Nature of the inhibitory effect of collagenase on phosphodiesterase activity

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Abstract The level of phosphodiesterase (PDE) activity is lower in collagenase-isolated human fat cells than in adipose tissue fragments. The inhibition is not species-specific since collagenase also inhibits PDE in rat adipose tissue and bovine heart. In subcellular fractions from isolated fat cells, the PDE activities were lowest in the plasma membrane-enriched fractions and highest in the cytosolic fractions. This is opposite to PDE in subcellular fractions obtained from adipose tissue fragments. In dose-response experiments, collagenase inhibited particulate PDE to a much larger extent than it inhibited soluble PDE. The extracellular activities of PDE were completely eliminated by collagenase. Repeated washings or reincubation of the isolated fat cells did not restore the PDE activity. A purified collagenase with low specific protease activity reduced the PDE activity in isolated fat cells to a lesser extent than did a collagenase with high specific protease activities. Collagen and several protease inhibitors were ineffective in preventing the reduction of PDE after exposure to collagenase. It is concluded that nonspecific proteases in the collagenase preparations used for fat cell isolation interact with particulate and soluble PDE causing an irreversible inhibition of PDE activity in isolated fat cells. Of the various forms of PDE, plasma membrane-associated PDE seems most sensitive to collagenase. —**Engfeldt, P., P. Arner, and J. Östman.** Nature of the inhibitory effect of collagenase on phosphodiesterase activity. *J. Lipid Res.* 1985. **26:** 977–981.

Supplementary key words adipose tissue • protease inhibitors • fat cell • cyclic AMP

Fat cells isolated with collagenase by Rodbell's method (1) are often used when the hormonal regulation of metabolic processes is studied. It has recently been reported (2) that collagenase-isolated human fat cells display a lower cyclic AMP phosphodiesterase (PDE) activity than adipose tissue fragments from the same donor; the decrease in PDE activity in the isolated fat cells is accompanied by increased cAMP and lipolytic responses to catecholamines, as compared to adipose tissue fragments. It was suggested that the fall in PDE activity in the isolated fat cells was caused by collagenase or by some of the proteases or other contaminants in the collagenase preparations. It has also been reported (3) that the proteases present in crude collagenases can activate adenylate cyclase. This, in combination with the changed PDE activity, may be the cause of the observed increased cAMP levels and lipolytic responses to catecholamines in isolated fat cells as compared to adipose tissue fragments. Thus, results obtained from isolated fat cells may not necessarily reflect the physiological conditions. The present investigation was conducted to further analyze the mechanism of the inhibitory effect of collagenase on PDE in human fat cells and to determine whether this effect is also present in fat cells of other species.

MATERIALS AND METHODS

Subcutaneous adipose tissue was obtained at operation from patients undergoing elective abdominal surgery. None of the patients had jaundice, metabolic disorders, or malignant diseases. No attempt was made to select the patients on the basis of age, sex, or body weight. General anesthesia was induced with a short-acting barbiturate and maintained by neuroleptanalgesia (fentanyl and droperidol) in combination with N_2O and O_2. The patients fasted overnight and only saline was infused intravenously before the adipose tissue was removed at the beginning of surgery. The specimen of adipose tissue was transported to the laboratory in saline.

The study was approved by the Ethics Committee of the Karolinska Institute.

Epididymal fat pads were obtained from Sprague-Dawley rats (150–300 g) that were fed ad libitum.

Isolation and subcellular fractions of fat cells

The adipose tissue specimen was cut into fragments, each weighing about 10 mg. The cells were isolated from the stroma by incubation for 60 min with various concentrations of collagenase, specified elsewhere, in Krebs-Henseleit bicarbonate buffer containing 40 mg of bovine serum albumin per ml. Rodbell's method (1), as modified by Smith, Sjöström, and Björntorp (4) was used. To pre-

Abbreviation: PDE, phosphodiesterase.

pare an extract for the PDE assay, the fat cells were re-suspended in 100 mM Tris buffer (pH 7.5) containing 3 mM Mg^{2+}. The procedure was then the same as that for the tissue fragments (see below), except that the homogenization step was omitted. About 5 ml of packed fat cells was re-suspended in 25 ml of 0.25 M sucrose and homogenized by six up-and-down strokes in a glass Potter-Elvehjem homogenizer fitted with a Teflon pestle driven at 1200 rpm. The crude extract was defatted by centrifugation (1000 *g* for 60 sec at 20°C), and the infranatant was aspirated with a syringe from below the fat plug. This solution was centrifuged in the manner described by Jarett (5) to obtain three fractions: a plasma membrane-enriched fraction, a microsomal-enriched fraction, and a cytosolic fraction. The protein content was estimated by the method of Lowry and co-workers (6).

Preparation of tissue extracts for the PDE assay

About 100 mg of adipose tissue was cut into 10-mg pieces and incubated in Krebs-Henseleit bicarbonate buffer with 40 mg/ml of bovine serum albumin for 60 min at 37°C. The fragments were then washed in buffer and homogenized in a glass homogenizer (Termo Glas AB, Sweden) with 100 mM Tris buffer (pH 7.5) containing 3 mM Mg^{2+}. The homogenate was sonicated for 10 sec and centrifuged at 800 *g* at 4°C for 10 min. The liquid phase was removed with a syringe for the PDE assay and the lipids were extracted by the method of Dole and Meinertz (7) for determination of the lipid weight. The tissue extracts from adipose tissue fragments, isolated fat cells, and subcellular fractions were kept frozen at −80°C for subsequent assay.

Extracellular degradation and cellular uptake of ^{32}P-labeled cAMP

Isolated fat cells from human subjects were incubated in Krebs-Henseleit bicarbonate buffer with 40 mg per ml albumin and 0.1 μM ^{32}P-labeled cAMP. After different time periods, 0.15 M $ZnSO_4$ was added to an aliquot of the medium to stop the reaction and thereafter 0.15 M $Ba(OH_2)$ was added to precipitate nucleotides other than ^{32}P-labeled cAMP. After centrifugation the radioactivity in the supernatant was determined. The isolated fat cells were centrifuged through silicone oil for determination of the uptake of radioactivity into fat cells.

Determination of PDE activity

The PDE activity was measured by Pöch's method (8). Usually, the substrate was ^{32}P-labeled cAMP in a concentration of 0.1 μM. In the enzyme kinetic studies, however, the following concentrations of ^{32}P-labeled cAMP were used: 0.0125, 0.025, 0.05, 0.1, 0.2, 6.25, 12.5, 25, and 50 μM. Tris buffer, 100 mM, containing 3 mM Mg^{2+} was employed. The reaction was initiated by adding ^{32}P-labeled cAMP and terminated with 0.15 M $ZnSO_4$, when 10–20% of the cAMP had been degraded (after 5–120 min). To precipitate nucleotides other than ^{32}P-labeled cAMP, 0.15 M $Ba(OH)_2$ was added. By incubating at least four aliquots of a particular concentration of cAMP for four different periods, the initial speed of degradation was calculated, using a computerized curve adjustment. In the enzyme kinetic analysis, the Lineweaver-Burk (9) plots of the PDE activities showed that each curve could be approximated to two straight lines, which suggested the presence of two forms of the enzyme with different K_m values. This is in accordance with previous findings in human adipose tissue (2, 10). When the data are calculated from Lineweaver-Burk plots, the activity of the high K_m form may influence that of the low K_m form. It is possible to correct for this influence by using the method described by Spears, Sneyd, and Loten (11). This method was employed for calculating a "corrected" K_m and V_{max} of the high and low affinity forms of PDE. Various models for the expression of the PDE activity are now available (9, 12). Some investigators have employed a model based on one form of the enzyme with negative cooperative properties (12). The model we used is based on two non-interacting forms of the enzyme with different affinities for the substrate. It is not clear at present which model is more accurate. The coefficient of variation for PDE was about 10% when multiple tissue samples from one individual were assayed, and about 5% when one tissue sample was used for multiple enzyme determination.

Chemicals

Bovine serum albumin, fraction V, was purchased from the Armour Pharmaceutical Company, Eastbourne, England. Collagenase prepared from *Clostridium histolyticum* was of Sigma type I (Lot no. 70 F-0036), unless otherwise stated. Trypsin was of Sigma type III (bovine pancreas). Collagen type I (bovine achilles tendon), antipain, bacitracin, phospholipase C type I, and crude bovine heart PDE were all from Sigma. Aprotinin and trypsin inhibitor (hen egg white) were from Boehringer-Mannheim, West Germany. [^{32}P]Adenosine 3',5'-monophosphate (ammonium salt, sp act 2–20 Ci/mmol) was obtained from Radiochemical Centre, Amersham, England.

Statistical methods

The values presented are the mean and the standard error of the mean. The Student's *t*-test (13) was used for statistical comparisons of the experimental results.

RESULTS

When the PDE activity was compared in crude extracts from isolated fat cells and adipose tissue fragments, it was

found that the apparent V_{max} of the low and high K_m forms of PDE was significantly higher ($P < 0.01$) in adipose tissue fragments (**Table 1**). Similar findings were obtained with adipose tissue from the human subjects and rats. The apparent K_m values were also significantly ($P < 0.05$) higher in the adipose tissue fragments than in isolated fat cells of both species. In Table 1, the PDE activities were expressed in terms of lipid weight; similar results were obtained if the activities were expressed in terms of the amount of protein. It is also evident from Table 1 that the apparent V_{max} was higher in the rat tissue preparations than in the human tissue preparations, except for the high K_m form of PDE in isolated fat cells.

Isolated fat cells were prepared from human adipose tissue, using a low (0.5 mg/ml) and a high (3 mg/ml) collagenase concentration; thereafter subcellular fractions were prepared from the adipocytes and the fractions were submitted to PDE analysis (**Fig. 1**). It was found that the PDE activity was lower in the plasma membrane- and microsome-enriched fractions than in the soluble fraction. The PDE activities were also lower in all the subcellular fractions obtained from the cells isolated with a high collagenase concentration compared to cells isolated using a low collagenase concentration ($P < 0.01$). However, the reduction of PDE activity induced by a high collagenase concentration was much less pronounced in the cytosolic fraction (30%) than in the plasma membrane-enriched and microsome-enriched fractions (55% and 90%, respectively).

In other experiments (data not shown) isolated fat cells from human subjects were incubated in Krebs-Henseleit bicarbonate buffer with albumin and ^{32}P-labeled cAMP as described in Materials and Methods. It was found that the amount of ^{32}P-labeled cAMP in the medium decreased with time in a linear fashion; about 7 pmol of ^{32}P-labeled cAMP disappeared per min per g lipid. This indicated that isolated fat cells may possess an extracellular capacity that leads to degradation of ^{32}P-labeled cAMP. The degradation was abolished 60 min after the addition of collagenase (5 mg/3 ml) to the buffer. The uptake of ^{32}P-labeled cAMP into the cells was less than 3% of the reduction of ^{32}P-labeled cAMP.

In another set of experiments (not shown), the effects of various fat cell isolation procedures on PDE activity were investigated. Repeated washes (10 vs 3 times) of collagenase-isolated fat cells led to a small but insignificant increase of the reduced PDE activity (expressed in terms of lipid weight) in the cells as compared to adipose tissue fragments incubated in parallel but without collagenase. Furthermore, isolation of the cells in the presence of excess amounts of collagen (20 mg/ml) or reincubation of the cells for different periods (10–60 min) in buffer without collagenase did not change the PDE activity.

Fat cells were also isolated using a very pure collagenase preparation (Sigma type VII), which contained a small amount of proteolytic activity and a high concentration of collagenase. The PDE activity in fat cells was inhibited to a much lower degree with this collagenase preparation than with a crude type I collagenase, which has a high proteolytic activity and low collagenase activity (**Table 2**).

To further examine the mechanisms behind the PDE inhibitory effect of collagenase, a partly purified PDE extract from bovine heart was used. It was noted (data not shown) that the inhibitory effect of collagenase after 60 min of incubation was dose-dependent. It increased with increasing concentrations of collagenase. The PDE activity was about 15% of the control at 1.5 mg/ml of collagenase and 50% at a concentration as low as 0.05 mg/ml of collagenase. The protease inhibitors antipain (0.1 mM), aprotinin (500 × 10^3 U/ml), bacitracin (2 mg/ml), and trypsin inhibitor (10 mg/ml) did not reverse the inhibitory effect of collagenase. Trypsin (0.1 mg/ml) strongly inhibited bovine heart PDE (to 10% of the control); this inhibition was only partly reversed by trypsin inhibitor (10 mg/ml, to 60% of the control). The addition of an excess amount of collagen (20 mg/ml) to the enzyme extract did not prevent the inhibition by collagenase. Phospholipase C in a high concentration (100 units/ml) inhibited bovine heart PDE to about 90% of the control.

TABLE 1. Comparison between phosphodiesterase (PDE) activity of isolated fat cells and of tissue fragments obtained from human subcutaneous adipose tissue ($n = 7$) and rat epididymal adipose tissue ($n = 6$)

	Low K_m PDE				High K_m PDE			
	K_m		V_{max}		K_m		V_{max}	
	Human	Rat	Human	Rat	Human	Rat	Human	Rat
	μM		*pmol/min per g lipid*		*μM*		*pmol/min per g lipid*	
Isolated fat cells	0.11 ± 0.03	0.14 ± 0.03	9 ± 3	60 ± 9	78 ± 10	28 ± 3	2810 ± 460	2770 ± 650
Adipose tissue fragments	0.32 ± 0.07	0.37 ± 0.09	90 ± 21	240 ± 23	95 ± 8	62 ± 14	8500 ± 480	12450 ± 1480
P	< 0.05	< 0.05	< 0.01	< 0.01	< 0.05	< 0.05	< 0.01	< 0.01

Fragments and isolated fat cells were prepared from the same adipose tissue specimen. The fat cells were obtained after isolation with collagenase (5 mg/3 ml). Kinetic data were obtained from Lineweaver-Burk plots with ten different concentrations of ^{32}P cAMP ranging from 0.0125 μM to 50 μM. Values are mean ± standard error of mean. *P* denotes the statistical significant difference between isolated fat cells and adipose tissue fragments obtained with Student's paired *t*-test.

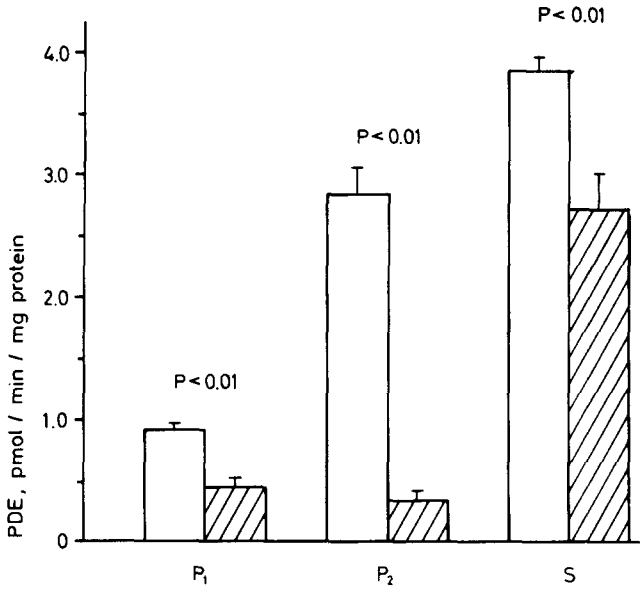

Fig. 1. Phosphodiesterase (PDE) activity in subcellular fractions from isolated fat cells obtained after isolation with 0.5 mg/ml (open bars) or 3 mg/ml (hatched bars) of collagenase. The PDE activity was assayed in quadruplicate with 0.1 μM cyclic AMP as substrate. The plasma membrane-enriched fractions are designated P_1, the microsome-enriched fractions P_2, and the cytosolic fractions S. One representative experiment of two is shown. The Student's unpaired *t*-test was used for the statistical analysis. Values are mean ± SE.

DISCUSSION

This study has demonstrated that collagenase inhibits various forms of PDE. The inhibitory effect was present in both isolated human and rat fat cells, as well as in a preparation from bovine heart. Thus the effect of collagenase was not species-specific. It should be noted that collagenase was a very potent inhibitor, since the PDE activity could be almost completely inhibited by the enzyme. It has been reported by Schönhöfer et al. (14) that the PDE activity was higher in the adipose tissue fragments than in isolated fat cells in the rat. In the latter study, however, the PDE activity was measured with only one rather high concentration of cAMP, which reflected mainly the high K_m form of the enzyme.

One may wonder whether the very low PDE activity in isolated fat cells (10–30% of the activity in adipose tissue fragments) is due to the loss of stroma cells from the isolated fat cells during the isolation procedure. However, this was probably not the case since we found earlier that the PDE activity in stroma cells represents only 1–2% of the total activity in human adipose tissue (15). Thus the effect of collagenase is probably directly associated with the PDE activity in the fat cells.

In the experiment with subcellular fractions obtained from isolated fat cells, the lowest specific activities of PDE were found in the plasma membrane- and microsome-enriched fractions and the highest activities in the cytosolic fractions. There were even greater differences between membrane-bound activity and soluble activity when high concentrations of collagenase were used for the isolation of the fat cells. These findings were in contrast to our previous findings with subcellular fractions obtained from adipose tissue fragments, in which we found a reverse relationship (15). This indicates that the major inhibitory effect of collagenase is on the low K_m particulate form of PDE located at the plasma membrane. However, soluble high K_m PDE was reduced in isolated adipocytes. Furthermore, when these cells were prepared using a high collagenase concentration (3 mg/ml), PDE in the microsomal enriched fraction was markedly reduced. These findings support the idea that the inhibitory effect of collagenase on PDE is also in the interior of the cell, especially when high concentrations of collagenase are used. The finding that isolated fat cells possess extracellular cAMP degradation activity may depend on an uptake of ^{32}P-labeled cAMP by the fat cells through passive diffusion, as has been reported for other tissues (16). However, the uptake of ^{32}P-labeled cAMP into the fat cells was found to be small and thus it is probable that the observed reduction of ^{32}P-labeled cAMP indeed represented extracellular degradation. Thus the collagenase-isolated fat cells washed free of collagenase possess a plasma membrane PDE activity (16, 17). This PDE activity was completely inhibited when collagenase again was added. Together with the above mentioned results, this indicates that a small part of the inhibitory effect of collagenase was reversible but the main inhibitory effect was irreversible.

It is well known that the collagenase preparations that are used for isolation of fat cells contain some contaminants, which are primarily different proteases and phospholipases (18). It has been stated that a small amount of protease activity must be present to obtain complete cell isolation (5, 19). In the experiments where the effect of a collagenase preparation with a high collagenase activity

TABLE 2. Enzymatic activities in two different types of collagenase and the phosphodiesterase (PDE) activity in fat cells isolated with each type

	Collagenase	Clostripain	Caseinase	Tryptic Activity	PDE Activity
Collagenase type I	290	0.08	84	0.017	36
Collagenase type VII	630	0.06	0.14	0.003	79

The enzymatic activities were obtained from the figures given by the manufacturer (Sigma) and are expressed in units per ml. The PDE activity was analyzed with 0.1 μM [^{32}P]cAMP as substrate and the results are expressed as the per cent of the control. Control values represent PDE activity in adipose tissue fragments incubated in parallel but without collagenase (n = 4).

but relatively low protease activity was tested, the reduction in the PDE activity was less than with the collagenase preparation that had low collagenase activity but high protease activity. Incubation of the isolated fat cells in buffer without collagenase and isolation in the presence of high concentrations of collagen did not reverse the reduction in PDE activity. However, extensive washings of the isolated fat cells led to a smaller loss of PDE activity. These findings favor the idea that the reduced PDE activity in isolated fat cells was at least partly due to contaminating proteases in the collagenase preparations rather than to the collagenase enzyme per se, and that the inhibition was mainly irreversible. It is possible that other impurities, such as phospholipases, may also be responsible for the reduced PDE activity in isolated fat cells. However, it is unlikely that phospholipases are of major importance in this respect since we found that very high concentrations of phospholipase C—much higher than the small amounts that can be present in crude collagenase preparations—reduced bovine heart PDE activity by only 10%.

In an attempt to further explore the mechanisms behind the inhibitory effect of collagenase on PDE, we examined the effect of different protease inhibitors in a partly purified PDE (bovine heart). It was found that protease inhibitors effective against serine and/or thiol proteases were unable to reverse the inhibitory effect of collagenase on PDE. Furthermore, it was observed that trypsin strongly inhibited PDE and that this inhibition could be partly reversed by a high concentration of trypsin inhibitor. It is, however, unlikely that collagenase-induced inhibition of PDE is due to trypsin contamination, since the addition of trypsin inhibitor to collagenase did not change the PDE activity. Thus it would appear from the present study that the inhibited PDE activity in collagenase-isolated fat cells probably was dependent on the nonspecific proteases in the collagenase preparation. These proteases act mainly on the particulate high-affinity form of PDE, including the PDE located at the plasma membrane, leading to an inhibition of PDE. The inhibitory effects of the proteases are also evident on PDE located in the interior of the cell, especially when high concentrations of collagenases were used.

The findings emphasize the importance of using as low a concentration of collagenase as possible in order to avoid as far as possible collagenase-induced changes in the metabolic processes of collagenase-isolated fat cells; otherwise there may be misinterpretations of results obtained from experiments with collagenase isolated fat cells.

This study was supported by grants from the Swedish Medical Research Council, the Swedish Diabetes Association, the Karolinska Institute, and the Osterman, Groschinsky, Tore Nilsson, Hierta, Folksam and Wiberg Foundations.

Manuscript received 3 December 1984.

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