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EFFECTS OF ALLOXAN-DIABETES ON THE SODIUM-POTASSIUM ADENOSINE TRIPHOSPHATASE ENZYME SYSTEM IN DOG HEARTS

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SUMMARY

The effects of alloxan-diabetes on the partial reaction of $(Na^+ + K^+)$-ATPase, K^+-activated para-nitrophenylphosphatase, and on ouabain binding were studied in isolated adult dog heart myocytes. The Km of K^+-activated para-nitrophenylphosphatase for K^+ activation was increased from 2.5 to 7.7 mM with no change in Vmax. The Scatchard plots for ouabain binding between control and diabetic animals were indistinguishable. These results indicate that in acute diabetes induced by alloxan, the number of Na^+-K^+ pumping sites in the heart is not altered but the affinity of the system for K^+ is decreased. It is suggested that the decrease in K^+ affinity of the $(Na^+ + K^+)$-ATPase enzyme system is at least in part responsible for the altered K^+ homeostasis in the diabetic state.

INTRODUCTION

A derangement in K^+ homeostasis is frequently seen in diabetes (1, 2). From the work of Zierler and his co-workers (3-5), it is clear that this derangement is due, at least in part, to a lack of insulin action on the cells (6). Although attempts have been made to relate altered K^+ homeostasis to insulin action on the Na^+-K^+ pump (4, 7-11), the mechanism by which insulin regulates K^+ homeostasis has yet to be delineated. In this report, we have examined the effect of alloxan-diabetes on the $(Na^+ + K^+)$-ATPase enzyme system in isolated adult heart myocytes by studying the kinetic characteristics of K^+-activated para-nitrophenylphosphatase (K^+-PNPPase) and ouabain binding. K^+-PNPPase activity was used as a measure of the $(Na^+ + K^+)$-ATPase enzyme system because it represents the dephosphorylation

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[1]Abbreviation used: K^+-PNPPase, K^+-activated para-nitrophenylphosphatase.

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phase of the $(Na^+ + K^+)$-ATPase system on the outer surface of the cell membrane (12, 13) and its activity is readily measurable in intact myocytes (14). Ouabain binding was used as a means of quantifying the number of enzyme molecules (15).

METHODS

Induction of diabetes and isolation of heart myocytes. Experiments were carried out on mongrel dogs of either sex weighing from 15 to 20 kg. All animals were fasted overnight with access to water prior to the experiments. Diabetes was induced by a single injection of 80 mg/kg alloxan monohydrate intravenously. Five to 8 days after alloxan injection, those dogs with a fasting plasma glucose concentration of 300 mg/100 ml or higher were used. Myocytes were isolated from hearts of control and alloxan-injected dogs by the method of Liu and Spitzer (16).

Measurement of K^+-PNPPase activity. K^+-PNPPase activity in isolated myocytes was measured as described by Skou (17) with modification. Freshly prepared myocytes (2.4 mg protein) were incubated in the following media in a final volume of 2 ml: Medium 1 contained 0.25 M sucrose and 20 mM Tris-HCl (pH 7.4); Medium 2 contained, in addition to the ingredients in Medium 1, different concentrations of KCl. The reaction was inititated by addition of 5 mM $MgCl_2$ and 5 mM para-nitrophenylphosphate, proceeded for 6 min at 37°C, and was terminated by addition of 0.2 ml of 50% trichloroacetic acid. After removal of protein precipitate by centrifugation, 1 ml of supernatant was removed and mixed with 2 ml of 0.5 M Tris base. The reaction product, para-nitrophenol, was measured spectrophotometrically at 410 mμ. The activity obtained in Medium 2 (0.65 μmoles/mg·hr at 10 mM K^+) minus that of Medium 1 (0.33 μmoles/mg·hr at 10 mM K^+) is defined as K^+-PNPPase activity (0.32 μmoles/mg·hr at 10 mM K^+). Preliminary experiments showed that K^+-PNPPase activity was linear with incubation time (0-10 min) and myocyte protein (0-4.8 mg).

Assay of ouabain binding. Binding of ouabain in isolated myocytes was carried out in 20 ml polyethylene vials. The assay mixture in a final volume of 2 ml contained 135 mM choline chloride, 2.5 mM glucose, 5.0 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.4) and 32 nM [^{3}H]-ouabain containing a radioactivity of approximately 1.25 μCi in the presence of different concentrations (0-10^{-3} M) of unlabeled ouabain. The reaction was started by addition of freshly isolated myocytes containing 2.4 mg protein, proceeded for 60 min at 37°C, and was terminated by addition of 15 ml of ice-cold washing buffer (140 mM choline chloride, 20 mM Tris-HCl, pH 7.4). The vials were then centrifuged at 4,500 g for 8 min at 4°C. The resulting myocyte pellets were washed 3 times with 10 ml of washing buffer. The final pellets were dissolved in 1 ml of NCS tissue solubilizer, heated at 50°C overnight, and then suspended in 10 ml of PCS for radioactive determination. [^{3}H]-toluene was used as an internal standard for quenching correction. The specific binding was defined as the bound radioactivity not displaced by 10^{-3} M unlabeled ouabain. The non-specific binding was 5% of the total binding. No dissociation of bound [^{3}H]-ouabain was found to occur during the entire washing procedure (4°C). Preliminary experiments showed that the specific binding for ouabain reached a steady state by 60 min. A tissue linearity was observed for myocyte protein from 1.2 to 4.8 mg.

Determination of myocyte protein. The protein content of myocyte preparations was determined by Bio-Rad Protein Assay Kit (Bio-Rad laboratories)

based on the method of Bradford (18) after myocyte preparations were disrupted by a Takmar Tissumizer. Bovine gamma globulin was used as a standard.

Materials. Alloxan monohydrate was obtained from ICN Pharmaceuticals, Inc. Ouabain and para-nitrophenylphosphate (tris salt) were purchased from Sigma Chemical Co. [^{3}H]-ouabain and [^{3}H]-toluene were products of New England Nuclear Corp. NCS (Nuclear Chicago Solvent) and PCS (Phase Combining System) were supplied by Amersham Corp. Other chemicals and reagents were of analytical grade.

RESULTS

Fig. 1 shows the effect of alloxan-diabetes on the K^+ activation pattern of K^+-PNPPase in isolated adult dog heart myocytes. In control dogs, K^+-PNPPase activity was increased hyperbolically by increasing concentrations of K^+ and appeared to reach a plateau at 10 mM. The Vmax and Km for K^+ activation were 0.45 μmoles/mg·hr and 2.5 mM, respectively, for control dogs. In alloxan-diabetic dogs, the enzyme activity was decreased significantly at low K^+ concentrations (0.5 to 5 mM); the Km for K^+ activation was increased to 7.7 mM (a three-fold increase) with no change in Vmax. These

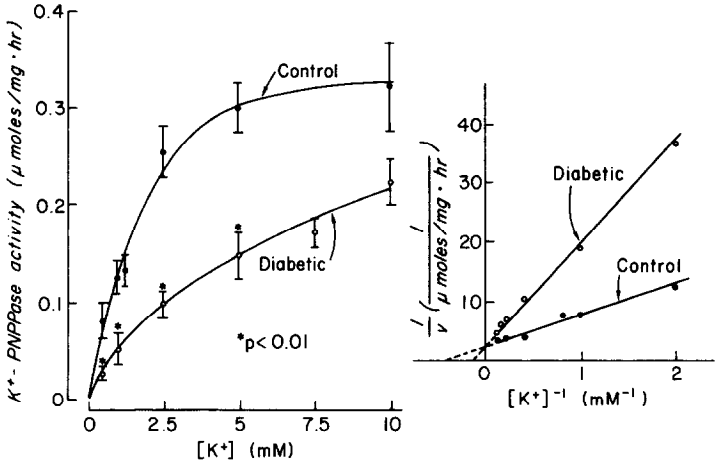

Fig. 1 Effect of alloxan-diabetes on the K^+ activation pattern of K^+-PNPPase in isolated adult dog heart myocytes.

K^+-PNPPase activity was assayed as described in Methods. Left panel illustrates the substrate-velocity relationship while right panel shows the double reciprocal plots using the same data presented in left panel. The ordinate (left panel) indicates enzyme activities in μmoles per mg protein per hr. The abscissa represents various K^+ concentrations. Vertical bar indicates standard error of the mean. Number of experiments was 9 for the control (filled circles) and 9 for the alloxan-injected (empty circles) experiments. The statistical significance was evaluated based on Student's t test.

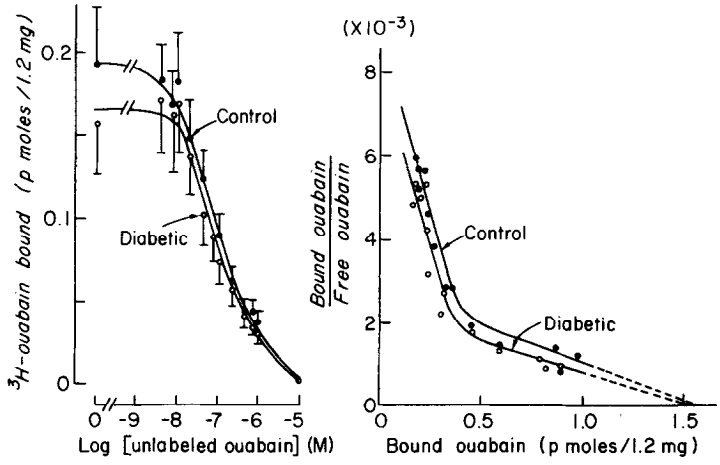

Fig. 2. Effects of alloxan-diabetes on ouabain binding in isolated adult dog heart myocytes.

Ouabain binding was assayed as described in Methods. Left panel illustrates the inhibition of [^{3}H]-ouabain binding by different concentrations of unlabeled ligands; right panel, the Scatchard plots. Vertical bar indicates standard error of the mean. Number of experiments was 9 for both the control (filled circles) and alloxan-diabetic (empty circles) experiments.

results indicate that although there is no change in the maximal velocity for K^+ activation, the affinity of the enzyme for K^+ was markedly decreased in alloxan-diabetes.

The increase in Km for K^+ activation without a corresponding change in Vmax as observed above suggests that the number of enzyme molecules remains unchanged but that the affinity of the K^+ activation site for K^+ may be decreased. Accordingly, the number of enzyme molecules, which is equivalent to the number of Na^+-K^+ pumping sites, was determined by measuring ouabain binding in isolated myocytes and the results are shown in Fig. 2. In control dogs, the binding of [^{3}H]-ouabain was inhibited by increasing concentrations of unlabeled ouabain; the inhibition was 53% at 10^{-7} M and 99% at 10^{-5} M. In diabetic dogs, the pattern of inhibition was identical to that of controls except that the binding of [^{3}H]-ouabain to isolated myocytes in the absence of unlabeled ouabain was decreased by 16.6% (the decrease was statistically not significant) (left panel of Fig. 2). The Scatchard plots between control and diabetic animals were indistinguishable (right panel

of Fig. 2). These results indicate that the number of Na^+-K^+ pumping sites remained unchanged in alloxan-diabetes.

DISCUSSION

Assuming that K^+-PNPPase represents the final step of the enzyme cycle for the (Na^++K^+)-ATPase system, the reaction sequence of K^+-PNPPase can be expressed in the following equation:

$$K^+ + E\text{-}P \underset{k_2}{\overset{k_1}{\rightleftharpoons}} K^+ \cdot E\text{-}P \xrightarrow{k_3} K^+ \cdot E + Pi$$

where k_1 and k_2 represent the rate constants for K^+ binding, k_3 the turnover number of the enzyme, E-P the phosphorylated enzyme, and $K^+ \cdot E$ the dephosphorylated enzyme. Since Vmax for K^+ activation and the Scatchard plot for ouabain binding (in which the number of enzyme molecules is calculated) were found to be unaltered as reported in this study, k_3 therefore remained unchanged. Since Km is defined as $(k_2 + k_3)/k_1$ and since k_3 was not altered, the increase in Km as reported here would be the result of increased k_2/k_1. An increase in k_2/k_1 means that the dissociation constant for K^+ binding to the phosphorylated enzyme is increased. Based on these results, it is concluded that the affinity of the (Na^++K^+)-ATPase enzyme system for K^+ is decreased in alloxan-diabetes.

K^+ homeostasis is known to be impaired in experimental (19) and clinical (1, 2) diabetes. Pettit and Vick (19) reported that pancreatectomy caused a transfer of K^+ from the intracellular to the extracellular fluid compartment. Perez et al. (1) found that in diabetic patients, the maximal values and increments of serum K^+ were higher than those in controls after K^+ loading. Our finding that alloxan-diabetes decreased the affinity of the (Na^++K^+)-ATPase enzyme system for K^+ has led us to hypothesize that a lack of insulin impairs the proper functioning of the Na^+-K^+ pump. This hypothesis is supported by the observations that insulin had a stimulatory effect on (Na^++K^+)-ATPase activity (7) and on the active sodium transport system (8, 10).

It should be mentioned that insulin affects certain phosphoprotein phosphatase reactions: it activates glycogen synthetase and pyruvate dehydrogenase by accelerating the dephosphorylation process (20, 21). Our finding that alloxan-diabetes (or lack of insulin) decreases the affinity of the $(Na^+ + K^+)$-ATPase enzyme system for K^+ suggests that the K^+-activated dephosphorylation phase of the enzyme cycle may be regulated by insulin.

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