Effectiveness of Insulin Therapy on Altered Renal Calcium Transport in Diabetic Rats

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SUMMARY

The uptake of ^{45}Ca was measured in slices of kidney cortex from normal rats, streptozotocin-diabetic rats, and streptozotocin-diabetic rats treated early and late with insulin. Insulin therapy was performed such that blood glucose levels were controlled in half the treated diabetic animals but not in the others.

Considerably earlier than evidence of nephropathy (i.e., proteinuria and increased BUN levels) in streptozotocin-diabetic rats, there was a significant decrease in active uptake of calcium by the kidney. Insulin therapy, begun immediately upon diagnosis of diabetes, maintained normal calcium transport even when blood glucose levels were not controlled. On the other hand, insulin therapy, begun 1 mo after diabetes was confirmed but before evidence of nephropathy, did not restore calcium transport to normal whether or not blood glucose was controlled.

We conclude that this biochemical mechanism, which possibly may be implicated in the pathophysiology of diabetic nephropathy, is clearly influenced by duration of insulin deficiency and not by the degree in hyperglycemia. DIABETES 28:1088–1094, December 1979.

Nephropathy is one of the most important and most serious of the long-term vascular complications of diabetes mellitus in man. It is a major cause of death of diabetics, especially among those who develop the disease early. However, proteinuria, the first clinical evidence of nephropathy, is seldom recorded in young patients during the first 10 yr of diabetes. After proteinuria first indicates the presence of nephropathy in the diabetic, its progress may be slow. However, death from uremia is a common end to diabetes that starts early in life despite attempts to retard the renal lesions. Evidence that the control of blood glucose will retard the progress of diabetic nephropathy is lacking, although such attempts are reasonable in the absence of a clear understanding of the pathogenesis of the nephropathy.

Bloodworth et al.[1] concluded that diabetic nephropathy is the result of an abnormal biochemical environment produced by the diabetic state, regardless of the cause of diabetes. This conclusion is supported by the work of Mauer and his colleagues.[2–6] For example, streptozotocin-induced diabetes in the rat is associated with a nephropathy that is apparently related to the diabetic state rather than to toxic effects of the chemical itself. Many of the renal changes observed in streptozotocin-diabetic rats are present in human diabetes.

Fraser[7] suggested that the second messenger of insulin is calcium and proposed a "unitary mechanism for the cellular action of insulin" based on experiments using isolated fat cells. If the actions of insulin are mediated by alterations in calcium transport, then the absence of insulin, i.e., the diabetic state, should produce significant changes in calcium transport. In support of this, Schneider and associates have shown that diabetes induced by either streptozotocin or alloxan is associated with decreased duodenal calcium absorption.[8–11] Furthermore, they reported that insulin treatment of streptozotocin-diabetic rats restores calcium transport to control levels.[12]

We report here studies designed to (1) determine if streptozotocin-induced diabetes alters calcium transport in the kidney, (2) answer whether such alterations are due to a direct nephrotoxic action of streptozotocin or to the diabetic state, (3) assess the time sequence of effects of the diabetic state on calcium uptake by the kidney, (4) determine if insulin therapy of streptozotocin-diabetic rats will prevent alterations in calcium uptake or restore altered uptake to normal levels, and (5) determine whether or not insulin's effect on calcium uptake is dependent on its control of blood glucose levels.

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MATERIALS AND METHODS

Animals. Age-matched male Sprague-Dawley rats were used throughout the study. All rats were maintained ad libitum on tap water and Purina rat chow.

Induction of diabetes. Rats to be made diabetic received intraperitoneal injections of 60 mg/kg streptozotocin (supplied by Upjohn, Kalamazoo, Michigan) freshly dissolved in citrate buffer (pH 4.5) on two successive days. Induction of the diabetic state was confirmed by finding persistent glucosuria of 4+ using Diastix (Ames). The presence of protein in urine was detected using Bili-Labstix (Ames).

Insulin treatment regimens. At the first evidence of persistent glucosuria (for 2 or 3 days after injection of streptozotocin), the diabetic animals were divided into three groups as follows: (1) untreated diabetic, (2) diabetic treated immediately with daily injections of pork insulin (Iletin, NPH, Eli Lilly) subcutaneously, and (3) diabetic treated with daily injections of insulin beginning 4 wk after confirmation of diabetes.

Groups 2 and 3 were subdivided into two groups. In subgroup A, injections of insulin (0.5–2.0 U) were given at times such that normoglycemia was maintained in each animal.

In subgroup B, injections of insulin (1.0 U) were given at times such that control of blood glucose was not maintained and hyperglycemia was present when the animals were killed, and, determination of calcium uptake by slices from their kidneys was made.

Subgroups A and B were employed to determine the importance of control of blood glucose for insulin's action on calcium. Weights of all rats were recorded before killing them.

Sequence of experiments. Rats were killed by decapitation as follows: untreated diabetic animals (group 1) and those treated early with insulin (group 2) were killed 1, 2, 4, 6, and 8 wk after confirmation of diabetes; diabetic rats treated late with insulin (group 3) were studied after 3 days or 1, 2, and 4 wk of insulin therapy. Control rats were killed throughout the study.

Blood was collected at the time of decapitation, and plasma was separated for determination of plasma glucose levels using the enzymatic method of Raabo and Terkildsen,[13] for colorimetric determination of urea nitrogen (BUN) using a diagnostic kit obtained from Sigma Chemical Company, which is based on the methods of Faucett and Scott[14] and of Chaney and Marbach,[15] and for determination of sodium and potassium concentrations using flame photometry.

Kidneys were immediately removed and weighed.

Determination of ^{45}Ca uptake in kidney cortex slices. Uniform slices of kidney cortex (0.5-mm thick) were prepared using a Stadie-Riggs tissue slicer. Three slices from kidneys of each rat were placed in each of nine beakers containing 3.0 ml of incubation solution, the composition of which included 126 mM NaCl, 5 mM KCl, 1.1 mM $MgSO_4$, 2 mM $CaCl_2$, 11 mM glucose, and 5 mM Na phosphate buffer (pH 7.4). The pH remained stable throughout the incubation. ^{45}Ca (0.06 μCi) in 0.1 ml of incubation solution was added to beakers 1–8, and 0.1 ml of ^{14}C-inulin (1.25 μCi/mg/ml) was added to the ninth beaker. Incubation was carried out in a Dubnoff metabolic shaker at 37°C under an oxygen atmosphere.

At 15-min intervals for 2 h, slices were removed from beakers 1–8, blotted, weighed, and homogenized in 2.0 ml of glass-distilled, deionized water. At the end of the 2-h incubation, slices were removed from the ninth beaker, blotted, weighed, and placed in an oven at 100°C for as long as 48 h, until a constant weight was obtained. After final dry weights were recorded, the dried slices were transferred to plastic tubes containing 5 ml of 0.1 N nitric acid, covered, and were allowed to stand for 48 h. Then, 1.0 ml of the nitric acid extract was transferred to counting vials and prepared for counting of radioactivity due to labeled inulin by the addition of 10 ml of Aquasol (New England Nuclear). Samples of media from all beakers and homogenates of slices from beakers 1–8 were prepared for counting of radioactivity by liquid scintillation techniques.

Studies in vitro. Uptake of ^{45}Ca by slices obtained from control rats was measured under the following conditions in vitro: (1) in glucose-free medium, (2) in the presence of insulin (50 μU/ml), (3) in the presence of 2,4-dinitrophenol (10^{-4} M), (4) in the presence of streptozotocin (1 mg/ml), and (5) in the presence of streptozotocin (1 mg/ml) + insulin (50 μU/ml).

Additionally, ^{45}Ca uptake by slices from rats diabetic for 10 wk was measured in the presence of insulin (50 μU/ml).

Calculation of data. Contents of beakers 1–8 were used to calculate the ^{45}Ca slice-to-medium (S/M) ratios, i.e., cpm/g tissue ÷ cpm/ml medium, for each time interval. Contents of the ninth beaker were used in calculations of percent tissue water and extracellular space. Percent tissue water (% H_2O_t) was calculated as the difference between the tissue wet weight (WW) and tissue dry weight (DW) divided by tissue wet weight (WW) × 100: % H_2O_t = [(WW − DW/WW] × 100.

Extracellular space (E), measured with labeled inulin, was expressed as percent of tissue water by dividing the counts of tissue WW by the counts per milliliter of medium contained in the ninth beaker and multiplying by % H_2O_t: % E = [(counts/g WW)/(counts/ml med)] × % H_2O_t.

Data were analyzed by Student's *t* test and are presented as means ± SE. Differences were considered significant at $P < 0.05$.

RESULTS

Figure 1 shows that both kidney and body weights are depressed significantly by the diabetic state (curve 1). Insulin therapy of diabetic animals resulted in significantly increased kidney and body weights over those for untreated diabetic animals. This increase was much greater when insulin was administered early (curves 2A and 2B) than when treatment was begun late (curves 3A and 3B) and when the treatment maintained normoglycemia (curves 2A and 3A) than when hyperglycemia persisted (curves 2B and 3B). The body and kidney weights of all diabetic animals remained significantly lower than those of control animals, except for the group that received insulin immediately upon diagnosis of diabetes such that normoglycemia was maintained (curve 2A). This group of rats had significantly higher body weights, after receiving insulin for 4 wk, than did paired controls. Ratios of kidney weights to body weights for all animals did not differ significantly from those of controls (0.0040 ± 0.0005).

Throughout the study, S/M ratios for ^{45}Ca were deter-

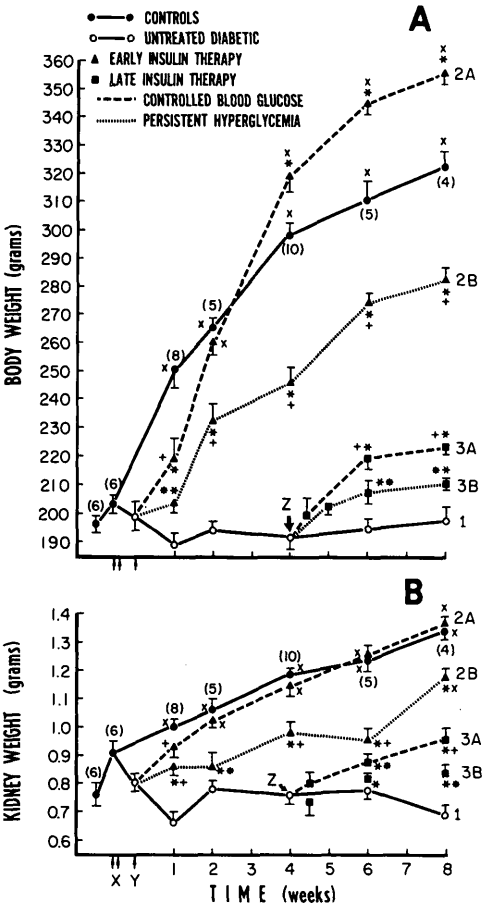

FIGURE 1. Graph A: Body weights of all animals used in study. Graph B: Kidney weights of all animals used in study. X signifies days of streptozotocin injections; Y indicates time of diagnosis of diabetes and institution of early insulin therapy; Z indicates time of institution of late insulin therapy. Labeling of curves (e.g., 2A) corresponds with that of treatment groups (see text). Data are presented as mean ± SE. Numbers in parentheses are numbers of control animals for given time. For numbers of animals in other groups, consult Table 1. Asterisk indicates a dignificant difference ($P < 0.01$) from controls, white double asterisk is significant difference ($P < 0.05$) from untreated diabetics, plus sign indicates significant difference ($P < 0.01$) from untreated diabetics and x marks a significant difference ($P < 0.0001$) from untreated diabetics.

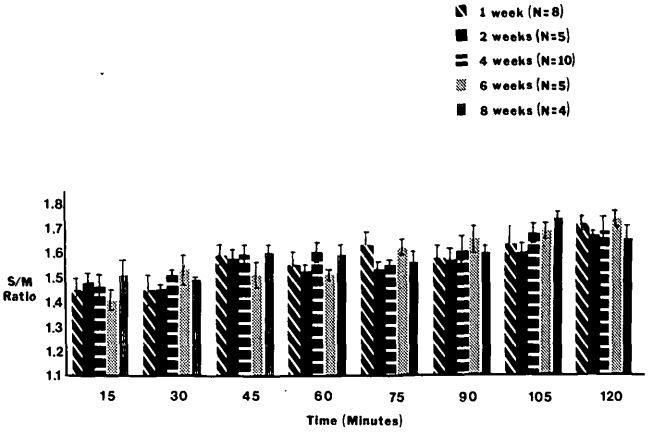

FIGURE 2. S/M ratios for ^{45}Ca in kidney cortex slices from control rats as a function of aging. Weekly intervals represent weeks after initiation of study. Minutes on the abscissa correspond to incubation periods. N = number of animals used.

mined in slices from control rats at each experimental period. Figure 2 shows that the calcium uptake by slices of kidney cortex from control animals did not change as the rats' age and weight increased but was constant throughout the study.

Table 1 is a summary of the nonfasting plasma glucose concentrations measured on all animals used in this study. The data show that untreated streptozotocin-diabetic rats were severely hyperglycemic, having glucose concentrations in excess of 300 mg/dl of plasma. Those diabetic rats that received insulin injections at intervals, which allowed for control of blood glucose (subgroup A), were normoglycemic during both the early and late regimens of insulin therapy, while the animals in subgroup B had plasma glucose concentrations significantly higher than those of controls.

Tests for proteinuria in diabetic rats detected no protein excretion greater than that of control rats for 6 wk. Mild proteinuria (<30 mg/dl) was detected in roughly 10% of the untreated diabetic animals for the remainder of the study. These data are in agreement with those of Weil et al.,[16] who showed no significant increase in urine protein concentrations for diabetic rats before 12 wk of diabetes.

Analyses of plasma urea nitrogen (BUN) and sodium and potassium levels gave values for all animal groups that were not significantly different from those of control animals: BUN = 30.43 ± 2.4 mg/100 ml; Na^+ = 144.2 ± 3.5 meq/L; K^+ = 7.4 ± 0.5 meq/L.

Effect of diabetes on ^{45}Ca uptake. Streptozotocin-induced diabetes did not affect calcium uptake by kidney cortex slices in the first week. However, after 2, 4, 6, or 8 wk, calcium uptake was significantly decreased in all diabetic animals (Figure 3).

Effects of early insulin therapy on ^{45}Ca uptake. Figure 4 shows that, when insulin therapy was started immediately after detection of glucosuria, calcium uptake by slices of kidney cortex from diabetic rats was not significantly different from that of slices from control rats after 2 wk of therapy,

TABLE 1
Blood glucose levels for all animals*

Animal groups		Plasma glucose (mg/dl)
Controls		118 ± 10 (36)
Diabetic groups†		
1. Untreated		
Duration of diabetes		
1 wk		490 ± 35 (10)
2 wk		530 ± 95 (10)
4 wk		575 ± 90 (7)
6 wk		716 ± 78 (9)
8 wk		901 ± 33 (9)
10 wk		987 ± 56 (3)
2. Early insulin therapy	Subgroup A	Subgroup B
Duration of therapy		
1 wk	94 ± 10 (4)	352 ± 34 (4)
2 wk	109 ± 6 (4)	395 ± 59 (4)
4 wk	104 ± 8 (5)	435 ± 77 (4)
6 wk	117 ± 5 (5)	433 ± 26 (4)
8 wk	112 ± 10 (4)	624 ± 41 (3)
3. Late insulin therapy		
Duration of therapy		
3 days	—	404 ± 21 (4)
1 wk	104 ± 6 (6)	—
2 wk	106 ± 7 (4)	543 ± 40 (4)
4 wk	111 ± 7 (5)	666 ± 71 (4)

* Number in parentheses are numbers of animals in each group. Data are presented as mean values ± SE.
† See text for description.

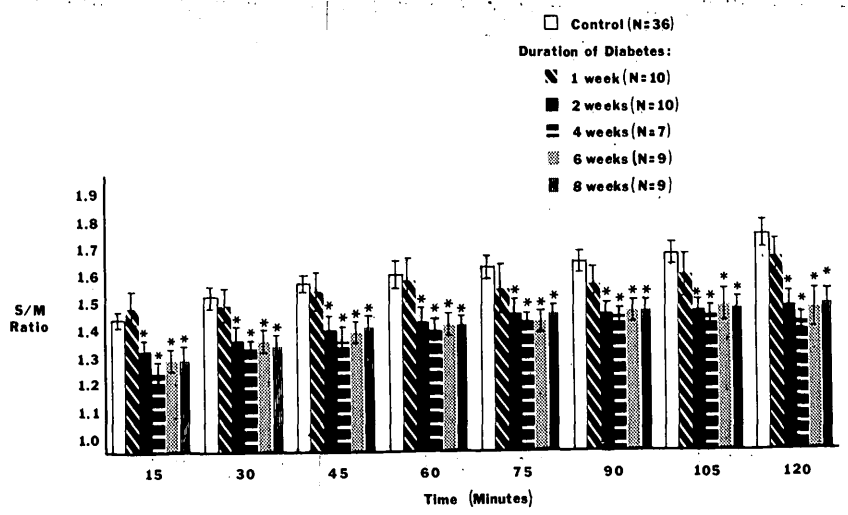

FIGURE 3. Effects of streptozotocin diabetes on ^{45}Ca uptake (S/M ratios) by kidney cortex slices from rats with diabetes of 1, 2, 4, 6, and 8 wk. N = number of animals. Asterisk indicates a significant difference ($P < 0.05$) from controls.

whether or not control of blood glucose was maintained. When insulin injections were given so that normoglycemia was maintained in diabetic animals, uptake of calcium into slices from their kidneys throughout 8 wk of diabetes never differed significantly from uptake into slices from control rats (Figure 4, panel A). However, when insulin injections did not maintain control of blood glucose levels, calcium uptake was normalized only after 2 wk of insulin (Figure 4, panel B).

Effects of late insulin therapy on ^{45}Ca uptake. Figure 5 shows that, when insulin therapy was begun 4 wk after the diagnosis of diabetes, it failed to normalize calcium uptake by kidney cortex slices from the diabetic rats. When injections were timed to control blood glucose, calcium uptake

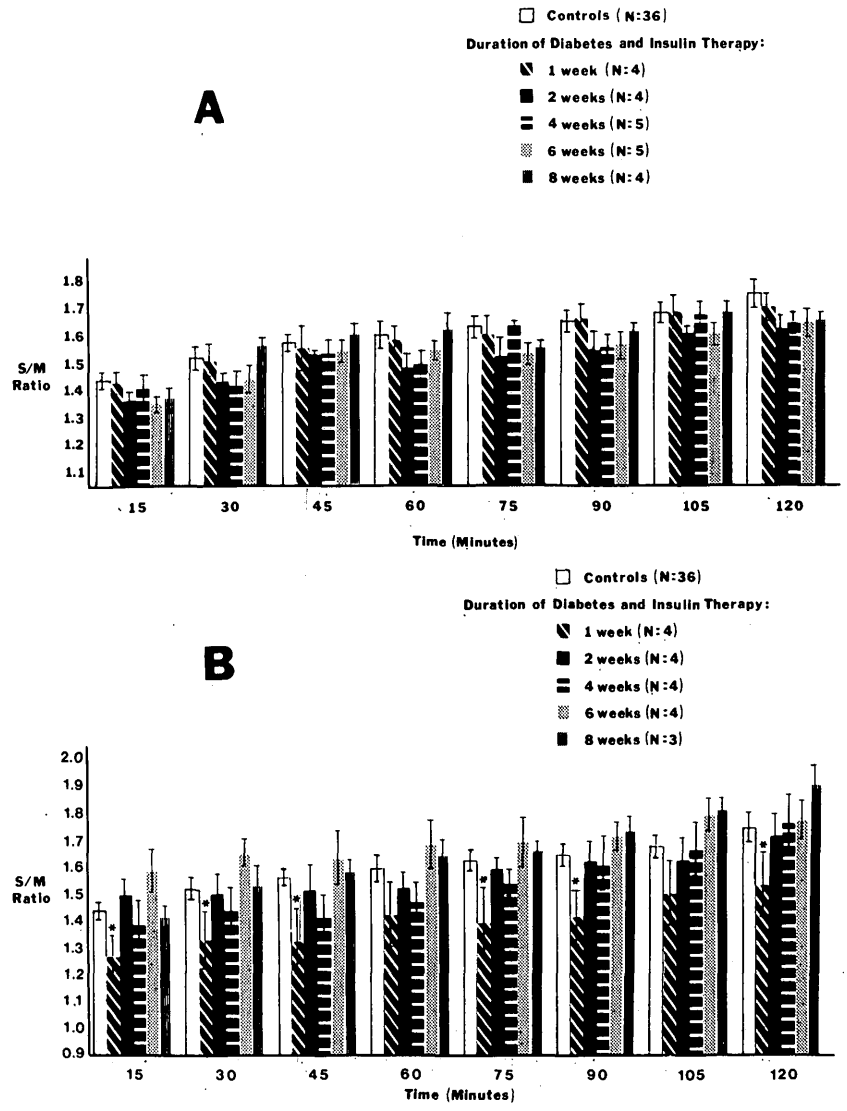

FIGURE 4. S/M ratios for ^{45}Ca in slices of kidney cortex from diabetic rats in which insulin therapy was begun immediately after confirmation of diabetes. Part A: Control of blood glucose was maintained. Part B: Control of blood glucose levels was not established. N = number of animals. Asterisk indicates a significant difference ($P < 0.05$) from control values.

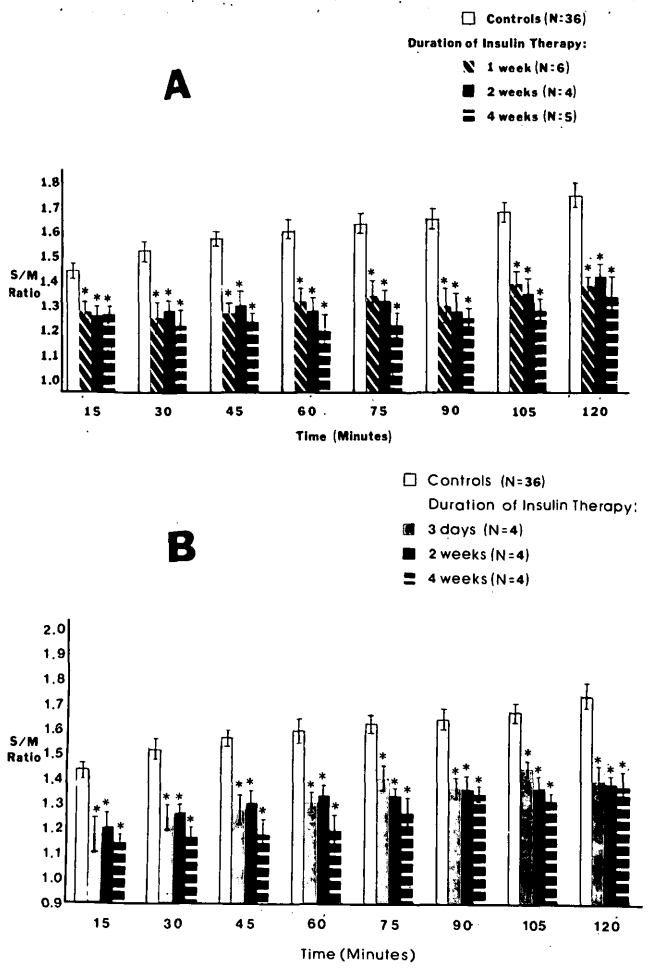

FIGURE 5. S/M ratios for ^{45}Ca in kidney cortex slices from diabetic rats in which insulin therapy was begun 4 wk after diagnosis of diabetes. Part A: Control of blood glucose was maintained. Part B: Control of blood glucose levels was not established. N = number of animals. Asterisk indicates significant difference ($P < 0.05$) from control values.

was significantly less than in controls at 1, 2, and 4 wk of insulin therapy, corresponding to 5, 6, and 8 wk of the diabetic state (panel A). When injections of insulin were timed so that blood glucose was not controlled, calcium uptake was significantly less than uptake into control slices at 3 days and 2 and 4 wk of insulin therapy, corresponding to 4½, 6, and 8 wk of diabetes (panel B).

Renal cortical slices from all rats had similar water content and extracellular space, as was determined using inulin as a marker of the extracellular compartment. There were no animal groups with values significantly different from those of controls: % H_2O_t = 73.4 ± 3.5; % E = 38.2 ± 2.9.

Studies in vitro. The data presented in Figure 6 show that, when kidney cortex slices from control rats were incubated in glucose-free media, ^{45}Ca uptake was no different from that measured when glucose was present. The presence of insulin (50 μU/ml) in the medium produced significant increases in S/M ratios after 1 h of incubation. In the presence of 2,4-dinitrophenol (10^{-4} M), the S/M ratios for ^{45}Ca decreased significantly, to values of 1.0 ± 0.05. When streptozotocin was present in the incubation medium at a concentration shown by Orci et al. to alter significantly islet cell membranes after 15 min of incubation,[17] there was no effect on ^{45}Ca uptake by normal kidney cortex slices. However, the presence of insulin in addition to streptozotocin caused significant increases in ^{45}Ca uptake after 1 h of incubation.

Finally, when slices of kidney cortex from rats with streptozotocin-induced diabetes of long duration (10 wk) were incubated in the presence of insulin, the uptake of ^{45}Ca was not significantly different from that measured in the presence of 10^{-4} M dinitrophenol.

DISCUSSION

It has been established that nearly all calcium from the glomerular filtrate is reabsorbed in the renal tubules.[18-20] Renal transport of calcium was studied from a biochemical point of view by Janda using slices of kidney cortex.[21] These studies indicated that the luminal membrane in the slices is accessible to the incubation medium and that calcium passes through both the luminal and the basal membranes in the slices; transport across the luminal membrane is energy de-

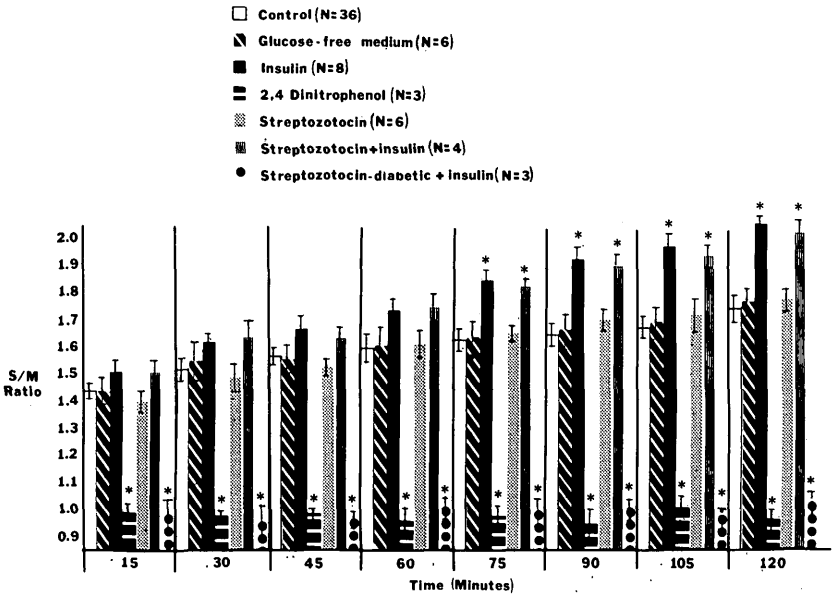

FIGURE 6. Effects of various conditions in vitro on S/M ratios of ^{45}Ca in slices of renal cortex. N indicates number of animals used. Slices from normal animals were incubated under the following conditions: Control, i.e., in the medium described in METHODS section; in the same medium, except without glucose being present; in the presence of insulin (50 μU/ml) with and without glucose in the medium (since the presence of glucose made no difference in ^{45}Ca uptake, these data are combined in the figure); in the presence of 2,4-dinitrophenol (10^{-4} M); and in the presence of streptozotocin (1 mg/ml). Slices from rats diabetic for 10 wk were incubated in the presence of insulin (50 μU/ml). Asterisks indicate significant difference ($P < 0.05$) in S/M ratios from those found under the first set of conditions, i.e., those of control.

pendent, while that across the basal is passive. To our knowledge, the conclusions reached by Janda have not been confirmed using such sophisticated techniques as the isolated perfused renal tubule. However, the use of kidney slices to investigate renal transport processes has been widespread, and there is general agreement that S/M ratios greater than unity are indicative of an active transport process.[22] Our experiments in vitro, in which the presence of 10^{-4} M 2,4-dinitrophenol, known to be an inhibitor of active transport (vide infra), reduced normal S/M ratios to about 1.0, support this.

The present study shows that streptozotocin diabetes significantly reduced the active uptake of calcium by slices of kidney cortex. This may mean that luminal transport of calcium, corresponding to tubular reabsorption of calcium, was depressed, consistent with the study of Janda;[21] however, as pointed out above, such a conclusion may be premature at this point.

Schneider et al. reported that streptozotocin-diabetic rats showed a significant increase in urinary calcium excretion compared with matched controls.[8,12] Furthermore, urinary calcium excretion in diabetic rats treated early with insulin remained almost three times greater than controls, even though urine volume was only twice as great.[12] On the basis of doubled BUNs in the diabetic animals, they concluded that streptozotocin directly damaged the mechanism(s) of calcium transport in the renal tubule. Streptozotocin is tumorigenic to human [23–25] and rat[26] kidneys and has been presumed by Myerowitz et al.[27] to cause injury to the kidney tubule cells in man, since its administration was followed by proteinuria with progressive azotemia. However, most studies in rats have not demonstrated any evidence of direct nephrotoxicity at doses of streptozotocin required to produce diabetes, i.e., less than 100 mg/kg.[28] For example, Weil et al.[16] produced severe diabetes in rats by giving a single intravenous injection of 65 mg/kg. No evidence of kidney damage was found (e.g., BUN was 28 ± 5 mg/dl for controls and 40 ± 10 mg/dl for diabetic animals). Schneider et al.,[12] on the other hand, used intraperitoneal injections of 100 mg/kg and 25 mg/kg on two successive days, the first dose being high enough to be nephrotoxic.

In the present studies, in which lower doses of streptozotocin were used, it is unlikely that the renal defect observed was caused by nephrotoxicity. Our reasons for this numbered six: (1) streptozotocin, added in vitro to kidney slices obtained from normal rats, had no effect on calcium uptake; (2) the effect in vivo did not occur until after 2 wk of diabetes; (3) early initiation of insulin therapy to streptozotocin-diabetic animals prevented any alteration of calcium uptake by the slices; (4) increased protein excretion did not follow streptozotocin administration; (5) plasma urea nitrogen and sodium and potassium levels were normal in all rats that received streptozotocin injections; (6) total water content and extracellular space of all kidney cortex slices from animals that received streptozotocin injections were normal throughout the study. Therefore, just as the association of experimental diabetes with a renal defect that depresses synthesis of 1,25-dihydroxycholecalciferol, resulting in decreased duodenal calcium absorption, is prevented by early insulin treatment,[12] the association of experimental diabetes with a renal defect that caused decreased renal slice accumulation of calcium was also prevented by early insulin treatment.

We observed that the significantly reduced reabsorption of calcium by the kidney occurred much earlier than did any evidence of nephropathy (e.g., proteinuria, increased BUN). Furthermore, the earliest effect on calcium uptake, i.e., a 66 ± 5% decrease in active transport, was not enhanced by longer durations of the diabetic state of up through 8 wk. Our observation of totally absent active uptake after 10 wk of untreated diabetes (Figure 6) may indicate further progression of the effect during long-term diabetes, since it is difficult to associate this additional depression with a direct effect of insulin.

If insulin treatment was begun at the first sign of glucosuria, renal calcium reabsorption was not affected by the diabetic state. On the other hand, if insulin therapy was delayed, it did not restore calcium transport to normal.

The results of this study clearly suggest that the effectiveness of insulin therapy in preventing diabetes-induced damage of calcium transport mechanisms depends on its early institution but is not related to control of blood glucose. Studies in vitro showed that calcium uptake by kidney slices from normal rats was not affected by the absence of glucose from the medium and that insulin's direct effect to increase ^{45}Ca uptake was the same whether glucose was present in or absent from the medium. Studies in vivo showed that early insulin therapy prevented abnormal calcium transport whether or not blood glucose was controlled. Furthermore, even when blood glucose concentrations were controlled in diabetic animals by insulin therapy started late, altered calcium movement persisted.

Body weights and corresponding kidney weights strongly suggest that early institution of insulin therapy is essential to maintain normal growth in diabetic animals. When control of blood glucose levels in diabetic rats was established early in the disease and maintained throughout the study, animals reached body weights roughly 10% greater than paired controls. These animals were found on autopsy to have obviously increased amounts of fat surrounding the viscera. Since kidney weights were not different from those of paired controls, the increased body weight appeared to be caused by a lipogenic action of maintenance doses of insulin.

Preliminary data from our laboratory show that intracellular calcium concentrations of kidney cortex slices are significantly reduced in patterns paralleling the reductions in ^{45}Ca uptake reported in this study. We believe, therefore, that the data presented here reflect alterations in net calcium transport.

Finally, although at this time we are unable to prove that any relationship exists between the observed defect in calcium transport and diabetic nephropathy, these data provide substantial justification for further investigation regarding the possible role of altered calcium metabolism and/or transport in the long-term degenerative complications of diabetes mellitus, the pathogeneses of which have thus far been poorly and/or incompletely defined.

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