Effects of Experimental Diabetes and Insulin on Cyclic AMP Phosphodiesterase and its Protein Activator in Rat Adipose Tissue

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SUMMARY

Streptozotocin-induced diabetes in the rat has been shown to decrease and insulin to increase the activity of cyclic AMP phosphodiesterase (PDE) in adipose tissue. Protein activator (PA), a substance of low molecular weight, is essential for full activity of some component phosphodiesterases. A significant decrease in protein activator level (60% of control) is found in the 13,000 × *g* boiled supernatant from streptozotocin-diabetic rat adipose tissue. When diabetic animals are treated with insulin 24 h following the induction of diabetes, protein activator is restored to 80% of the normal control level; when diabetic animals are immediately treated with insulin, the levels of protein activator are not reduced (98% of control). Hence, insulin is capable of reversing both the decrease in protein activator and phosphodiesterase activity seen with diabetes. When partially purified supernatant from diabetic animal fat is chromatographed on a DEAE Sephacel column, the untreated diabetics show inhibition, whereas insulin-treated diabetics show maximal stimulation of the low K_m cyclic AMP phosphodiesterase. Protein activator and an inhibitor are readily separated when adipose tissue homogenate is centrifuged at 13,000 × *g* and the boiled supernatant is passed over a DEAE Sephacel column.

Diabetes is associated with a decrease in both cyclic AMP phosphodiesterase and protein activator activity, both of which are restored to normal by administration of insulin. We also report the presence of an inhibitor of activator, which is increased in streptozotocin diabetes. All of these components appear to play a role in the pathophysiology of diabetes.

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Insulin was first shown to stimulate cyclic AMP phosphodiesterase (PDE-EC 3.1.4.C) activity from adipose tissue in 1970.[1] Subsequently, streptozotocin-diabetic rats were shown to have a decrease in PDE activity.[2,3] Insulin appears to stimulate a specific "low" K_m cyclic AMP PDE activity in adipose tissue separated by disc gel electrophoresis.[4] Cheung et al.[5] and Kakiuchi et al.[6] have also demonstrated a low molecular weight protein activator (PA) essential for full activation of some forms of PDE. This material is identical to calmodulin.[18] More recently, Wang and Desai[7] and Wallace and Cheung[8] demonstrated a competitive inhibitor of PA. In light of the above, we have studied the inactivation of PDE in experimental diabetes and its restoration by insulin. Furthermore, we have examined the time course of reduction of PA activity in experimental diabetes and the effects of insulin on PA. We also report the presence of an inhibitor(s) of PA in adipose tissue, substantially different from the one described in neural tissue.[7]

MATERIALS AND METHODS

Holtzman rats (250–300 g) were rendered diabetic by streptozotocin, 65 mg/kg body wt. The drug was supplied by the Upjohn Company (Kalamazoo, Michigan). Control animals received an injection of normal saline. Following injection, the rats were maintained on Purina laboratory chow in metabolic cages for 3 days during which time daily weights, urine volumes, and the absence or presence of glucose and ketone bodies in the urine were recorded. At the time the rats were killed, plasma glucose and immunoreactive insulin measurements were made.[2,9] In other experiments, animals rendered diabetic with streptozotocin were injected immediately with 8–12 U/day mixture of NPH and regular insulin. These animals were maintained for varying periods of time until they were killed, and fat pads were assayed for PDE or PA. In another experiment, animals rendered diabetic by streptozotocin were allowed to develop their diabetes for 24 h, and were treated with a mixture of regular and NPH insulin, (8–12 U/day) and then killed at the times indi-

cated. PDE and PA were prepared from the adipose tissue, and PDE assay was performed as outlined elsewhere.[2,4,9,10] The heat-stable, calcium-sensitive protein activator or calmodulin was prepared from rat fat pads by homogenization, sonication, adjustment of pH to 5.9, and centrifugation at 13,000 × *g*. The resultant supernatant was boiled for 5 min, rapidly cooled, and centrifuged as above. The last step of this procedure was repeated. The supernatant contained PA (60 μg protein/ml).[2] Cyclic AMP PDE was assayed by a modification[10] of the method of Thompson and Appleman.[11] PDE activity in adipose tissue was measured in the 100,000 × *g* supernatant prepared from rat fat pads as described elsewhere.[9] PDE activity was measured at high and low K_m cyclic AMP and cyclic GMP substrate concentrations (low, 5×10^{-7} M; high, 5×10^{-5} M).

Protein activator was assayed by measuring the stimulation of PA-deficient PDE prepared by DEAE cellulose column chromatography of partially purified beef heart PDE (Sigma, St. Louis, Missouri) in the absence and presence of added calcium. PA prepared from several rat tissues consistently produced a four- to fivefold stimulation of activator-deficient beef heart PDE. PA levels were measured at two concentrations (1:1, 60 μg/ml; and 1:5, 12 μg/ml) to determine the optimal level of stimulation. The amount of protein activator was determined by its ability to activate the activator-deficient beef heart PDE and was expressed as relative activator activity per milligram of protein in diabetics compared with control. Protein concentration refers to total protein of the boiled extracts. PA activity was assayed in the linear range of the dose response curve. In light of inhibitor, subsequently identified and discussed later in this article, activator-like activity is relative and represents at a minimum the contribution of both activator and inhibitor. The total amount of protein activator present in the adipose tissue was also determined per weight of tissue.

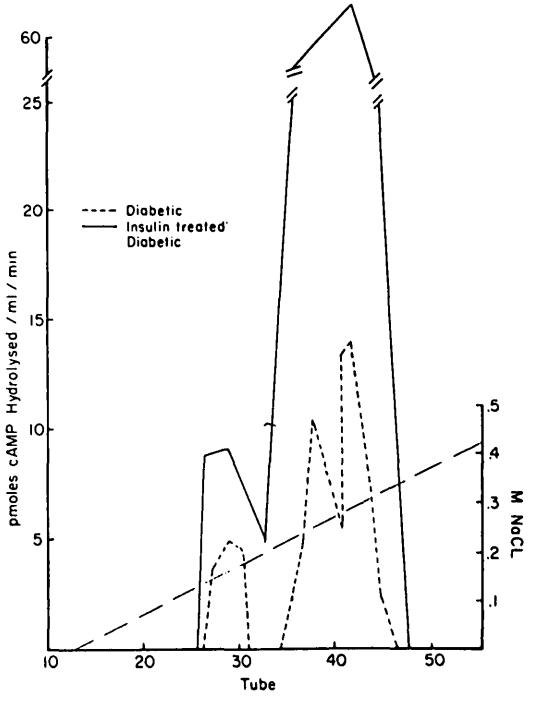
Column chromatographic procedures were done using DEAE Sephacel and a standard salt gradient.[12] When the 100,000 × *g* supernatant of rat adipose tissue from normal or diabetic animals was placed on a column "low" and "high" K_m cyclic AMP PDE and "low" K_m cyclic GMP PDE were separated on the column. Similarly, when boiled homogenate, prepared as outlined above, was placed on the ion exchange column and salt gradient was applied, protein activator and inhibitor were readily identified by PDE assay in the absence and presence of activator, respectively.[2,8] Inhibitor "content" was quantitated by separation and comparison made after DEAE Sephacel chromatography of five individual normal or "streptozotocin-diabetic" rat fat pads. Characterization of the PA and inhibitor was accomplished by hydrolysis with 6 N HCl and reaction with ninhydrin[13] and chromatography on Sephadex G-50 and G-75.[19] Kinetics for activator and inhibitor were conducted as outlined earlier.[2,10] Statistical analyses were done by Student's *t* test.[15]

RESULTS

The clinical data of normal and diabetic animals, which allow us to assess the severity of the streptozotocin-induced diabetes, are not presented again.[2] By day 3, diabetic animals had lost substantial weight, whereas diabetic animals treated with insulin had not lost as much weight. In contrast to both these groups, control rats had gained 15 g. In addition, increased urinary output with positive urine glucose and acetone were found in the diabetic animals, but not in the controls. The insulin-treated diabetic rats exhibited glycosuria but no ketonuria. Elevated plasma glucose and decreased immunoreactive insulin values in the diabetic rats further confirm the differences between control, streptozotocin-diabetic, and insulin-treated streptozotocin-diabetic rats. Although plasma insulin levels were equal in control and insulin-treated diabetic rats, plasma glucose remained elevated in these insulin-treated diabetic rats.

When normal rat adipose tissue is prepared and the supernatant is chromatographed on a DEAE Sephacel column, the elution pattern separates specific component cyclic nucleotide phosphodiesterases and PA (data not shown). Figure 1 illustrates chromatography of 100,000 × *g* supernatant prepared from diabetic and insulin-treated diabetic adipose tissue. In these experiments, comparable amounts of material (mg protein) were applied to the column for each experimental group. Figure 1 demonstrates the activity of low K_m cyclic AMP PDE. A marked stimulation (10-fold) of low K_m cyclic AMP PDE activity in the insulin-treated diabetic animals compared with diabetic animals alone is observed. In similar preparations, no stimulation by insulin of high K_m cAMP or low K_m cGMP was observed (data not shown). Data shown in Figure 2 illustrate levels of rat adipose tissue PA in control, diabetic (day 3), and insulin-treated diabetic rats killed 5 days after induction of the diabetes. The data demonstrate a decrease in protein activator in the diabetic animals ($P < 0.02$, compared with controls) and restoration toward normal after administration of insulin by day 5 ($P < 0.05$, compared with untreated diabetic). Similar data are presented in Figure 3, where insulin

FIGURE 1. DEAE Sephacel column chromatography of 100,000 × *g* supernatant of rat adipose tissue from streptozotocin-diabetic and insulin-treated streptozotocin-diabetic animals. PDE activity is shown as picomoles cAMP hydrolyzed/ml/min. The concentration of cAMP used in these experiments is 5×10^{-7} M identifying the "low" K_m component.



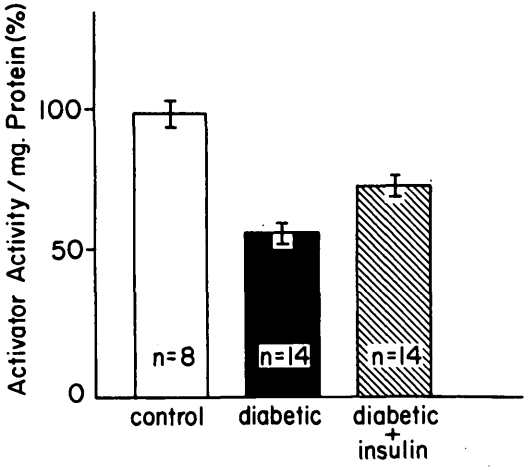


FIGURE 2. Measurement of protein activator (PA) /mg protein in adipose tissue from normal, streptozotocin-diabetic, and insulin-treated streptozotocin-diabetic. PA was measured in the 13,000 × *g* supernatant and was assayed by its ability to activate PDE at a cAMP concentration of 5×10^{-7} M (see METHODS). Control animals values were set at 100% ± SEM. Statistical comparisons are shown, with PA from diabetic lower than control ($P < 0.02$) and insulin-treated diabetic higher than diabetic alone ($P < 0.05$).

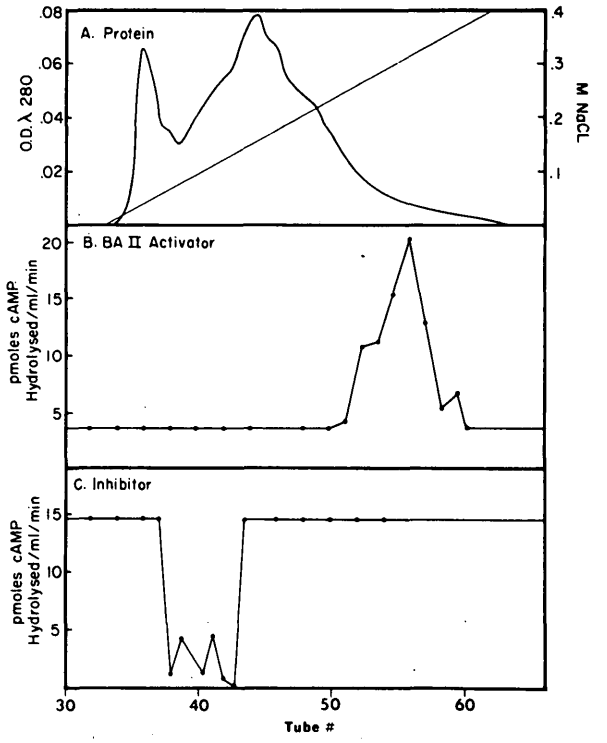
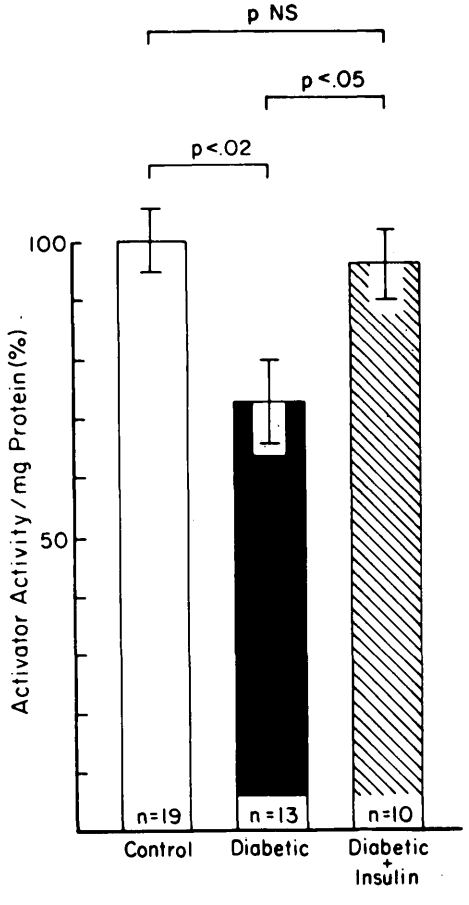


FIGURE 4. DEAE Sephacel column chromatography of rat adipose tissue protein activator preparation (see METHODS) using a salt gradient—(A) protein, λ280; (B) PA activity; and (C) inhibitor activity. Assays performed as described in the text. This is a typical experiment of three experiments all showing the same qualitative data.

FIGURE 3. Measurement of PA/mg protein in adipose tissue from normal, streptozotocin-diabetic, and insulin-treated streptozotocin-diabetic rats. Please see METHODS and the legend for Figure 2 for further details. Statistical comparisons are shown with PA from diabetics lower than controls ($P < 0.02$) and insulin-treated diabetics higher than untreated diabetics ($P < 0.05$). Insulin-treated diabetic rats do not differ significantly from control (P = NS).



has been administered immediately after induction of streptozotocin diabetes. Protein activator level is maintained at 98% of control when clinical diabetes has not been allowed to develop (P = NS, treatment from control). Clinical data on these animals shows control of blood glucose to normal (160 mg/dl) in insulin-treated diabetic rats compared with blood glucose of 350 mg/dl in these untreated diabetic rats. Column chromatography of the PA preparation is illustrated in Figure 4. Here PA activity has been assayed and inhibitor activity has been determined by assay of phosphodiesterase in the presence of PA. Two peaks, one of "activator activity" and the other of "inhibitor activity," are separated from each other by the salt gradient. Studies illustrated in Table 1 document the protein nature of the activator, which shows an increase in ninhydrin reactivity and loss of ability to stimulate PDE after acid hydrolysis. Acid hydrolysis of inhibitor failed to increase ninhydrin reactivity but did not destroy its biologic activity. Standard Sephadex chromatography on G-50 (Inhibitor) and G-75 (Activator) revealed mol.

TABLE 1
Partial characterization of activator and inhibitor

	Hydrolysis shows increase in ninhydrin reaction	Biologic activity after hydrolysis*	Mol. wt.‡
Activator†	+	−	≅ 20,000
Inhibitor†	−	+	≅ 2,500

* By respective assays for activator and inhibitor given in text.
† These materials are obtained as separate "single peaks" off the ion exchange column as described in the text.
‡ Sephadex G-50 and G-75.[19]

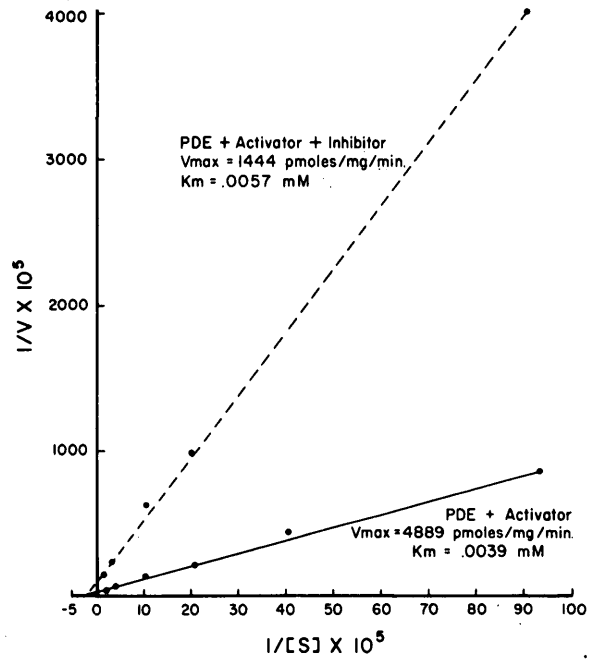


FIGURE 5. Lineweaver-Burke kinetics of rat adipose tissue activator-poor PDE; + PA; + PA + Inhibitor. Materials used in these studies were obtained from columns as described in the legend to Figure 4 (N = 3).

wt. ≅ 2500 and 20,000, respectively. Data shown in Figure 5 establish the ability of activator to stimulate activator-deficient PDE and inhibitor to inhibit the combination of PDE + activator. The changes that are observed reflect changes in activity (V_{max}) but not K_m of the PDE. Further studies (data not shown) indicate that the inhibitor activity can be overcome by addition of excess PA.

In preparing the inhibitor, activity was lost after dialysis. The inhibitor appears to be heat stable and of low mol. wt., possibly "carried" by another protein (data not shown). These data illustrate the presence of a distinct heat-stable, low molecular weight inhibitor of PDE by column chromatography in rat fat. Preliminary animal experiments comparing inhibitors from normal and streptozotocin-diabetic rat fat were performed. These data (Table 2) demonstrate increased ($P < 0.02$) inhibitor activity in diabetic animals compared with controls.

DISCUSSION

Insulin stimulates and streptozotocin-induced diabetes inhibits low K_m cAMP PDE activity.[9] The data presented in this paper support these results and explore the possible mechanisms involved. Diabetes is associated with a decrease in both PDE and PA in adipose tissue, which is reversed by the administration of insulin. However, the time course of reversing this decrease by insulin treatment is important. If insulin therapy begins after the clinical symptoms of diabetes appear, insulin tends to restore PA activity toward normal but does not fully restore it, but if the administration of insulin begins simultaneously with the induction of diabetes, there is no decrease in PA activity. The findings of decreased PA in diabetes and its reversal after 5 days of insulin administration are also consistent with data available on protein synthesis in diabetes.[16] Whether or not PA synthesis and turnover are more specifically regulated by insulin than other proteins has not yet been determined. Specific genetic determinants of PA in hereditary retinal degeneration support this concept.[17] There are several other possible explanations for the loss of PDE activity in diabetic tissues. Smoake and Solomon have recently demonstrated that in liver of diabetic rats, there is a relocation of low K_m cAMP PDE from the particulate fractions to the soluble components.[20] The net result of this would be to remove the enzyme from its control site, i.e., receptor sites on membranes.

We also found an inhibitor of PA activity by ion-exchange column chromatography. The inhibitor appears to be a PA binding substance and is different from the PDE inhibitors recently described in other tissues.[7,8,14] However, in contrast to the protein inhibitors in brain which have molecular weights of about 80,000,[8,14] the inhibitor in fat is a nonprotein with an approximate mol. wt. of 2500. Kinetically, PA stimulates the V_{max} of the low K_m cAMP PDE without altering its K_m. The inhibitor blocks this stimulation and has no apparent effect on PA-deficient PDE activity. These data support the idea that the inhibitor produces its effect by interacting with PA similar to those studies of the PA binding protein in brain.[7,8,14,15]

The effects of diabetes and insulin administration on the activity of low K_m cyclic AMP PDE are shown after column chromatography. Diabetes decreases and insulin increases PDE activity. These effects on PDE cannot be explained at present. Insulin produces a large number of effects in a given cell type, such as fat cell, but many of these effects would appear to be independent of each other. There may be a primary defect in the PDE enzyme itself. The decrease in PA activity could be due to decrease in protein synthesis, i.e., a decrease in total amount of PA, or it could be due to a change in PDE inhibitor content or activity. Data presented in this paper support the latter. Although the mechanism (s) is not understood, it is not unlikely that a role for the inhibitor as well as the activator as regulatory components in insulin action and diabetes will eventually be demonstrated.

TABLE 2
Inhibitor activity in normal and diabetic adipose tissue

	N	Inhibitor activity*
Normal	5	12.6 ± 0.7
Diabetic	5	22.8 ± 3.3†

* Inhibitor activity was quantitated as %/mg protein extracted from the individual normal or diabetic whole rat epididymal fat pad.
† $P < 0.02$.

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