The Effect of Streptozocin-induced Diabetes on the Plasma Membrane Calcium Uptake Activity of Rat Liver

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SUMMARY

The effect of streptozocin (STZ)-induced diabetes on the plasma membrane calcium uptake of rat liver was investigated. Plasma membrane preparations from diabetic rats showed a 2–3-fold increase in calcium uptake activity over controls 3–4 wk after the initial injections. Such an increase can be either reversed or blocked by treating the diabetic rats with exogenous insulin or administering nicotinamide 15 min before and 3 h after the STZ injection, respectively. The activity of 5′-nucleotidase and the [^{3}H]ouabain binding to the plasma membranes were similar in samples from both the control and diabetic rats. These findings made it unlikely that preferential enrichment of plasma membranes or increased proportion of inside-out vesicles was the cause of the enhanced calcium uptake activity in membranes from diabetic animals. In addition, the effect of diabetes on the calcium uptake activity did not diminish even when the assay was performed in the presence of 2.5 μM ruthenium red, an inhibitor of calcium uptake by mitochondria, or when oxalate was omitted from the assay, suggesting that the effect was specifically on the plasma membrane pump. The enhanced calcium uptake activity was a result of an increase in the V_{max} (58.8 versus 113.1 pmol calcium/mg protein/min for control and diabetic rats, respectively). No significant change in K_m for calcium was detected. DIABETES 1984; 33:1072–77.

In recent years, it has become increasingly apparent that changes in intracellular free calcium levels are associated with many hormone-sensitive cellular processes.[1] The regulation of intracellular calcium in some tissues, such as liver, can occur via a concerted effort of the calcium pumps in plasma membranes, mitochondria, and microsomes. There is a growing body of evidence suggesting that the activity of these pumps can be affected by a number of hormones.[2-8] The effects of cyclic AMP, glucagon, insulin, and glucocorticoid on the microsomal calcium uptake activity have been well documented.[2-4] The mitochondrial calcium uptake activity has also been described to be under the regulation of epinephrine, glucagon, thyroid, and growth hormones.[5-8] Recently, Prpic et al.[9] reported that rat livers perfused with vasopressin, angiotensin II, or epinephrine showed lower calcium uptake activity in subsequently isolated plasma membranes. In this communication, we report an elevation of the calcium uptake activity in isolated plasma membranes of STZ-induced diabetic rats. This effect of diabetes on calcium uptake could be either reversed by treatment of these diabetic rats with exogenous insulin or blocked by administration of nicotinamide 15 min before and 3 h after the STZ injection.

MATERIALS AND METHODS

Animals. Age-matched, male Sprague-Dawley rats, weighing 200–250 g, were obtained from Eldridge Laboratory Animals, Barnhart, Missouri. They were fed Purina Chow ad libitum and given free access to water.

Induction of diabetes. Diabetes was induced by a single intravenous (i.v.) injection of a freshly prepared solution of streptozocin (STZ, 65 mg/kg in 0.1 M citrate buffer, pH 4.5).

Groups of experimental animals. (1) Sham-injected controls: Animals received only the injection vehicle (citrate buffer, pH 4.5). (2) Nicotinamide controls: Animals in this group received nicotinamide (350 mg/kg, i.p., Sigma Chemical Co., St. Louis, Missouri) 15 min before and again 3 h after STZ administration as described by Schmidt et al.[10] (3) Diabetics: Diabetes was chemically induced with STZ as previously described and the animals received no further treatment. (4) Insulin: Two to three weeks after induction of diabetes, animals received single daily injection of 3.0–4.0 U of insulin/day (NPH, Eli Lilly and Company, Indianapolis, Indiana). Insulin dosage was adjusted daily to achieve urine volumes of <10–20 ml/day. They were killed 5–7 days after the insulin treatment.

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TABLE 1
Final body weight, plasma glucose levels, and plasma membrane 5′-nucleotidase activities for all animals

Experimental group	Final body wt (g)	Final plasma glucose (mg/dl)	Plasma membrane 5′-nucleotidase activity (nmol/mg/min)
Sham-injected controls	330 ± 7 (30)	138 ± 5 (32)	43.57 ± 3.08 (26)
Nicotinamide controls	308 ± 7 (27)	171 ± 6 (28)	44.54 ± 1.64 (23)
Diabetics	246 ± 5 (28)	525 ± 11 (36)	40.70 ± 2.52 (26)
Insulin-treated	328 ± 6 (16)	167 ± 17 (8)	Not assayed

Plasma glucose and 5′-nucleotidase activity were assayed as described in MATERIALS AND METHODS. Values are mean ± SEM. Numbers in parentheses are either numbers of animals that were monitored (for body weight and plasma glucose) or the total number of determinations that were taken throughout the study (5′-nucleotidase activity).

Monitoring of animals. All animals were killed by cervical dislocation at the end of 3–4 wk. Plasma glucose at the time of killing was analyzed with the YSI model 23A glucose analyzer (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio) or in the Astra 8 (Beckman Instruments, Fullerton, California).

Preparation of plasma membrane fractions from rat liver. Liver plasma membranes were prepared according to Pilkis et al.[11] or Touster et al.[12] and resuspended in 50 mM Tris-HCl, pH 8.0, 0.25 M sucrose as previously described,[13] and stored at −80°C. The calcium uptake activity was stable for at least 10 days under this storage condition. Approximately 70% of its original activity was retained after 1 mo. 5′-Nucleotidase was assayed according to Avruch and Wallach.[14] Protein was quantitated by the method of Lowry et al.[15]

Calcium uptake. Calcium uptake was assayed essentially as previously described with the exception that $^{45}CaCl_2$ was used to initiate the reaction.[13] Modifications, if any, are indicated in the figure legends.

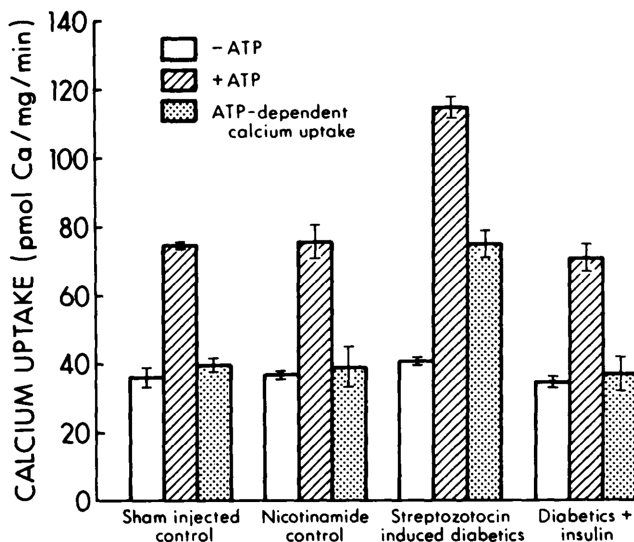

FIGURE 1. Effect of STZ-induced diabetes and insulin replacement on plasma membrane calcium uptake activity. The experimental groups were set up as described in MATERIALS AND METHODS. Three weeks after the initial injection, one-half of the rats in the diabetic group received single daily i.p. injection of 3.5–4 U of insulin. They were killed 5 days later. Calcium uptake was assayed as described in MATERIALS AND METHODS after 30-min incubation at 37°C. Results represent the mean ± SEM of 3 experiments.

Determination of [³H]ouabain binding. [³H]Ouabain binding by liver plasma membrane vesicles was performed at 37°C for 30 min according to Sips et al.[16] in a 500-μl medium containing 1 mM imidazole-EDTA, 1 mM dithiothreitol, 5 mM Tris-HCl, 5 mM $MgCl_2$, 30 mM imidazole HCl, pH 7.4, and 100–150 μg of plasma membrane proteins. In addition, 250 mM sucrose was present when ouabain binding to intact vesicles was to be measured. The reaction was started by the addition of [G-³H]ouabain to a final concentration of 1 μM (sp act 3000–4000 cpm/pmol). Aspecific binding was estimated by comparison with parallel incubations in the presence of an excess of 2.5 mM unlabeled ouabain. The binding assays were terminated after 30 min at 37°C by Millipore filtration.

Determination of total and free calcium concentrations. The total calcium in the reaction mixture was determined by atomic absorption spectroscopy. The free calcium concentrations were buffered by ethylene glycol bis-(β-aminoethyl ether)-N,N,N′,N′-tetracetic acid (EGTA) and were calculated using the MUSIC/FORTRAN version of the HALTAFALL program as described in detail by Ingri et al.[17] The association constants used in the calculation were identical to those described previously.[13]

RESULTS

Calcium uptake in diabetic and insulin-treated rats. After the induction of diabetes with STZ, there was a development of severe hyperglycemia, with plasma glucose concentrations in excess of 500 mg/dl and a substantial reduction in weight gain (Table 1). Insulin therapy of diabetic rats resulted in the restoration of normoglycemia and significant increase in body weight over those of untreated diabetic animals. On the other hand, nicotinamide treatment successfully blocked the development of severe hyperglycemia induced by STZ. The plasma glucose concentrations, however, were slightly higher than those of controls. The 5′-nucleotidase activity, which is used to estimate the purity of plasma membrane fractions, was similar in the plasma membranes from the different groups of animals.

The effects of STZ-induced diabetes on calcium uptake by plasma membrane vesicles are shown in Figure 1. Plasma membrane vesicles from these untreated diabetic rats showed a 213 ± 10% (SEM) increase in ATP-dependent calcium uptake activity without any effect on the basal or non-ATP-dependent process. When diabetic rats were treated with insulin for 4–7 days, calcium uptake by plasma mem-

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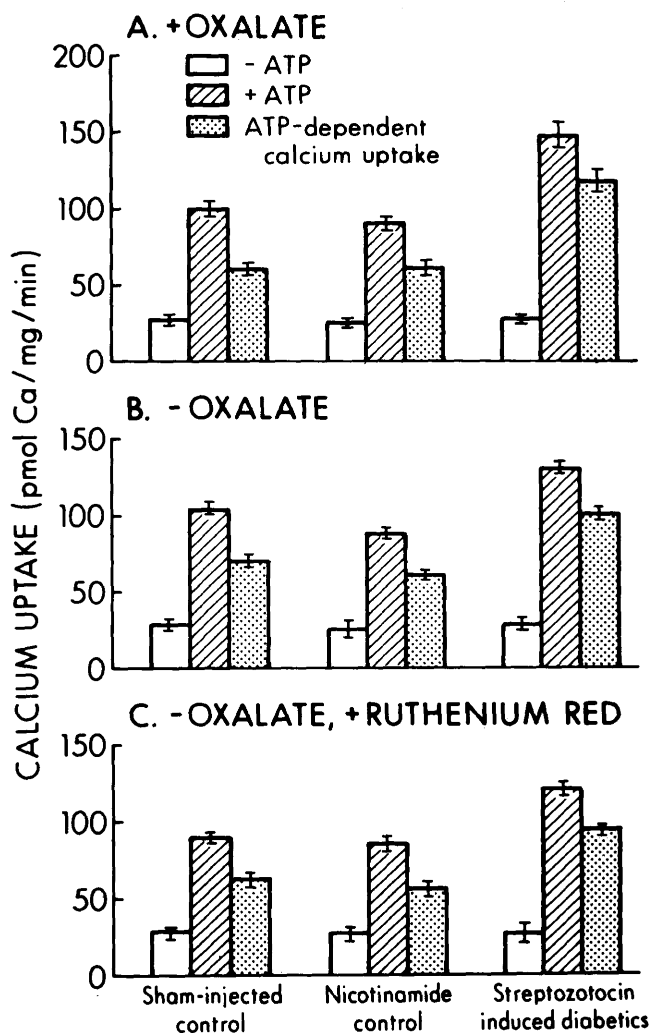

FIGURE 2. Effect of oxalate and ruthenium red on the plasma membrane calcium uptake activity. Plasma membrane vesicles were prepared from the 4 groups of experimental animals as described in legend to Figure 1. Calcium uptake activity was assayed as described in MATERIALS AND METHODS after 30-min incubation at 37°C, but in the presence or absence of oxalate and 2.5 μM ruthenium red. Results represent the mean ± SEM of triplicate determinations.

branes was restored to the level (92 ± 4%) similar to those for sham-injected control animals. Plasma membranes from nicotinamide control animals had ATP-dependent calcium uptake activity (102 ± 6%) similar to those of sham-injected rats.

Effect of oxalate and ruthenium red on calcium uptake. It was shown previously that the calcium uptake assay was specific for plasma membranes.[13] Nonetheless, since plasma membrane preparations are invariably contaminated with mitochondria and microsomes, we performed experiments to ensure that the observed effect of STZ-induced diabetes on calcium uptake was not due to the presence of these contaminants. This was achieved by performing the calcium uptake assay under conditions that are unfavorable for the mitochondrial or microsomal pumps, namely in the presence of 2.5 μM ruthenium red or in the absence of oxalate, respectively. There was only a slight decrease in the ATP-dependent calcium uptake activity in the three groups of animals, with preservation of the enhanced calcium uptake activity in the STZ-induced diabetic rats (Figure 2).

Binding of [³H]ouabain to rat liver plasma membrane vesicles. Estimation of the degree of sidedness of the plasma membrane vesicles prepared from the control and diabetic animals was derived from ouabain binding experiments performed under hypotonic and isotonic conditions. Ouabain, a specific inhibitor of the plasma membrane (Na^+ + K^+)ATPase, has been shown to bind to the enzyme at the extracellular side of the cell membrane only.[18,19] Thus, the binding of [³H]ouabain under isotonic conditions represents the binding to intact right-side-out and leaky vesicles, whereas the binding of [³H]ouabain under hypotonic conditions represents the total binding. From these, the percentage of inside-out vesicles can be estimated. Table 2 shows the comparison of the [³H]ouabain binding under isotonic and hypotonic conditions for membrane vesicles prepared from both control and diabetic animals. These membrane vesicles had essentially the same proportion of inside-out vesicles (42% versus 47% for control and diabetic animals, respectively).

Kinetics of calcium uptake. The kinetics of calcium uptake by plasma membrane vesicles from sham-injected and nicotinamide controls and untreated and insulin-treated diabetic rats were measured over the free calcium concentration of 2.5–9.4 nM (Figure 3). Calcium uptake was measured after only a 5-min incubation at 37°C to obtain an accurate determination of the initial rate. The Lineweaver-Burke plot for the data from each of the three groups of animals was linear over the entire concentration range tested (Figure 4). Our data, summarized in Table 3, indicate that the stimulation of calcium uptake after the induction of diabetes by STZ resulted from an increase in the V_{max} of the system from 58.8 to 113.1 pmol Ca·mg^{-1}·min^{-1}. When calcium uptake was measured using plasma membranes from diabetic animals treated with insulin, the V_{max} was restored to that of the control group. Rats injected with both nicotinamide and STZ showed little or no increase in the V_{max}. The K_ms remained unchanged for each of the three groups of animals.

TABLE 2
Sidedness of plasma membrane vesicles isolated from controls and diabetic rats

Experimental group	[³H]Ouabain binding (pmol/mg)*		% Inside-out†
	Isotonic	Hypotonic	
Control	0.77 ± 0.13	1.31 ± 0.17	41.9 ± 2.0
Diabetic	0.68 ± 0.03	1.28 ± 0.01	47.3 ± 2.4

*The values represent the mean ± SEM of three preparations.
†Percent inside-out = (1 − [binding in isotonic/binding in hypotonic]) × 100.

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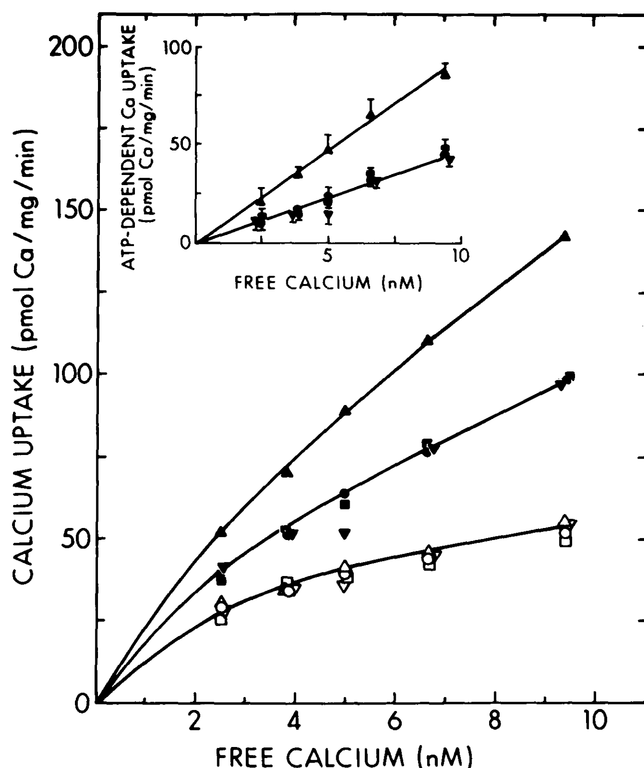

FIGURE 3. The relation between free calcium concentration and the calcium uptake activity of plasma membranes isolated from livers of sham-injected control (○,●), nicotinamide control (□,■), STZ-diabetic (△,▲), and insulin-treated (▽,▼) animals. Calcium uptake was measured as described in MATERIALS AND METHODS, but in the absence of oxalate and presence of 2.5 μM ruthenium red. The assay was carried out for only 5 min at 37°C. Solid and open symbols designate the calcium uptake activity in the presence and absence of Tris-ATP, respectively. The inset represents the ATP-dependent calcium uptake activity. Results are mean ± SD of duplicate experiments.

DISCUSSION

The results of this study demonstrate that, when diabetes was induced in rats by injection with STZ, there was an enhanced calcium uptake activity in the subsequently isolated plasma membranes. Because the plasma membrane calcium pump is spatially oriented to pump calcium out of cells, calcium uptake measurements can be made only with inside-out vesicles. Therefore, any changes in either the membrane purity or proportion of the inside-out vesicles will affect the calcium uptake activity. Results from the [^{3}H]ouabain binding experiment indicate that the effect of diabetes on calcium uptake was probably not due to the differences in the proportion of inside-out vesicles in membranes from control and diabetic animals. Likewise, the similarity in 5′-nucleotidase activities in these membranes also argues against any difference in plasma membrane contents being the cause. Moreover, plasma membranes are invariably contaminated with mitochondria and microsomes, and in vivo injections of glucagon and insulin are shown to be capable of altering the activity of the microsomal calcium pump.[4] It becomes necessary to eliminate the possibility that the apparent effect of diabetes on the plasma membrane calcium uptake was, instead, on the calcium pumps of these contaminants. As the marker enzyme for microsomes, namely glucose-6-phosphatase, is stimulated in diabetic animals,[20] this enzyme cannot be used to assess the magnitude of contamination of the various plasma membrane preparations by microsomes. Nevertheless, plasma membrane and microsomal pumps do exhibit different properties. First, they have different pH optima. At the pH 8 under which calcium uptake was measured, the microsomal pump showed little or no activity (personal observation). Second, the plasma membrane pump, unlike its counterpart in microsomes,[21,22] is less dependent on oxalate. Thus when calcium uptake was assayed at pH 8, in the absence of oxalate and also in the presence of 2.5 μM ruthenium red, a potent inhibitor of mitochondrial calcium transport, the plasma membrane activity was preferentially measured. When assayed under these conditions, plasma membranes from diabetic rats still showed a higher calcium uptake activity, suggesting that the effect of diabetes was indeed on the plasma membrane calcium pump.

We also demonstrate that the stimulatory effect of diabetes on calcium uptake was not caused by a direct hepatotoxic effect of the diabetogenic agent STZ. First, nicotinamide is known to have preventive and therapeutic effects on the induction of diabetes by STZ.[23] Administration of nicotinamide before and immediately after STZ injection prevented both the diabetogenic action of STZ and the enhanced calcium uptake by liver plasma membranes, while presumably allowing possible systemic toxic effects of STZ to be manifested. Second, STZ has a short biologic half-life and yet its

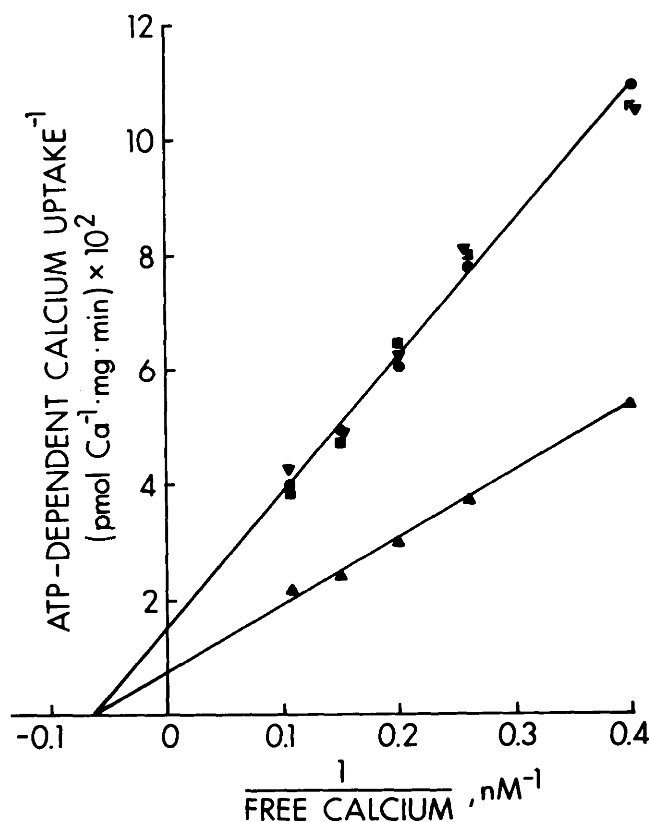

FIGURE 4. Double reciprocal plot of ATP-dependent calcium uptake by plasma membrane vesicles isolated from sham-injected control (●), nicotinamide control (■), diabetic (▲), and insulin-treated (▼) animals. Calcium uptake was measured for 5 min at 37°C over the free calcium concentration of 2.5–9.4 nM. The kinetic constants obtained are summarized in Table 2.

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TABLE 3
Kinetic constants of ATP-dependent calcium uptake activity by plasma membrane vesicles isolated from liver of controls, diabetic, and insulin-treated rats

Experimental group	K_m (nM)	V_{max} (pmol Ca·mg^{-1}·min^{-1})	V_{max}/K_m
Sham-injected controls (4)	15.2 ± 0.8	58.8 ± 3.9	3.9
Nicotinamide controls (3)	14.0 ± 0.4	70.9 ± 10.4	5.0
Diabetic (4)	15.5 ± 0.8	113.1 ± 10.6	7.3
Insulin-treated (2)	14.5 ± 0.9	58.9 ± 1.2	4.1

The procedure used to measure the kinetics of calcium uptake of 2.5–9.4 nM free calcium is discussed in the legend to Figure 3 and in the section under MATERIALS AND METHODS. Kinetic constants were calculated from the double reciprocal plots of the data in Figure 3 and expressed as mean ± SEM. Numbers in parentheses refer to the number of experiments.

in vivo effect on calcium uptake was not measured until several weeks after the development of diabetes. Third, institution of insulin therapy restored the calcium uptake activity to that of control animals. Finally, in vitro addition of STZ to the assay had no effect on the calcium uptake activity (personal observation).

Although our data indicate that the changes in the activity of the plasma membrane calcium pump were indeed a result of the development of diabetes in these animals, several questions remain. Whether the in vivo results could be caused by systemic changes secondary to the development of diabetes rather than a direct effect of insulin on the liver will have to be explored. Recently, Prpic et al.[9] demonstrated that after the perfusion of rat liver with vasopressin, angiotensin II, and α-adrenergic agents, the subsequently isolated plasma membranes had lower calcium uptake activity. Their findings are consistent with the proposed effects of these hormones on the cytolic free calcium concentration,[24–26] and strongly suggest the importance of this plasma membrane calcium pump in the action of these hormones. On the contrary, although calcium has been demonstrated to play a vital role in insulin action,[27–31] a direct effect of insulin on the intracellular free calcium concentration of hepatocytes has not been reported. Furthermore, by performing calcium uptake studies using Ca^{2+}-ATPases that were reconstituted into liposomes, many investigators demonstrated a functional relationship between the enzymic and calcium uptake activities.[32–35] The calcium transport system described in this study has been previously shown to be closely associated with the plasma membrane (Ca^{2+} + Mg^{2+})ATPase based on their similarities in substrate affinities and specificities.[13] Although we showed a profound increase in calcium uptake after the rats became diabetic, we failed to detect any consistent changes in the plasma membrane Ca^{2+}-ATPase activity (personal observation). Thus, the question of whether this (Ca^{2+} + Mg^{2+})ATPase is indeed the calcium pump ATPase, or whether the differential effect of diabetes on the enzymic and calcium uptake activities is a result of alteration in their coupling efficiency, deserves further attention.

To gain insight into the mechanism of the diabetes-induced changes, kinetic parameters of calcium uptake were measured. The changes in the V_{max} rather than the K_m in plasma membranes from diabetic rats are not surprising, as similar observations have also been noted with hepatic microsomal calcium uptake,[4] amino acid transport,[36,37] and glucose uptake.[38] Yet what appears to be confusing is that, despite the K_m for the plasma membrane pump being similar to those reported by others,[9,39] it is at least an order of magnitude below the micromolar free calcium concentration known to exist in the cytosol. It is conceivable that other, yet to be identified factors may be involved in regulating the affinity of this system in vivo. To be able to develop a hypothesis regarding possible physiologic significance of calcium uptake in diabetes, we would require a better understanding of the mechanism by which calcium uptake is regulated, as well as its relationship with regard to the transport of other substances and the metabolic state of the diabetic animal.

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