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MECHANISMS OF INCREASED DIGITALIS TOLERANCE IN STREPTOZOTOCIN-INDUCED DIABETIC RAT MYOCARDIUM*

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Abstract—Our earlier studies revealed that the inotropic and cardiotoxic responses to ouabain are depressed significantly in chronically-induced diabetic rats (Navaratnam S and Khatter JC, *Arch Int Pharmacodyn* **301**: 151–164, 1989). In the present study, we examined the Na^+-Ca^{2+} exchange mechanism in sarcolemmal membrane vesicles (right-side out orientation) isolated from chronically-induced diabetic rat hearts. The apparent initial rates of Na^+-dependent $^{45}Ca^{2+}$ uptake were substantially lower in vesicles from 6- and 12-week diabetic rat hearts when compared to non-diabetic controls. These rates were reduced further in vesicles of 24-week diabetic rat hearts. Associated with the progressive reduction in initial rates was also a progressive reduction in the maximum amount of $^{45}Ca^{2+}$ accumulated by the vesicles. A kinetic analysis revealed a significant reduction in the maximum initial rate of $^{45}Ca^{2+}$ uptake (V_{max}) in vesicles of 24-week diabetic rat hearts. Affinity for $^{45}Ca^{2+}$, however, was the same for both diabetic and control groups. The efflux rate of $^{45}Ca^{2+}$ was also depressed in these vesicles, and they retained significantly more $^{45}Ca^{2+}$ than controls after 2–4 min of initiation of Na^+-dependent $^{45}Ca^{2+}$ efflux. These data demonstrate that the trans-sarcolemmal Ca^{2+} flux through Na^+-Ca^{2+} exchange is depressed and may explain the observed increase in digitalis tolerance of the myocardium in diabetic rats.

Myocardial metabolic disorders [1–3] as well as functional abnormalities [4–6] of the heart in the diabetic state have been well documented. Using the streptozotocin (STZ)-induced diabetic rat model, McCullogh and McNeill [7] have shown that the inotropic response to ouabain of papillary muscle and left atria of chronic diabetic rat heart are depressed markedly. In an earlier study, Bailey and Dresel [8] also reported that the inotropic response to ouabain in left atria obtained from alloxan-induced diabetic rabbits is diminished. In a recent study from our laboratory [9] it was demonstrated that diabetic rats are highly resistant to arrhythmogenic toxicity induced by intravenous infusion of ouabain. The dose of ouabain necessary to produce arrhythmias increased more than 2-fold, after 8–12 weeks of induction of diabetes with a single injection of 60 mg/kg STZ.

Cardiac glycosides bind specifically to the membrane Na^+,K^+-ATPase [10] and inhibit the enzyme [11] in various tissues including cardiac muscle. Although rats have low sensitivity to cardiac glycosides, the basic mechanisms leading to inotropic and toxic actions of digitalis are not different from those of other species [12]. Several reports have suggested that the mechanism of digitalis-induced cardiac toxicity involves intracellular Ca^{2+} overload [13–15] resulting from the inhibition of the Na^+-K^+ pump [11], an elevation of intracellular Na^+ [16], and ensuing changes in Na^+-Ca^{2+} exchange [17, 18]. Alterations in some of these mechanisms in the STZ-induced diabetic rat heart have been studied recently in various laboratories. Thus, Ku and Sellers [19] have reported a significant decrease in the specific ouabain-sensitive ^{86}Rb uptake (an estimate of sodium pump activity) in 4- to 6-week STZ-induced diabetic rat heart. Pierce and Dhalla [20] have reported a decrease in the enzyme Na^+,K^+-ATPase activity in membrane preparation obtained from 8-week diabetic hearts. An inhibition of the Na^+-K^+ pump results in increased intracellular Na^+ [21] and, as a consequence, may alter Na^+-Ca^{2+} exchanger activity. Since the ultimate step in digitalis-induced cardiotoxicity involves intracellular Ca^{2+} overload [14] through Na^+-Ca^{2+} exchange [15], it is conceivable that the Na^+-Ca^{2+} exchanger activities are modified in the diabetic heart which might explain the observed increases in digitalis tolerance [9]. The present study was undertaken to examine the sarcolemmal Na^+-Ca^{2+} exchanger activities from the heart tissue of STZ-induced diabetic rats during progressive development of the disease. A preliminary report on the data obtained was presented earlier [22].

MATERIALS AND METHODS

Male Sprague–Dawley rats, weighing between 175 and 200 g, were used for the present study. The proposed investigations were carried out 6 weeks, 12 weeks and 24 weeks after the induction of diabetes.

* A preliminary report of this work was presented at the 30th Annual Meeting of the Canadian Federation of Biological Societies, Winnipeg, June 22, 1987.

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Induction of diabetes. Rats were made diabetic by a single intravenous injection of STZ. Streptozotocin solution was prepared in 0.1 M citrate buffer (pH 4.5) and used within 30–45 min of preparation. Animals were divided into two groups; one group was injected with 60 mg/kg STZ via the tail vein and the other group was injected with equal volume of citrate buffer. After the injection, both groups of animals were maintained on normal rat chow diet and water. Plasma glucose levels of the animals were measured randomly between 36 and 48 hr of injection (using glucose kit 510 purchased from Sigma) of streptozotocin. More than 95% of the animals were found diabetic within this period.

Membrane preparation. The method employed in the preparation of the sarcolemmal membrane vesicles was essentially the same as described by us earlier [23]. Briefly, after 6 weeks, 12 weeks and 24 weeks of induction of diabetes, rats were decapitated and the hearts quickly removed and chilled on ice. Blood was flushed out of the hearts with saline, the atria and connective tissue were trimmed, and the ventricles were weighed. Approximately 6 g of ventricular tissue was used for each preparation. The ventricles were minced in a small volume of ice-cold buffer (600 mM sucrose in 10 mM imidazole, pH 7.0). Then the minced tissue was homogenized three times in the above buffer (4 vol./g tissue) using polytron PT 20 with 15-sec cooling intervals. The homogenate was centrifuged at 10,000 *g* (4°) for 20 min in a Beckman J2-21 centrifuge (rotor JA 20). The supernatant was then diluted 5-fold with 160 mM NaCl, 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), (NaCl/MOPS), pH 7.4, and centrifuged at 96,000 *g* for 60 min in a Beckman L3-40. The pellet was suspended in 2 mL NaCl/MOPS and layered over 15 mL of 30% sucrose solution containing 0.3 M NaCl, 50 mM sodium pyrophosphate and 0.1 M Tris–HCl, pH 8.3, and centrifuged at 95,000 *g* for 75 min. The white band at the sample–sucrose interface was pipetted out, diluted with 5 vol. of NaCl/MOPS, and centrifuged at 100,000 *g* for 30 min. The pellet was resuspended in either NaCl/MOPS (for Na^+-loaded vesicles) or KCl/MOPS (for K^+-loaded vesicles) to a protein concentration of 1 mg/mL. The protein concentration in the final suspensions was determined by the method of Lowry *et al.* [24], using bovine serum albumin as standard. These membranes were characterized by us earlier using various marker enzymes for sarcolemma, mitochondria, sarcoplasmic reticulum and myofibrils and found to be relatively pure sarcolemmal membrane fraction [23].

Na^+-induced $^{45}Ca^{2+}$ uptake. Sarcolemmal vesicles prepared in NaCl/MOPS were incubated at 37° to allow Na^+ to enter vesicles by passive diffusion. After 1 hr of incubation, 20-μg aliquots of these vesicles were diluted to a final volume of 0.5 mL in a series of polystyrene tubes (12 × 7 mm) preincubated at 37° containing 160 mM KCl/20 mM MOPS (pH 7.4) and 50 μM $^{45}CaCl_2$ to initiate Na^+-Ca^{2+} exchange. Then the Na^+-dependent uptake of $^{45}Ca^{2+}$ was determined by the method described by us earlier [23].

To determine the kinetics of Na^+-induced $^{45}Ca^{2+}$ uptake, $^{45}Ca^{2+}$ concentrations of the medium were varied from 20 to 100 μM to initiate the uptake. The apparent initial rates were measured at 5 sec after the initiation of $^{45}Ca^{2+}$ uptake. The $^{45}Ca^{2+}$ uptake was found linear within the first 3–10 sec of initiation of uptake.

Na^+-dependent $^{45}Ca^{2+}$ efflux. Sodium-loaded vesicles (as described in the $^{45}Ca^{2+}$ uptake study) were allowed to accumulate $^{45}Ca^{2+}$ for 1 min in 160 mM KCl and 20 mM MOPS (pH 7.4) plus 50 μM $^{45}Ca^{2+}$ in a volume of 0.5 mL. Calcium efflux was then initiated by increasing the Na^+ concentration of the medium to 90 mM. To ensure that change in osmolarity was not a contributing factor, in some experiments equimolar choline chloride was substituted for Na^+ in the medium and efflux studies were carried out. The exchange was terminated at the desired time and filtered through the Millipore filters as in the $^{45}Ca^{2+}$ uptake study, and the quantity of $^{45}Ca^{2+}$ was counted by liquid scintillation.

Reagents. Except where otherwise noted, biochemical reagents were obtained from Sigma, St. Louis, MO, U.S.A.; radioisotopes were obtained from New England Nuclear, Montreal, Quebec, Canada. All reagents were of analytical grade, and all solutions were prepared in glass-distilled deionized water.

Data analysis. The data are expressed as means ± SE. The significance of differences between control and diabetic animals was ascertained by the use of analysis of variance. For each set of experimental data, regression lines were drawn, and V_{max} and K_m were calculated separately. Student's *t*-test was then carried out for each set of paired data using $P \leq 0.05$ as the level of statistical significance. For time–course studies, the means of pairs were compared using Tukey's and Duncan's tests at $P \leq 0.05$.

RESULTS

Na^+-dependent $^{45}Ca^{2+}$ uptake. Experiments carried out in non-diabetic control rats showed that initial rates and the amounts of Na^+-dependent $^{45}Ca^{2+}$ uptake were virtually similar in vesicles isolated from 10- and 16-week-old non-diabetic control rat hearts and were grouped as one set of data, representing non-diabetic controls for 6- and 12-week diabetic rats. A separate group of rats were age-matched and used as non-diabetic controls for 24-week diabetic rats. Figure 1 shows the time–course of Na^+-dependent $^{45}Ca^{2+}$ uptake in vesicles isolated from 6-, 12- and 24-week diabetic rat hearts and their respective controls. Vesicles from non-diabetic control rats accumulated a total of 2.98 ± 0.03 nmol/mg of $^{45}Ca^{2+}$ after 1 min. On the other hand, vesicles from 6-week diabetic rat hearts accumulated $^{45}Ca^{2+}$ at a significantly lower rate resulting in a significantly lower maximal accumulated $^{45}Ca^{2+}$. Calcium content of these vesicles at 1 min was 2.18 ± 0.03 nmol/mg. The $^{45}Ca^{2+}$ uptake in vesicles after 12 weeks of diabetes was reduced further (2.02 ± 0.04 nmol/mg/min) but was not significantly different from the 6-week diabetic rat. Vesicles from 24-week diabetic rat hearts showed even further reduction in Na^+-dependent $^{45}Ca^{2+}$ uptake (1.65 ± 0.03 nmol/mg) when compared to 6- and 12-week diabetic rat

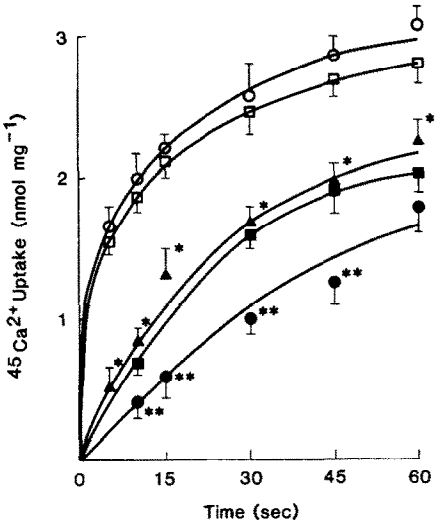

Fig. 1. Time-course of Na^+-dependent Ca^{2+} uptake in sarcolemmal vesicles. The figure shows net Ca^{2+} uptake in non-diabetic controls (○—○), age-matched controls for 24-week diabetics (□—□) and 6-week diabetic (▲—▲), 12-week diabetic (■—■) and 24-week diabetic (●—●) rat hearts. Each point is the mean ± SE of 6–8 experiments. Key: (*) significantly different from non-diabetic controls at $P < 0.05$; and (**) significantly different from age-matched controls at $P < 0.05$.

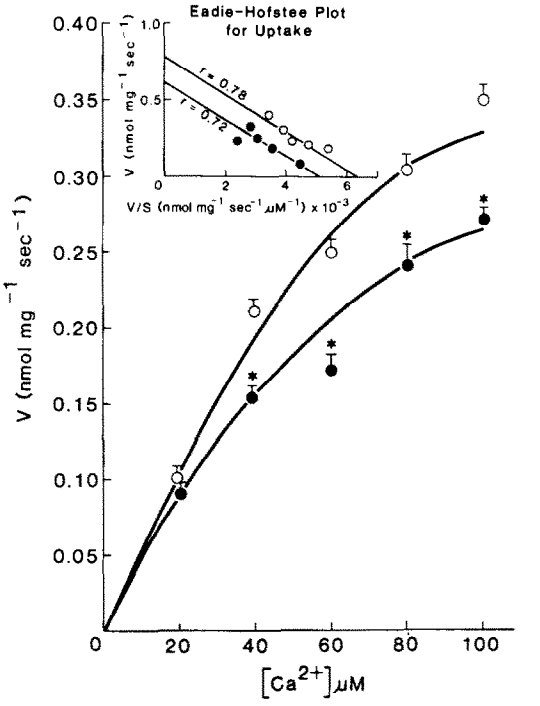

Fig. 2. Effects of increasing concentration of $[Ca^{2+}]_o$ on the initial rates (5 sec) of calcium uptake. The figure shows net initial rates of Ca^{2+} uptake in 24-week buffer-injected control (○—○) and 24-week diabetic (●—●) rat heart vesicles. Inset: Eadie–Hofstee plot of the calcium uptake data. Each point is the mean ± SE of 4–6 experiments. Key: (*) significantly different from control at $P < 0.05$.

hearts as well as 24-week control rat hearts. The reduction in Na^+-dependent $^{45}Ca^{2+}$ uptake observed in vesicles from diabetic hearts was further characterized by studying the kinetics of the exchanger as a function of $^{45}Ca^{2+}$ concentration from 20 to 100 μM. The data in Fig. 2 shows that there was a significant reduction in the initial rates of $^{45}Ca^{2+}$ uptake in vesicles of diabetic rat hearts in the presence of 20–100 μM $^{45}Ca^{2+}$. An Eadie–Hofstee plot of the data shown in Fig. 2 (inset) revealed that the apparent maximal rate of uptake of $^{45}Ca^{2+}$ (V_{max}) in 24-week diabetics was reduced by 29% (49 ± 1.8 vs 35 ± 2.4 nmol/mg/min). However, there was no significant difference in affinity for Ca^{2+} between diabetic and non-diabetic rat hearts.

Na^+-dependent $^{45}Ca^{2+}$ efflux. Sodium-loaded vesicles from hearts of three different diabetic groups of rats as well as controls were incubated with 50 μM $^{45}Ca^{2+}$ for 1 min to load the vesicles with $^{45}Ca^{2+}$ and then exposed to 90 mM $[Na^+]_o$ to initiate $^{45}Ca^{2+}$ efflux. Figure 3 illustrates the initial rates of Na^+-dependent $^{45}Ca^{2+}$ efflux (measured at 5 sec) from vesicles pre-loaded with $^{45}Ca^{2+}$ for 1 min. When the external concentration of Na^+ was increased to 90 mM in the medium, vesicles of control hearts extruded $^{45}Ca^{2+}$ with an apparent initial rate of 4.85 ± 0.15 nmol/mg/min (Fig. 3) and retained only 3.8% of its basal $^{45}Ca^{2+}$ content at the end of 1 min of incubation (Fig. 4). Alteration in osmolarity was not a contributing factor to the observed Ca^{2+} effluxes as replacement of Na^+ with choline chloride was ineffective. Vesicles from 6- and 12-week diabetic rat hearts, on the other hand, extruded $^{45}Ca^{2+}$ at a significantly slower rate (2.5 and 2.3 nmol/mg/min respectively) and retained 15–20% of their basal $^{45}Ca^{2+}$ at the end of 1–4 min. The rate of $^{45}Ca^{2+}$ efflux in vesicles of 24-week diabetic rats was reduced further (1.8 ± 0.12 nmol/mg/min) and was significantly lower than in control as well as 6- to 12-week diabetic rats.

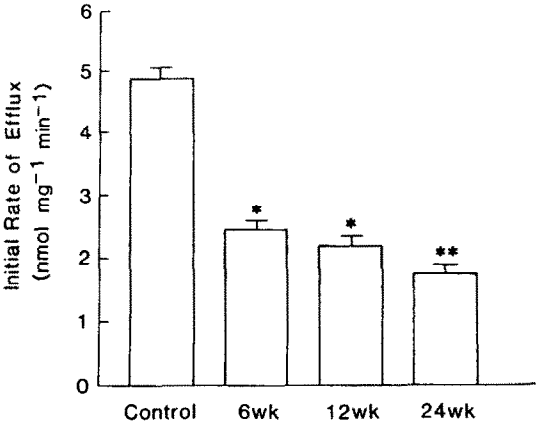

Fig. 3. Initial rates (measured at 5 sec) of vesicular Ca^{2+} efflux in vesicles pre-loaded with $^{45}Ca^{2+}$ and then exposed to 90 mM $[Na^+]_o$. The bar graphs represent the initial rates of Ca^{2+} uptake in non-diabetic controls and 6- to 24-weeks diabetic rat hearts. The data shown are the means ± SE of 4–6 experiments. Key: (*) significantly different from control, $P < 0.05$; and (**) significantly different from 6- to 12-week diabetics and controls, $P < 0.05$.

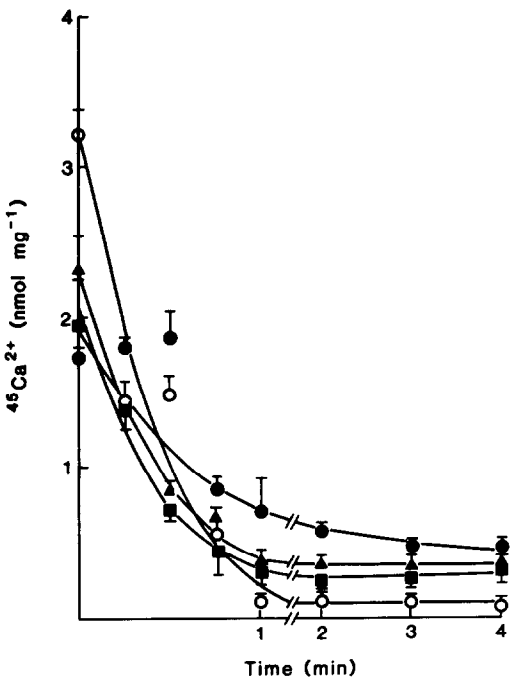

Fig. 4. Time–course of net vesicular calcium content during efflux. The figure shows Ca^{2+} content in non-diabetic controls (○—○), and 6-week diabetic (■—■), 12-week diabetic (▲—▲), and 24-week diabetic (●—●) rats. Each point is the mean ± SE of 4–6 experiments.

DISCUSSION

In this study, Na^+-dependent $^{45}Ca^{2+}$ uptake was found to be significantly lower in the membrane vesicles of diabetic rat heart. The initial rates of $^{45}Ca^{2+}$ uptake in hearts of 6- to 12-week diabetic rats was significantly lower than the rates in both non-diabetic controls. After 24 weeks of induction of diabetes, the initial rate of $^{45}Ca^{2+}$ uptake declined further such that the vesicles from 24-week diabetic hearts accumulated only 20% of the $^{45}Ca^{2+}$ taken up by non-diabetic heart vesicles after 5 sec of the initiation of the exchange. There was a 29% reduction in the V_{max} of $^{45}Ca^{2+}$ uptake without any significant change in affinity for $^{45}Ca^{2+}$. Thus, although the changes in initial rates of $^{45}Ca^{2+}$ uptake were seen as early as 6–12 weeks after the induction of diabetes, the magnitude of these changes was significantly greater after 24 weeks of the induction of diabetes than after 6–12 weeks, demonstrating that the change in the activity of Na^+-Ca^{2+} exchange is progressive and disease related and is not a direct effect of STZ injection. Furthermore, since there were no significant differences in Na^+-dependent Ca^{2+} uptake in non-diabetic age-related controls, the observed alterations in Na^+-Ca^{2+} exchange activities in 6- to 24-week diabetes are not a manifestation of maturational effect. The apparent initial rate of Na^+-dependent $^{45}Ca^{2+}$ efflux measured at 5 sec was also significantly lower in vesicles from diabetic hearts as compared to those from non-diabetic controls. The alteration in the rates of efflux progressed with time such that after 24 weeks of induction of diabetes, the rate of Ca^{2+} efflux in membrane vesicles was only 37% that of control heart vesicles. The reduced $^{45}Ca^{2+}$ uptake and efflux observed in our study may signify that the activity of the Na^+-Ca^{2+} exchanger progressively declines with the increasing duration of diabetes.

The role of Na^+-Ca^{2+} exchanger in the normal beat-to-beat regulation of cardiac contractile function has been reviewed recently [25, 26]. That digitalis acts through an increase in Ca^{2+} influx via Na^+-Ca^{2+} exchange mechanisms was postulated by Langer and his associates [27–29]. The central tenet of this theory is that increased Na_i^+, due to an inhibition of Na^+,K^+-ATPase by cardiac glycosides, stimulates an exchange of Na_i^+ for Ca_o^{2+} across the plasma membrane. In recent years, ample evidence has been accumulated to support this concept [22, 30–32]. In fact, it was recently suggested that Na^+-related Ca^{2+} influx may be important for excitation–contraction coupling even in normal beat-to-beat contractile function [25, 26]. A second theory essentially states that inotropic steroids first decrease Ca^{2+} efflux due to increased Na_i^+, presumably because of a reduction in the efficiency of the Na^+-Ca^{2+} exchange system [33]. As a consequence of this, the same intracellular compartment from which activator Ca^{2+} is released, presumably sarcoplasmic reticulum, is now able to accommodate a greater fraction of Ca_i^{2+} mobilized during each beat and release greater Ca^{2+} during additional beats. Wood and Schwartz [34] have reported that a toxic concentration of ouabain (10^{-5} M) reduces $^{45}Ca^{2+}$ efflux from minced guinea pig heart. However, whether or not the digitalis-induced decrease in Ca^{2+} efflux is, in fact, due to increased Na_i^+ has not been tested directly. Regardless of the mechanism, it is now clear that inhibition of Na^+, K^+-ATPase by cardiac glycosides stimulates Na^+-Ca^{2+} exchange in a direction that leads to an increase in intracellular Ca^{2+}. The reduced activities of Na^+-Ca^{2+} exchanger, observed in the present study, signify that diabetic hearts should be less susceptible to cellular Ca^{2+} overload and, as a consequence, to digitalis-induced arrhythmias. Significant increases in myocardial tolerance to ouabain due to diabetes in rat were reported recently [9]. These data demonstrate that in addition to a decline in receptor function [35], reduced Na^+-Ca^{2+} exchanger activities may contribute to an increased tolerance of digitalis cardiac glycosides in diabetic rat myocardium.

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