding section were suspended in 20 ml of KRHA buffer. This suspension was divided into 10-ml portions. Each 10-ml portion was transferred to a 100 ml polyethylene flask and incubated at 37 °C for 10 min. Insulin was then added to one of the two flasks to a final concentration of 3 nM. The flask was incubated for a further 10 min at 37 °C. After incubation, the mixture was transferred to a 50 ml polyethylene tube. The flask was washed twice with STS buffer (0.25 M sucrose, 10 mM *N*-tris(hydroxymethyl)-2-aminoethanesulfonic acid (Tes)–KOH, pH 7.0) and the washings were added to the tube. The tube was centrifuged at 400 × *g* for 1 min. STS buffer was removed by aspiration to obtain fat cells. The fat cells were washed twice with 8 ml of STS buffer. The fat cells after the addition of 15 ml of STS buffer were then homogenized in a glass homogenizer equipped with a Teflon pestle (5 strokes) to obtain the suspension of fat cells treated with insulin. As a control, the 10-ml portion to which no insulin was added was treated similarly. Care was taken to carry out all steps within 10 min from the end of the second incubation to the final step described above.

Preparation of Crude Microsomal Fraction This was performed by the method of Osegawa *et al.*[15] The fat cells suspension (15ml) obtained as described in the preceding section was centrifuged at 4000 × *g* for 10 min. The resulting supernatant was further centrifuged at 27000 × *g* for 30 min to obtain a crude microsomal fraction. The pellet (1 mg protein) was suspended in 1 ml of Tes–KOH buffer (pH 7.5) containing 0.25 M sucrose. This suspension is referred to as P-2 fraction. Thereafter, P-2 fraction, unless stated otherwise, means the P-2 fraction from noninsulin-treated (control) fat cells.

Solubilization of cAMP Phosphodiesterase from P-2 Fraction Three-hundredth ml of 10% Brij 58 was added to 1 ml of P-2 fraction (1 mg protein/ml). The mixture was stirred at 4 °C for 60 min and centrifuged at 100000 × *g* for 60 min. The supernatant (0.5 mg protein/ml) thus obtained is referred to as solubilized cAMP phosphodiesterase.

cAMP Phosphodiesterase Assay Assay of low-K_m cAMP phosphodiesterase activity was performed by the method of Kono *et al.*[16] with a slight modification. The reaction mixture (0.25 ml) containing 40 mM Tes–KOH (pH 7.5), 4 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 100 nM cAMP, 3.7 kBq [^{3}H]cAMP and 10 μg protein of P-2 fraction or 4 μg protein of solubilized cAMP phosphodiestease was incubated at 30 °C for 5 min. After incubation, the reaction mixture was mixed with 0.05 ml of a solution containing 5 mM cAMP and 5 mM AMP and heated at 70 °C for 4 min to stop the reaction. The mixture was then cooled in an ice-water bath, added with 200 μl of distilled water and 0.05 ml of 1 mg/ml snake venom, and incubated at 37 °C for 20 min. The hydrolysis was terminated by the addition of 0.05 ml of 200 mM EDTA containing 5 mM adenosine (pH 7.0). A 0.5 ml aliquot of the mixture was withdrawn and applied to a column of AG1-X2 (7.5 × 7 mm) which had been equilibrated with distilled water. The column was eluted with distilled water. The first 1.6 ml eluate was discarded and the next 1.6 ml collected. A 1.5 ml aliquot of the latter eluate was withdrawn to measure the radioactivity of [^{3}H]adenosine produced by the reaction. The radioactivity was assayed by a liquid scintillation counter with toluene–triton X-100 scintillator (toluene 1000 ml, triton X-100 500 ml and 2,5-diphenyloxazole 4 g). Thereafter, cAMP phosphodiesterase refers to low-K_m cAMP phosphodiesterase.

One unit of cAMP phosphodiesterase activity was defined as the amount of enzyme which caused the hydrolysis of 1 pmol cAMP per min under the above conditions.

In order to measure the stimulating and inhibiting factor activities, a stimulating or inhibiting factor was added to the reaction mixture for cAMP phosphodiesterase assay.

One unit of stimulating and inhibiting factor activities was defined as the amount of factor which produced 100% stimulation of 10 μg of cAMP phosphodiesterase activity of P-2 fraction and 50% inhibition of 4 μg of solubilized cAMP phosphodiesterase activity, respectively, under the above conditions.

Protein Determination This was performed by the method of Lowry *et al.*[17] using bovine serum albumin as standard.

Results

Effect of Crude Extract from Rat Liver on cAMP Phosphodiesterase Activity As shown in Fig. 1, the maximal stimulation of cAMP phosphodiesterase activity of P-2 fraction from noninsulin-treated fat cells was observed by the addition of 30 μl of the crude extract. When P-2 fraction from insulin-treated fat cells was used, somewhat different results were obtained. The maximal stimulatory effect was attained by the addition of 20 μl of the crude extract. With the addition of the crude extract less than 30 μl, the phosphodiesterase activity of P-2 fraction

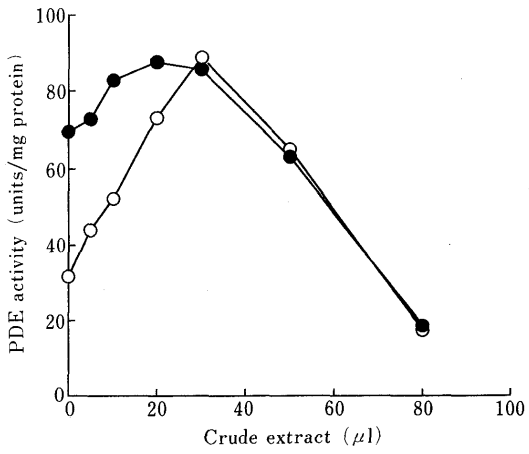

Fig. 1. Effect of Crude Extract from Rat Liver on cAMP Phosphodiesterase (PDE) Activity of P-2 Fraction from Rat Fat Cells

P-2 fraction was prepared from noninsulin- and insulin-treated fat cells and assayed for cAMP phosphodiesterase activity in the presence of varying amounts of crude extract prepared from rat liver particulate fraction. ○, P-2 fraction (10 μg protein) from noninsulin-treated fat cells; ●, P-2 fraction (10 μg protein) from insulin-treated fat cells. Data are shown for a typical experiment.

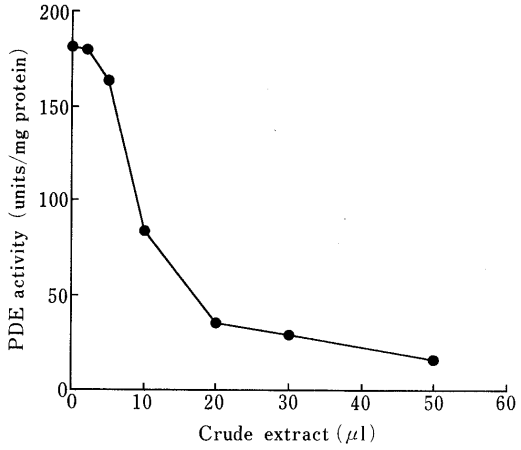

Fig. 2. Effect of Crude Extract from Rat Liver on Solubilized cAMP Phosphodiesterase Activity from P-2 Fraction

cAMP phosphodiesterase was solubilized by the addition of 0.3% Brij 58 to P-2 fraction. Solubilized enzyme activity was assayed in the presence of varying amounts of the crude extract prepared from rat liver particulate fraction. Data are shown for a typical experiment.

from insulin-treated fat cells was larger than that of P-2 fraction from noninsulin-treated fat cells. When more than 30 μl of the crude extract was added, however, almost the same amount of enzyme activity was observed in P-2 fractions from fat cells treated with and without insulin.

Effect of crude extract on the activity of solubilized cAMP phosphodiesterase from P-2 fraction from noninsulin-treated fat cells is shown in Fig. 2. The solubilized enzyme activity was progressively decreased by the addition of the crude extract and almost 50% inhibition of the enzyme activity was achieved by the addition of 10 μl of the crude extract.

These results suggest that the crude extract from rat liver particulate fraction contains two factors, one of them having a stimulating and the other an inhibiting effect on the activity of cAMP phosphodiesterase.

Separation of Stimulating and Inhibiting Factors from Rat Liver Particulate Fraction The crude extract from noninsulin- and insulin-treated rat livers was applied to a column of Sephadex G-25 (1.5 × 30 cm) equilibrated with 50 mM NH_4HCO_3 (pH 7.5). The column was eluted with the same solution. The typical elution pattern is shown in Fig. 3. Two peaks were detected; the first peak (fractions 12—21) contained a stimulating factor for the phosphodiesterase activity of P-2 fraction and the second peak (fractions 23—25) contained an inhibiting factor for the activity of solubilized cAMP phosphodiesterase. The levels of stimulating and inhibiting factors from insulin-treated rat liver were larger than those from noninsulin-treated rat liver.

Effect of Heat Treatment and Trypsin Digestion on Stimulating and Inhibiting Factors The results of heat stability of both factors are shown in Fig. 4. Heat treatment of the stimulating factor at 100 °C for less than 20 min had little effect on its ability to stimulate the cAMP phosphodiesterase activity of P-2 fraction although the ability of this factor was decreased significantly by the heat treatment at 100 °C for 30 min. Heat treatment of the inhibiting factor at 100 °C had no effect on its ability to inhibit the solubilized cAMP phosphodiesterase.

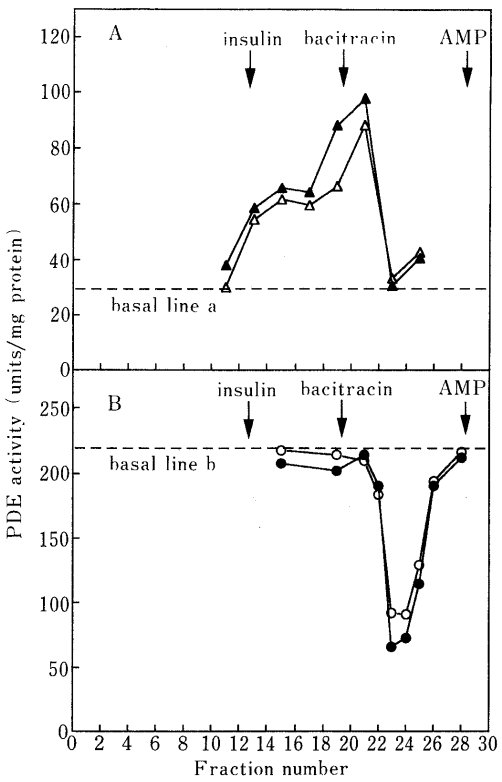

Fig. 3. Sephadex G-25 Chromatogram of the Crude Extract from Rat Liver

The crude extract (3 ml) prepared from noninsulin-(○, △) and insulin-treated (●, ▲) rat liver particulate fractions was loaded on a Sephadex G-25 (1.5 × 30 cm) equilibrated with 50 mM NH_4HCO_3 (pH 7.5) and the column was eluted with the same solution. Fractions of 2 ml were collected, lyophilized and dissolved in 0.2 ml of 1 mM formic acid. Each fraction (10 μl) was assayed for the effects on the cAMP phosphodiesterase activity of P-2 fraction (△, ▲) (A) and on the activity of solubilized cAMP phosphodiesterase (○, ●) (B). Insulin (5700), bacitracin (1420) and AMP (347) were used as molecular weight standards. Dotted lines indicate the cAMP phosphodiesterase activity of P-2 fraction (basal line a) and solubilized enzyme activity (basal line b) without the addition of the crude extract.

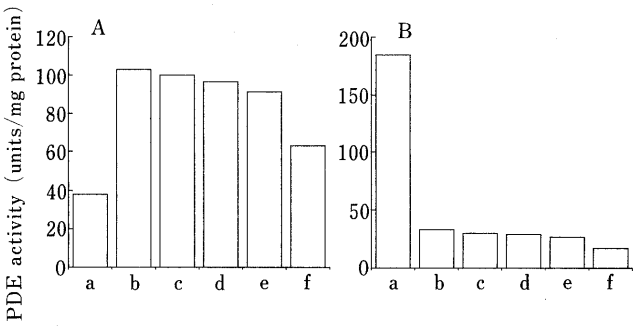

Fig. 4. Effect of Heat Treatment on Stimulating (A) and Inhibiting (B) Factors from Rat Liver

Stimulating and inhibiting factors prepared as described in the text concerning Fig. 3 were heated at 100 °C for the specified periods. (A): cAMP phosphodiesterase activity of the P-2 fraction (10 μg protein) was assayed without stimulating factor (a) and with stimulating factor (1.5 units each) heated for 0 min (b), 5 min (c), 10 min (d), 20 min (e) and 30 min (f). (B): Activity of solubilized cAMP phosphodiesterase (4 μg protein) was assayed without an inhibiting factor (a) and with an inhibiting factor (1.5 units each) heated for 0 min (b), 5 min (c), 10 min (d), 20 min (e) and 30 min (f). Data are shown for a typical experiment.

As shown in Fig. 5, digestion of the stimulating and inhibiting factors by trypsin had no effect on their ability although the enzyme activity of the P-2 fraction was increased significantly by 100 μg trypsin treatment of the stimulating factor. The reason for the latter case will be

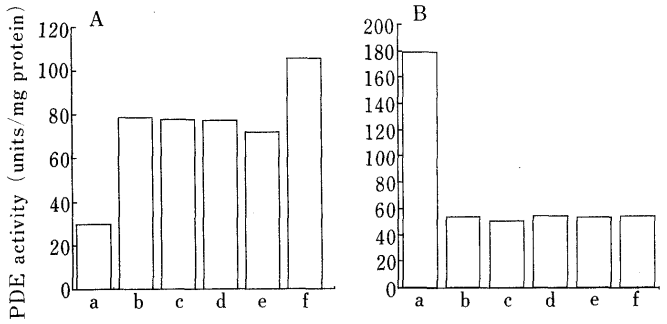

Fig. 5. Effect of Trypsin Digestion of Stimulating (A) and Inhibiting (B) Factors from Rat Liver

The stimulating and inhibiting factors dissolved in Tes–KOH (pH 7.5) were digested with trypsin at pH 7.5 for 60 min at 37 °C and then heated for 5 min at 100 °C to destroy trypsin. (A): cAMP phosphodiesterase activity of P-2 fraction (10 μg protein) was assayed without a stimulating factor (a) and with a stimulating factor (1.5 units each) treated with 0 μg/ml trypsin (b), 0.1 μg/ml trypsin (c), 1 μg/ml trypsin (d), 10 μg/ml trypsin (e) and 100 μg/ml trypsin (f). (B): Activity of solubilized cAMP phosphodiesterase (4 μg protein) was assayed without an inhibiting factor (a) and with an inhibiting factor (1.5 units each) treated with 0 μg/ml trypsin (b), 0.1 μg/ml trypsin (c), 1 μg/ml trypsin (d), 10 μg/ml trypsin (e) and 100 μg/ml trypsin (f). Data are shown for a typical experiment.

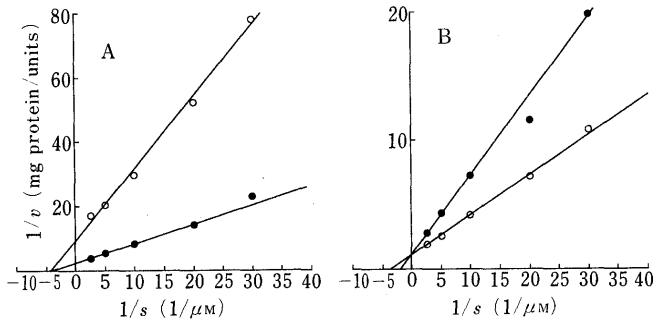

Fig. 6. Lineweaver–Burk Plot for cAMP Phosphodiesterase Activity of P-2 Fraction (A) and for the Activity of Solubilized cAMP Phosphodiesterase (B) in the Presence and Absence of a Stimulating or Inhibiting Factor

v, velocity of enzyme action; s, substrate (cAMP) concentration. A, with (●) and without (○) 1.5 units of a stimulating factor; B, with (●) and without (○) 1.5 units of an inhibiting factor. Data are shown for a typical experiment.

discussed later.

Kinetic Studies of the Action of Stimulating and Inhibiting Factors The mechanism of action of the stimulating and inhibiting factors was analyzed by means of double-reciprocal plots. As shown in Fig. 6, the stimulating factor did not significantly change the apparent K_m value of cAMP phosphodiesterase of P-2 fraction, but increased the maximal velocity of the reaction. The inhibiting factor raised the K_m value of the solubilized cAMP phosphodiesterase without affecting the maximal velocity of the reaction.

Comparison of Levels of Stimulating and Inhibiting Factors in Liver Particulate Fraction of Normal and Diabetic Rats As shown in Fig. 7, 171 and 245 units of total stimulating factor activities were contained in normal and diabetic rat livers, respectively. Thus, the level of stimulating factor was larger in the diabetic rat than in the normal rat. Total inhibiting factor activities of normal and diabetic rats were 57.1 and 28.6 units, respectively. Therefore, the inhibiting factor level of the diabetic rat was smaller than that of the normal rat.

Discussion

Our data show that stimulating and inhibiting factors are present in the rat liver particulate fraction; the former factor stimulates cAMP phosphodiesterase activity of P-2 fraction and the latter inhibits the activity of solubilized cAMP phosphodiesterase.

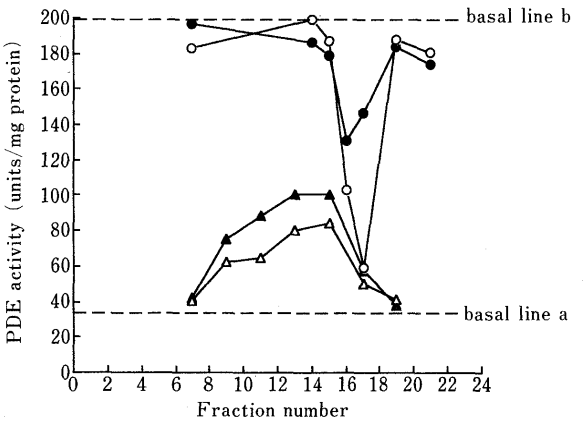

Fig. 7. Comparison of Levels of Stimulating and Inhibiting Factors in Liver Particulate Fraction of Normal and Diabetic Rats

The crude extract (3 ml) prepared from liver particulate fraction (150 ml) of normal (○, △) and diabetic (●, ▲) rats was fractionated by gel filtration on a Sephadex G-25 (1.2×35 cm) as described in the legend to Fig. 3. Each fraction (10 μl) was assayed for the effect on the cAMP phosphodiesterase activity of P-2 fraction (△, ▲) and on the activity of solubilized cAMP phosphodiesterase (○, ●). Dotted lines indicate the cAMP enzyme activity of P-2 fraction (basal line a) and solubilized enzyme activity (basal line b) without the addition of the crude extract. Typical elution patterns are given. Total stimulating and inhibiting factor activities of normal and diabetic rats were determined as follows: Fractions 9—15 (stimulating factor) and 16—17 (inhibiting factor) of both rats were pooled separately. cAMP phosphodiesterase activity in P-2 fraction was reassayed with or without 0.01 ml of the pooled fractions (1.4 ml) containing the stimulating factor of both rats. The addition of stimulating factor of normal and diabetic rats increased this enzyme activity (basal line a) by 122 and 175%; the corresponding units were 1.22 and 1.75 units, respectively. Total stimulating factor activity (units) of normal rat is $1.22 \times 1.4/0.01 = 171$ and that of diabetic rat is $1.75 \times 1.4/0.01 = 245$. Solubilized cAMP phosphodiesterase activity was reassayed with or without 0.007 ml of the pooled fractions (0.4 ml) containing the inhibiting factor of normal rat and 0.014 ml of the pooled fractions (0.4 ml) containing the inhibiting factor of diabetic rat. The addition of inhibiting factor of normal and diabetic rats resulted in 50% inhibition of solubilized cAMP phosphodiesterase activity (basal line b), which corresponded to 1 unit. Total inhibiting factor activity (units) of normal rat is $1 \times 0.4/0.007 = 57.1$ and that of diabetic rat is $1 \times 0.4/0.014 = 28.6$

Seals and Czech[18] have reported that digestion with proteases (100 μg/ml of trypsin, chymotrypsin or papain) of low molecular weight stimulating factor released from rat adipose tissue plasma membrane after insulin treatment blocks the stimulation of pyruvate dehydrogenase activity suggesting that this factor may be a peptide compound. However, Saltiel and Cuatrecasas[10] repoted that a stimulating factor generated from bovine liver particulate fraction treated with insulin was not affected by proteolytic enzyme. Our stimulating and inhibiting factors were not influenced by trypsin although the cAMP phosphodiesterase activity of P-2 fraction was increased significantly by 100 μg/ml trypsin treatment of the stimulating factor. The difference in the cAMP phosphodiesterase activities of P-2 fraction between the additions of 100 μg/ml trypsin- and nontrypsin-treated stimulating factor seems to be due to the activation of the enzyme activity of the P-2 fraction by the remaining trypsin. The stimulation of the cAMP phosphodiesterase activity of P-2 fraction by trypsin may be the result of the action of trypsin on the membrane but not on the enzyme itself because 100 μg/ml trypsin treatment of the inhibiting factor produced no effect on its inhibiting ability against the solubilized cAMP phosphodiesterase. Therefore, our results suggest that both stimulating and

inhibiting factors may not have a peptide structure, at least, necessary for their effects and susceptible to trypsin. Saltiel *et al.*[19] have suggested that the stimulating factors generated from bovine liver particulate fraction by insulin appear to be complex related carbohydrate-phosphate substances containing glucosamine and inositol. In order to clarify the structure of our stimulating and inhibiting factors, it is necessary to purify them. A low molecular weight compound in a soluble fraction from rat liver and adipose tissue has been shown to inhibit Ca^{2+}-calmodulin dependent cAMP phosphodiesterase.[20] As many cAMP phosphodiesterases are found to be present in a soluble fraction from rat adipose tissue[21] and liver,[22] it is of interest to examine whether our inhibiting factor inhibits these enzymes. Our stimulating factor stimulated the cAMP phosphodiesterase activity of P-2 fraction but had no demonstrable effect on the activity of the cAMP phosphodiesterase solubilized from this fraction with 0.3% Brij 58. These results suggest that component(s) of membrane or membrane itself may be required to exert the stimulation on cAMP phosphodiesterase activity by the stimulating factor. It is of great interest to elucidate the mechanism of stimulation of cAMP phosphodiesterase activity of P-2 fraction by this stimulating factor.

The inhibiting factor inhibited the activity of the solubilized cAMP phosphodiesterase but did not influence the cAMP phosphodiesterase activity of P-2 fraction. From these results, we can imagine the possibility that the inhibiting factor contributes to the prevention of some undesirable action by cAMP phosphodiesterase released from membrane fraction as a result of some membrane disorder.

The stimulating factor level in the liver particulate fraction of the diabetic rat was larger than that of the normal rat while the inhibiting factor level of the diabetic rat was smaller than that of the normal rat. These results suggest that the levels of both factors are regulated by insulin. Makino *et al.*[23] reported that stimulation by insulin of cAMP phosphodiesterase activity of P-2 fraction from adipose tissue of the diabetic rat was higher than that of the normal rat. Because we prepared the stimulating and inhibiting factors from rat liver particulate fraction after incubation of the fraction with 10 nM insulin for 10 min at 37 °C, one possible explanation is that the sensitivity of the particulate fraction in response to insulin to elevate the stimulating factor level and also to drop the inhibiting factor level may be increased by the diabetic state.

Acknowledgement The authors express their deep gratitude to Dr. H. Makino, Second Department of Internal Medicine, Chiba University School of Medicine, for teaching the cAMP phosphodiesterase assay method associated with the insulin treatment of fat cells.

References

1) R. W. Butcher, J. G. T. Sneyd, C. R. Park and E. W. Sutherland, *J. Biol. Chem.*, **241**, 1651 (1966).
2) V. C. Manganiello, F. Murad and M. Vaughan, *J. Biol. Chem.*, **246**, 2195 (1971).
3) E. G. Loten and J. G. T. Sneyd, *Biochem. J.*, **120**, 187 (1970).
4) V. Manganiello and M. Vaughan, *J. Biol. Chem.*, **248**, 7164 (1973).
5) C. M. Heyworth, A. V. Wallance and M. D. Houslay, *Biochem. J.*, **214**, 99 (1983).
6) J. Larner, G. Galasko, K. Cheng, A. A. DePaoli-Roach, L. Huang, P. Daggy and J. Kellogg, *Science*, **206**, 1408 (1979).
7) L. Jarett and J. R. Seals, *Science*, **206**, 1407 (1979).
8) F. L. Kiechle, L. Jarett, D. A. Popp and H. Kotagal, *Diabetes*, **29**, 852 (1980).
9) A. R. Saltiel, S. Jacobs, M. Siegel and P. Cuatrecasas, *Biochem. Biophys. Res. Commun.*, **102**, 1041 (1981).
10) A. R. Saltiel and P. Cuatrecasas, *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 5793 (1986).
11) A. Junod, A. E. Lambert, W. Stauffacher and A. E. Renold, *J. Clin. Invest.*, **48**, 2129 (1969).
12) A. Genba, M. Yanaka, K. Odaki and K. Kondo, *Rinsho Byori*, **11**, 116 (1963).
13) M. Rodbell, *J. Biol. Chem.*, **239**, 375 (1964).
14) H. Makino, A. Kanatsuka, M. Osegawa and A. Kumagai, *Biochim. Biophys. Acta*, **704**, 31 (1982).
15) M. Osegawa, H. Makino, A. Kanatsuka and A. Kumagai, *Biochim. Biophys. Acta*, **721**, 289 (1982).
16) T. Kono, F. W. Robinson and J. A. Sarver, *J. Biol. Chem.*, **250**, 7826 (1975).
17) O. H. Lowry, N. J. Rosebrought, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **191**, 265 (1951).
18) J. R. Seals and M. P. Czech, *J. Biol. Chem.*, **255**, 6529 (1980).
19) A. R. Saltiel, J. A. Fox, F. Sherline and P. Cuatrecasas, *Nature* (London), **233**, 967 (1986).
20) R. H. Rao, M. Palazzolo, L. Sander, J. A. Smoake and S. S. Solomon, *Life Sci.*, **35**, 2155 (1984).
21) H. W. Webere and M. M. Appleman, *J. Biol. Chem.*, **257**, 5339 (1982).
22) B. E. Lavan, T. Lakey and M. D. Houslay, *Biochem. Pharmacol.*, **38**, 4123 (1989).
23) H. Makino, M. Osegawa, A. Kanatsuka and T. Suzuki, *Endocrinology*, **113**, 646 (1983).