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BINDING AFFINITY OF BETA-ADRENOCEPTORS IN ATRIA OF STREPTOZOTOCIN-INDUCED DIABETIC AND NORMAL RATS

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BINDING AFFINITY OF BETA-ADRENOCEPTORS IN ATRIA OF
STREPTOZOTOCIN-INDUCED DIABETIC AND NORMAL RATS

By

EDWARD JAMES MCKENNA

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
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1983

MASTER OF SCIENCE THESIS

OF

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1983

Abstract

Diabetes mellitus is associated with a reduced responsiveness to catecholamines. The reduced responsiveness may be attributable to a reduction in beta-adrenoceptor sensitivity to catecholamines. Radioligand binding studies demonstrate that chemically-induced diabetes reduces beta-adrenoceptor number without altering beta-adrenoceptor drug binding affinities. The present study re-examines the effect of chronic (10 weeks) streptozotocin-induced diabetes in the rat on beta-adrenoceptor drug binding affinity, using pharmacological techniques, to test the results of the binding studies. The study employed two different methods: partial irreversible receptor blockade and the use of a partial agonist, to determine beta-adrenoceptor agonist binding affinity and the method of competitive antagonism to determine beta-adrenoceptor antagonist binding affinity. Diabetes produced no significant differences in the dissociation constants (1/affinity) for isoproterenol or for metaproterenol and no significant differences in the pA_2 values for timolol maleate which correlates to antagonist binding affinity. Therefore, the study confirms the results of radioligand binding studies by using intact tissue that diabetes does not alter beta-adrenoceptor drug binding affinity in cardiac tissue.

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DEDICATION

Special thanks are extended to my parents,
Edgar G. and Doris L. McKenna
for their support, encouragement, and constant concern
throughout my educational training.

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Diabetes mellitus is associated with a higher incidence of morbidity and mortality from cardiac disease than is present in the non-diabetic population (Kannel, 1979). The data suggest that diabetes is another discrete cause of congestive heart failure and that a cardiomyopathy is associated with diabetes, as a result of small vessel disease, metabolic disorders, or both. These disorders produce structural, functional, and biochemical changes in cardiac tissue (Ledet et al., 1979).

Vascular disease associated with diabetes mellitus is well known (Colwell et al., 1979; Zoneraich et al., 1980). However, diabetes may produce a cardiopathy which is independent of vascular abnormalities. In some cases, pathological studies of diabetic human hearts revealed patent and atherosclerosis-free large coronary arteries (Hamby et al., 1974). Autopsies failed to detect significant obstructive disease of the proximal arteries in some diabetic patients succumbing to heart failure (Regan et al., 1977). Several experimental studies (Regan et al., 1974; Miller, 1979; Fein et al., 1980; Penpargkul et al., 1980; Vadlamudi et al., 1982) demonstrated impaired muscle function in both chronic (greater than two weeks duration) and acute (usually less than one week) chemically-induced diabetic rat hearts. Some of these studies, also, suggest that diabetes decreases diastolic ventricular compliance and the rate of relaxation (Regan et al., 1974; Miller, 1979). Streptozotocin-induced diabetes in the rat produces no significant cardiac macrovascular disease (Chobanian et al., 1982), but impairs cardiac performance (Vadlamudi et al., 1982). These results suggest that diabetes produces a direct alteration of the rat myocardium.

A possible consequence of diabetic cardiopathy might be the appearance of an altered sensitivity to the positive chronotropic and positive inotropic effects of catecholamines (Cavaliere et al., 1980). Experimental and clinical evidence suggests that diabetes may also alter autonomic control of the myocardium. Clinically, diabetic patients exhibit a supersensitivity to cholinomimetic agents and to catecholamines; the supersensitivity to the former is more pronounced than to the latter (Lloyd-Mostyn and Watkins, 1975). However, hearts of two week streptozotocin-diabetic rats are subsensitive to both acetylcholine and catecholamines (Foy and Lucas, 1978). Diabetic hearts are less sensitive than non-diabetic hearts to carbachol 100 days after the induction of diabetes by streptozotocin, but they are supersensitive to carbachol at 180 days (Vadlamudi and McNeill, 1983). The supersensitivity observed both clinically and experimentally may be related to the well known autonomic neuropathy associated with chronic diabetes in humans (Watkins and Edmonds, 1983) and experimentally diabetic rats (Schmidt et al., 1981). The possible mechanism for diabetes-induced cardiac subsensitivity is less clearly defined.

The effect of diabetes on cardiac responsiveness to catecholamines may vary with the severity of the diabetic state, although the evidence is not at all clear. In humans, tachycardia is often associated with chronic diabetes, primarily due to vagal dysfunction (Lloyd-Mostyn and Watkins, 1975). In the rat, experimental chronic diabetes most often produces bradycardia (Savarese and Berkowitz, 1980), but acute diabetes has no effect on heart rate. A reduction

in beta-adrenoceptor sensitivity to catecholamines can explain the lowered heart rate of the diabetic rat. Beta-adrenoceptor number or density and drug-binding affinity determine beta-adrenoceptor sensitivity to catecholamines. Radioligand binding techniques provide a method of assessing beta-adrenoceptor density (B_{max}) and drug-binding affinity. Diabetes produced a 28% decrease in beta-adrenoceptor density accompanied a 24% in heart rate without any alterations in antagonist (H^3 -dihydroalprenolol) binding affinity (Savarese and Berkowitz, 1980).

Ingebretson et al. (1981a), Heyliger et al. (1982), and Ramanadham and Tenner (1983) confirm that diabetes decreased beta-adrenoceptor density without altering antagonist affinity in rat hearts. No direct in vitro studies of the positive chronotropic responsiveness of diabetic hearts to catecholamines have been performed. Diabetes had no effect on competitive [3H]-DHA binding curves by isoproterenol in membrane homogenates suggesting that agonist binding affinity is unaltered (Williams et al., 1983). However, no studies on beta-adrenoceptor drug-binding affinities of intact tissue have been performed.

Diabetes alters the mechanical function of the heart, as previously indicated, but apparently not the inotropic responsiveness to catecholamines. Ingebretson et al. (1981b) reported that acute alloxan diabetes had no effect on the inotropic response of isolated rat hearts to isoproterenol. Heyliger et al. (1982) report that diabetic papillary muscle exhibited a decreased ability to respond to

beta-adrenergic stimulation, based upon the rates of tension development (positive dF/dt) and relaxation (negative dF/dt). In control preparations, isoproterenol produced marked increases in both the positive dF/dt and negative dF/dt , whereas, in diabetic preparations, the positive dF/dt was unresponsive to isoproterenol and the negative dF/dt responded only marginally.

The questions of cardiac beta-adrenoceptor sensitivity in experimental diabetes has not been fully resolved. The depressed responses of the papillary muscle preparations from diabetic rats discussed above may be explained as an alteration in beta-adrenoceptor sensitivity. A reduced beta-adrenoceptor sensitivity can also explain the bradycardia which accompanies experimental diabetes. As previously shown, diabetes reduces beta-adrenoceptor sensitivity by reducing beta-adrenoceptor density. The possibility of reducing beta-adrenoceptor sensitivity by the reduction of agonist binding affinity needs to be reexamined. The functional differences between agonist and antagonist binding kinetics (Weiland et al., 1979; 1980) suggest that diabetes may alter agonist binding affinity without altering antagonist binding affinity. Agonist-induced desensitization of beta-adrenoceptors may reduce the apparent agonist binding affinity without altering antagonist binding affinity (Tse et al., 1978; Hoffman and Lefkowitz, 1980; Harden, 1983). Response is a function of agonist binding, complete assessment of beta-adrenoceptor sensitivity requires characterization of agonist binding affinity (Wessels et al., 1978).

The original hypothesis of the present study stated that diabetes reduces agonist affinity for beta-adrenoceptors in the rat heart. The recent observations by Williams et al., 1983) that eight weeks of streptozotocin-induced diabetes had no effect on beta-adrenoceptor agonist binding lead to a re-evaluation of the hypothesis. The present study reexamines the recent findings that diabetes does not alter beta-adrenoceptor drug-binding affinity using alternative experimental techniques. A decrease in beta-adrenoceptor number plays a major role in the diabetic subsensitivity to catecholamines and can help to explain the bradycardia which accompanies experimental diabetes in the rat; however, the possible role of a reduction in agonist affinity remains unclear. This study provides a systematic determination of beta-adrenoceptor agonist and antagonist affinities in normal and experimentally-diabetic rat atria. The use of isolated atria allows characterization of both the positive chronotropic and positive inotropic effects of catecholamines.

Several pharmacological procedures exist for the determination of agonist affinity. The availability of the beta-adrenoceptor antagonist Ro 03-7894 (1-(5-chloroacetylaminobenzfuran-2-yl)-2-isopropylaminoethanol), which acts irreversibly and selectively with beta-adrenoceptors (Nicholson and Broadley, 1978; Rankin and Broadley, 1982) allows pharmacological characterization by the method of partial irreversible receptor blockade (Furchgott and Bursztyrn, 1967). The method of partial agonists described by Waud (1969) affords an alternative technique for the determination of agonist affinity for beta-

adrenoceptors. Construction of Schild plots, using several concentrations of a competitive beta-adrenoceptor antagonist, allows calculation of antagonist affinities, expressed as pA_2 values. The present study statistically compares agonist dissociation constants ($1/\text{Affinity}$) or antagonist pA_2 ($-\log K_B$) values from control and diabetic rat atria tests for affinity changes produced by diabetes.

LITERATURE REVIEW

Induction of Diabetes

Alloxan and streptozotocin are the most extensively used agents for induction of diabetes because the diabetogenic dose is 1/4 to 1/5 times the lethal dose (Grotsky et al., 1982). The dose varies considerably among species and with the age and metabolic state of the animal. Both alloxan and streptozotocin produce beta-cell necrosis in the rat (Ganda et al., 1976). Streptozotocin appears to be more selective than alloxan (Rerup, 1970); the possible reason for this might be the high capacity of beta cells to accumulate this agent (Srivasta et al., 1982). Streptozotocin models are thought to be more relevant to the human diabetic state than alloxan models, due to metabolic profiles, enzyme concentrations, and histopathology. Alloxan-induced diabetes is more ketotic than streptozotocin-induced diabetes (Mansford and Opie, 1968).

Due to its instability, streptozotocin is dissolved in 0.1 M citrate buffer, pH 4.5 just prior to injection. Streptozotocin is

optimally stable at around pH 4 (Rerup, 1970) and its biological half-life is about 5 minutes, which necessitates an intravenous injection. For induction of diabetes, streptozotocin is conventionally administered as a single injection (Like and Rossini, 1976). Maximal elevation of plasma glucose is achieved with 60 mg/kg (Ganda et al., 1976), but significant increases in plasma glucose occur with 40 mg/kg.

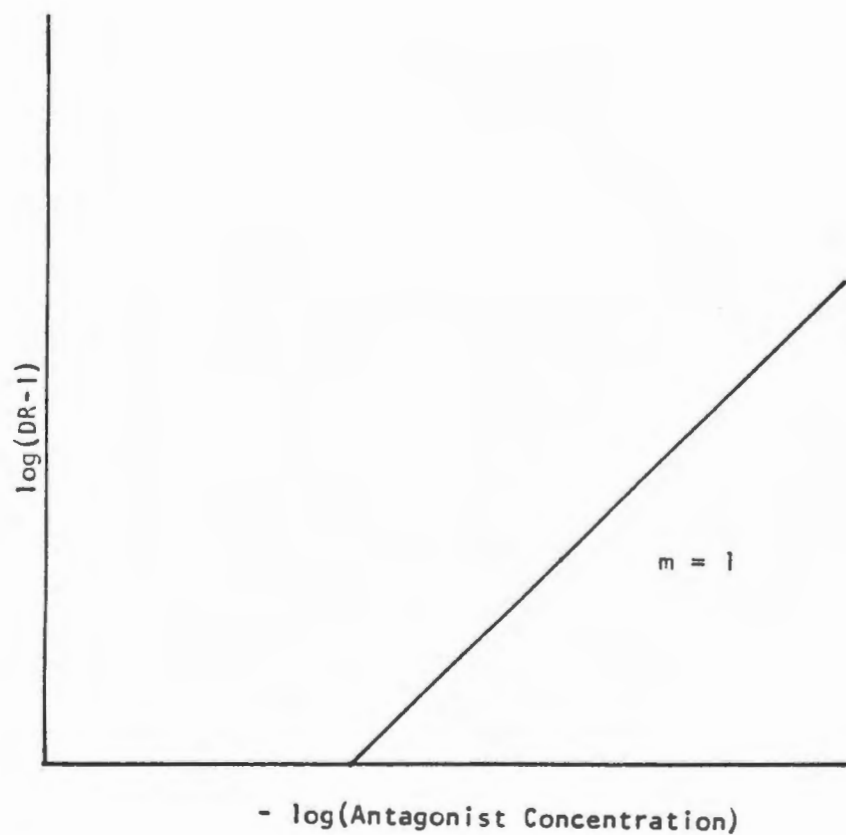
Pharmacological vs. Radioligand Binding Techniques for Assessing Receptor Characteristics

Recent reviews (Furchgott, 1978; Tallarida 1981; 1982) compare the strengths and drawbacks of pharmacological and radioligand binding procedures. In summary, both procedures estimate drug-binding affinities for a specific receptor and the rate and extent of receptor inactivation by irreversible antagonists. However, only a radioligand yields an estimate of receptor number or density (B_{max}), whereas, a pharmacological procedure permits evaluation of the relative efficacies of agonists acting upon a receptor to produce a response. Radioligand binding procedures require the demonstration of specific binding to tissue sites and no effect is measured. Thus, receptor is defined differently in each technique. The pharmacological definition of receptor has an "operational" meaning, i.e., drug-binding produces an effect; whereas the radioligand binding technique requires demonstration of a specific binding site or receptor. Typically, pharmacological procedures employ isolated tissue and radioligand techniques use membrane fractions.

The pA_2 and the Schild Plot

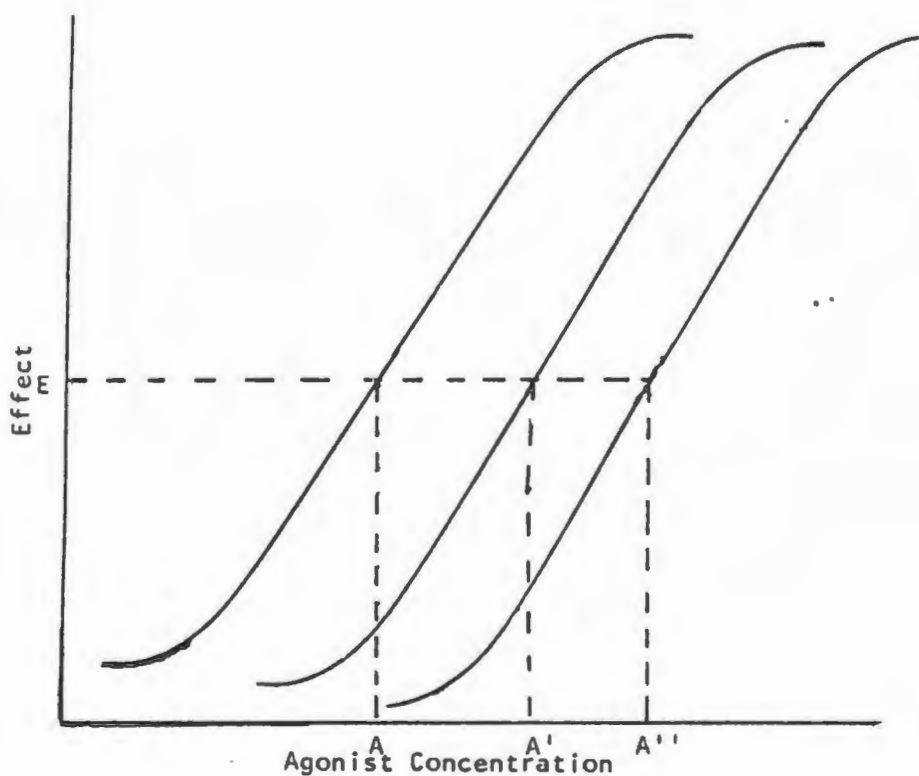
The Schild regression (Arunlakshana and Schild, 1959), theoretically, can yield the binding constant (K_B) of a competitive antagonist for a drug receptor using pharmacological techniques. The x-intercept of the Schild regression (pA_2) provides an estimate of the binding constant under certain conditions (Furchgott, 1972). The pA_2 is important in classifying receptors (Kenakin, 1982). The pA_2 is defined as the negative logarithm of the molar concentration of an antagonist which reduces the effect of a dose of agonist by half (Tallarida et al., 1979). Figure a shows a Schild plot constructed from cumulative dose-response curves in the presence of various concentrations of a competitive antagonist (see figure b).

Figure a: Theoretical Schild Plot Depicting the pA_2 Value



A theoretical Schild Plot, $\log(DR-1)$ versus $-\log[B]$, consisting of a straight line of slope unity and an x-intercept ($-\log K_B$ or pA_2). Several antagonist concentrations $[B]$ yield the points for drawing the line.

Figure b: Cumulative Dose-Response Curves of a Full Agonist Illustrating Competitive Antagonism



As the antagonist concentration $[B]$ increases, the dose-response curve shifts to the right. The degree of the shift is indicated by the dose ratio (DR) of A'/A . Thus, it takes a higher agonist concentration, A' , to produce the same effect in the presence of antagonist than the concentration A , producing the same effect in the absence of antagonist. All curves achieve the same maximum effect, since the antagonism is surmountable and the curves should be parallel due to the competitiveness of the antagonism. The dose ratios (DR), converted to $\log (DR-1)$, for each antagonist concentration $[B]$, expressed as $-\log [B]$ yield points for a Schild plot.

Based upon the occupation theory (Furchgott, 1972) stating that the effect produced by an agonist depends upon the concentration of the agonist-receptor complex, the following equation can be derived:

$$\frac{[A']}{[A]} - 1 = \frac{[B]}{K_B} \quad \text{or} \quad \log (DR-1) = -\log K_B + \log [B]$$

where DR (dose ratio) is defined as $[A']/[A]$, B is the antagonist concentration and K_B is the equilibrium binding constant of the antagonist.

A plot of $\log (DR-1)$ versus the negative logarithm of the antagonist concentration ($-\log [B]$) in molar units yields a straight line with a slope of 1 (Figure a). The x-intercept yields the pA_2 values or the negative logarithm of the equilibrium binding constant of the antagonist.

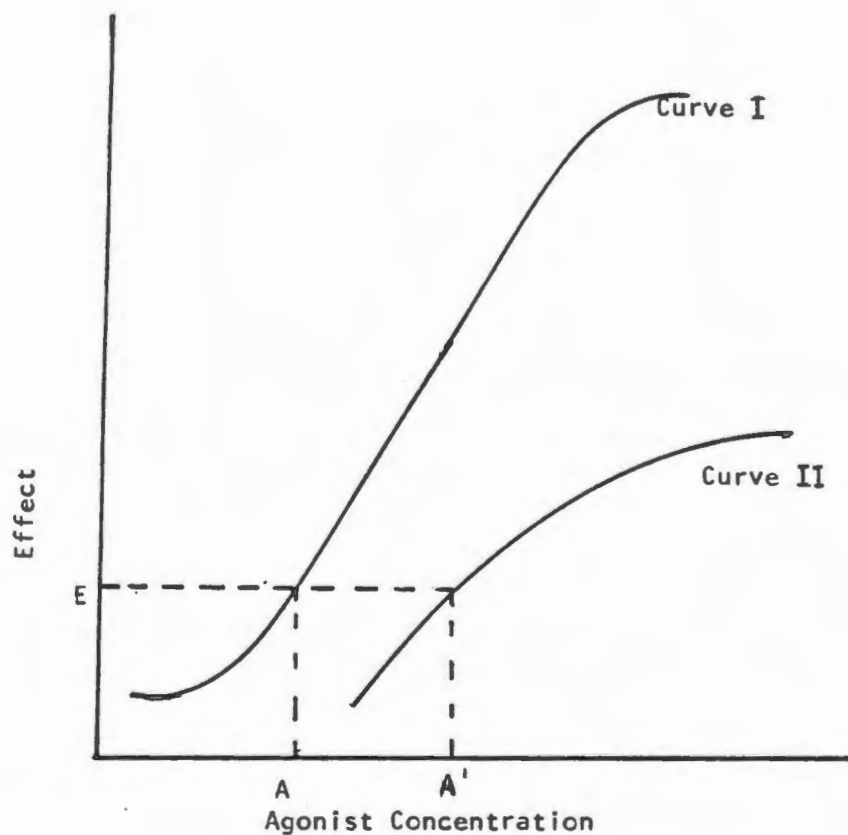
Agonist Affinity ($1/K_A$): Method of Partial Irreversible Receptor Blockade

The method of partial irreversible receptor blockade (Furchgott and Bursztyn, 1967) avoids the assumption of classical receptor theory that the full effect requires full receptor occupancy. The method requires an antagonist that combines with the same receptor as the agonist. The antagonist, by combining irreversibly and selectively with the receptor, reduces the free receptor population. If a sufficient number of receptors become inactivated, then the maximum response diminishes, based upon the occupation theory.

Figure c illustrates the dose-response curves obtained from an idealized experiment using partial irreversible receptor blockade.

Partial irreversible receptor blockade displaces the dose response curve to the right and reduces the maximum response in a dose-dependent manner. This method requires proper washout of the antagonist to achieve an equilibrium receptor blockade.

Figure c: Equal Response to a Full Agonist in the Absence and Presence of Irreversible Receptor Blockade



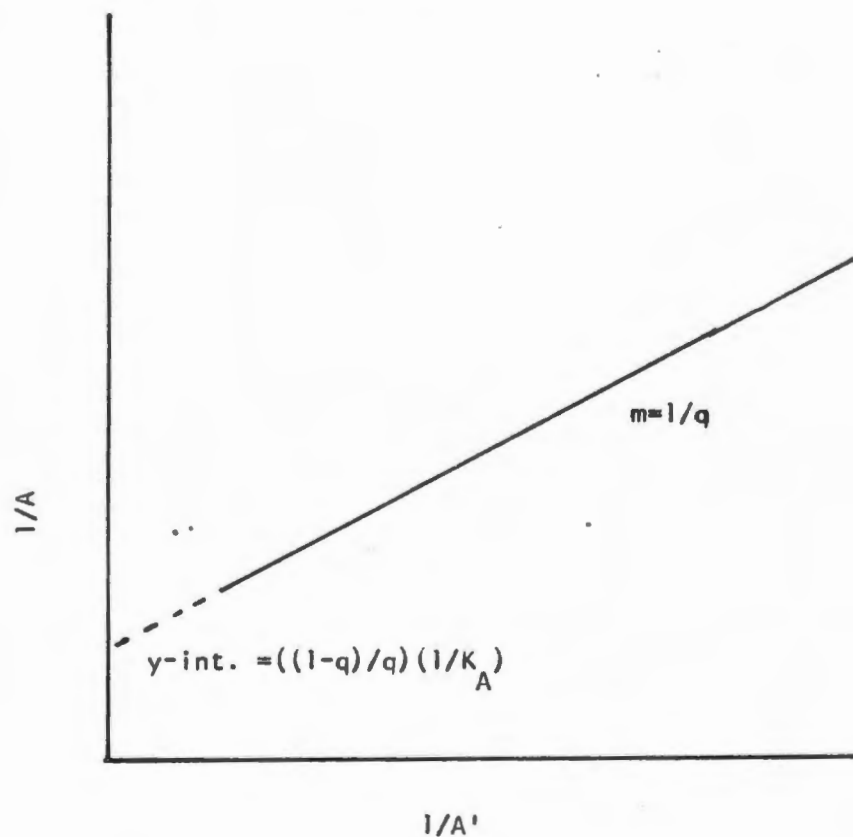
Curve II represents the dose-response curve for agonist after washout of the irreversible antagonist. Curve II does not achieve the maximum response of curve I (pre-antagonist). The irreversible antagonist displaces the dose-response curve to the right. Equiactive effects from the linear portion of each line E_1 and E_4 etc., yield pairs of agonist concentrations, (A_1, A'_1) , (A_2, A'_2) , etc. which produce each effect. Plotting the reciprocal values of each pair of agonist concentration, $(1/A_1, 1/A'_1)$, etc. yields a straight-line.

By taking equiactive effects lying on the linear portions of both curves (see figure c), one obtains pairs of agonist concentrations corresponding to each effect; i.e., (A_1, A'_1) , (A_2, A'_2) , ..., (A_N, A'_N) . Plotting each pair of agonist concentrations, expressed as their reciprocals $(1/A, 1/A')$, theoretically yields a straight line (figure d). After irreversible inactivation of a fraction of receptors, a small fraction (q) of receptors remain active. Also, equal effects require the same amount of receptor occupancy ($E = f(AR)$). From these conditions, the following equation for the line obtained in the double reciprocal plot is derived:

$$1/A = 1/q \cdot 1/A + 1 - q/q \cdot 1/K_A$$

where K_A is the dissociation constant of the agonist. The slope of the line is the reciprocal value of the fraction of remaining receptors (q). The dissociation constant may be determined by subtracting one from the slope and dividing the resulting value by the y-intercept (see figure d).

Figure d: Double Reciprocal Plot of Equipotent Agonist Concentrations in the Absence and Presence of Irreversible Receptor Blockade



Theoretical double reciprocal plot with slope of $1/q$ and y-intercept of $(1-q/q)(1/K_A)$, obtained from partial irreversible receptor blockade experiments. It follows that the K_A is slope-1/y-intercept.

Agonist Affinity ($1/K_A$): Method of Partial Agonists

The method of partial agonists (Waud, 1969) makes use of the large cardiac spare receptor capacity (Venter, 1979) and the fact that partial agonists require greater receptor occupancy than full agonists. Irreversible antagonists make full agonists act like partial agonists, and thereby allow estimation of the full agonist's receptor affinity. Since a larger receptor occupancy is required, a partial agonist may or may not elicit the tissue's maximum response.

In this method, one constructs dose-response curve for a full agonist followed by a dose-response curve for the partial agonist, after restoration of resting levels. Figure 4 shows the concentration of each agonist which produce equal responses yield agonist concentration pairs, $(A_1, P_1) \dots (A_N, P_N)$ with A_1 representing the full agonist concentration and P_1 representing the partial agonist concentration from only the linear portions of each line (Thron, 1970). Plotting the reciprocal values of each agonist pair, $(1/A, 1/P)$, yields a straight-line (figure f) having the following equation:

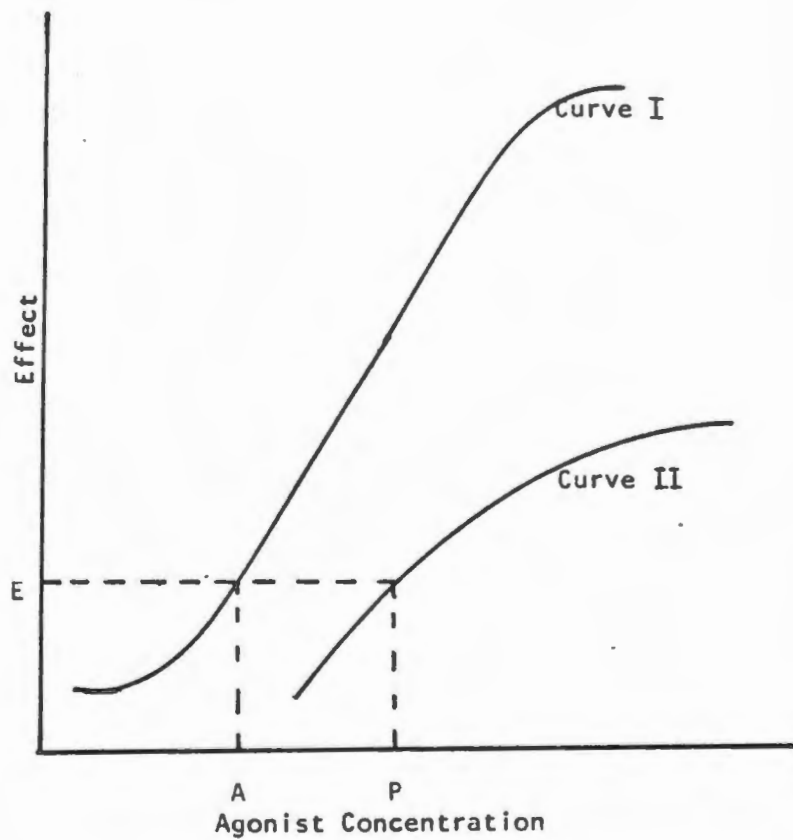
$$1/A = e_A/epK_A + e_A/K_A \cdot K_p/ep \cdot 1/p$$

where A and P represents the full agonist and partial agonist concentration, respectively. The two terms K_A and K_p represent equilibrium dissociation constants for the full and partial agonists. The value obtained by dividing the slope by the y-intercept yields the equilibrium dissociation constant of the partial agonist (K_p):

$$\text{slope/y-intercept} = \frac{e_A/K_A \cdot K_p/\epsilon_p}{e_A / \epsilon_p K_A} = K_p$$

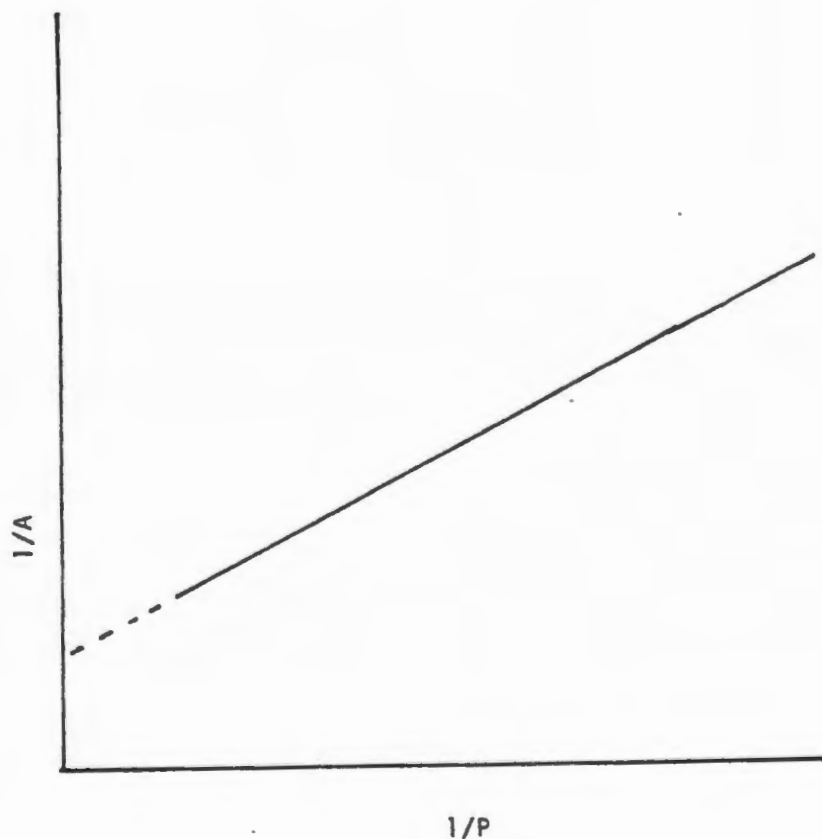
after cancelling out like terms.

Figure e: Equal Response to a Full and Partial Agonist



Curve I represents a dose-response curve to a full agonist. Agonist concentrations, A_1, A_2 etc. produces an effect, E_1, E_2 etc. Partial agonist concentrations, P_1, P_2 etc. from curve II¹ yield the same effects, E_1, E_2 etc. Thus, for each effect, there exists a pair of agonist concentrations which can produce the same effect.

Figure f: Double Reciprocal Plot of Equipotent Agonist Concentrations Using Agonists with Different Efficacies



Equipotent agonist concentrations (A_1, P_1) yield reciprocal pairs ($1/A_1, 1/P_1$) which yield the following line;

$$1/A = e_A/e_P K_A + e_A/K_A \cdot K_P/e_P \cdot 1/P$$

The quotient obtained by dividing the slope by the y-intercept yields the partial agonist dissociation constant (K_P).

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (7 weeks old) were obtained from Charles River Breeding Labs (Wilmington, Mass.). Unless otherwise indicated, all animals were supplied with food (Purina Rat Chow) and water ad libitum and housed under identical conditions throughout the 10-week experimental period.

Experimental Grouping

The rats were divided into three main groups, designated control (CT), streptozotocin-diabetic (STZ), and food restricted control (FR). The CT and STZ groups were divided into eight subgroups (1-8) and the FR group into two subgroups (7-8) of at least 4 rats each. The food restricted rats received two pellets of rat chow daily. Five of the subgroups (1-5) were used for the study of competitive beta-adrenoceptor blockade with each subgroup representing a different concentration of timolol maleate. The sixth subgroup (6) was used for the study of beta-adrenoceptor activation by the partial agonist, metaproterenol. The remaining subgroups (7-8) were used to study the effect of the irreversible beta-adrenoceptor antagonist, Ro 03-7894. The number of rats within each subgroup are shown in Table 1.

Table 1. Experimental Grouping of Rats

Group \ Subgroup	Timolol Maleate Concentration(M)					Partial Agonist	Irreversible Antagonist	
	10 ⁻⁹	10 ^{-8.5}	10 ⁻⁸	10 ^{-7.5}	--	Metaproterenol	Ascorbate	Ro 03-7894
	1	2	3	4	5	6	7	8
(CT)	6	6	6	4	6	6	4	5
(STZ)	4	4	6	4	6	4	6	4
(FR)	-	-	-	-	-	-	4	4

This table shows the grouping and number of rats used in the study of beta-adrenoceptors. CT = Age-matched controls fed ad libitum; STZ = streptozotocin-induced diabetic animals; FR = age-matched food restricted controls fed 40 grams of rat chow daily. Subgroups 1-4 represent timolol concentrations used in the study of reversible antagonism. Subgroup 5 was used as controls for both the reversible antagonism and partial agonist study. The partial agonist, metaproterenol, was used with animals in subgroup 6. The remaining subgroups (7-8) were used for the study of irreversible antagonism, ascorbate control and Ro 03-7894.

Induction of Diabetes

Diabetes was induced in rats with a single intravenous injection of streptozotocin or STZ (40 mg/kg) into a tail vein (Rerup, 1970; Like and Rossini, 1976; Ganda et al, 1976). The STZ was prepared in 0.1 M citrate buffer, pH 4.5 (40 mg/ml) immediately before injection. About 75% of the animals injected with STZ became diabetic and exhibited glycosuria. Animals which were injected with STZ, but did not subsequently become diabetic, were omitted from the study. Age-matched controls were injected with the citrate vehicle. Four to seven days after injection, weekly metabolism and blood pressure recordings were initiated. Ten weeks after injection, the animals were sacrificed.

Metabolism Studies

Beginning one week prior to injection, all rats were placed singly into metabolism cages once a week for a period of 24 hours. The following measurements were recorded: urine output, the extent of glycosuria using enzymatic test strips (Tes-Tape®, Lilly) body weight, and food and water consumption.

Indirect Blood Pressure and Pulse Rate Measurement

Beginning two weeks prior to injection, systolic blood pressure and pulse rates were measured weekly using an indirect tail-cuff method. The measurements were made after warming the rat at 34°C for 20 minutes in a temperature-controlled box. An inflatable cuff was placed around the base and a small bulb was placed on the distal portion of the tail. The bulb was attached to a pneumatic pulse trans-

ducer (MK III) which was coupled to an electrospigmograph coupler (Narco 7211) and an E & M type 4 physiograph. Systolic arterial pressure was obtained by inflating the tail cuff at pressures exceeding 180 mm Hg, then noting the point at which the pulsations reappeared during slow pressure reduction. Pulse rates were recorded simultaneously by determining the number of pulses per centimeter at a set paper speed on the physiograph. The mean of at least three measurements was recorded for each animal. The pressure was calibrated at frequent intervals using a mercury column manometer.

Isolated Atria

Ten weeks after injection, the rats were killed by a blow to the head. The chest cavity was opened and the heart was rapidly removed and placed in oxygenated buffer at room temperature. A blood sample was taken from the chest cavity for analysis of serum thyroxine and glucose. The blood sample was frozen in liquid nitrogen for later analysis. The left and right atria were surgically removed from the ventricles. The right atria were tied to tissue hangers by cotton thread and the left atria were clamped to stimulating electrodes. Both atria were vertically suspended in a 100 ml organ bath containing Krebs-Henseleit buffer (composition in mM: NaCl, 120; KCl, 5.6; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 2.4; NaH_2PO_4 , 1.21; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.33; Na_2EDTA , 0.20; NaHCO_3 , 25; and glucose, 10) gassed with 5% CO_2 in oxygen at 37°C. The atria were connected by cotton thread to a tension transducer; Narco type A (0.1 - 3 gram sensitivity), and Narco type B (0.1 - 10 gram sensitivity) for right and left atria, respectively. The

resulting tensions were recorded on an E & M type six physiograph. Initial diastolic resting tensions of 0.8 and 0.5 grams were applied to the left and the right atria, respectively. Resting tensions were determined from preliminary length-tension determinations. The left atria were driven at 2 HZ with square wave pulses (5 msec) at 1.5 times the threshold voltage by a Narco stimulator and the right atria were allowed to beat spontaneously. Tension (g) and rate (bpm) changes were measured from the left and right atria, respectively.

Serum Analysis

Blood samples were thawed at room temperature and were allowed to clot. The clot was sedimented by centrifugation at 5000 g for 5 minutes at 4°C. The supernatant (serum) was decanted for analysis of thyroxine and glucose. Hypothyroidism often accompanies the diabetic state. An Amerlex™ T-4 RIA kit was used to determine serum thyroxine levels; the kit has a total range of 0 to 25 ug thyroxine/100 ml. Serum was deproteinized with equinormal amounts of barium hydroxide and zinc sulfate solutions prior to glucose determination. The solution fraction was obtained by centrifugation at 5000 g for 10 minutes and used for analysis of glucose. Glucose was determined enzymatically with glucose oxidase and peroxidase (Sigma Kit No. 510). Serum glucose was used to estimate the degree of the diabetic state.

Drug Addition

Left and right atria from non-diabetic (CT) and diabetic (STZ) rats were allowed to stabilize for thirty minutes with frequent buffer changes. The following protocol of drug administration was employed.

Three cumulative dose-response curves were generated consecutively per atrium. Each curve was obtained by adding dl-isoproterenol directly to the buffer, yielding concentrations ranging from 10^{-10} to 10^{-6} M, in 0.5 log molar increments (Van Rossum, 1963). The response to each concentration of isoproterenol was allowed to stabilize prior to the addition of the succeeding concentration (this stabilization period never exceeded 60 seconds). After the maximum responses were obtained, the atria were washed with drug-free buffer solution, at 5 minute intervals for at least 30 minutes, to restore the tension or rate to resting levels prior to the generation of the subsequent curve. When appropriate, the third curve was generated using the partial beta-adrenoceptor agonist dl-metaproterenol, at concentrations between 10^{-8} and 10^{-4} M, instead of dl-isoproterenol. Only the results of the second and third curves were utilized, because previous studies have shown that the slope of the first dose-response curve is different from those of subsequent curves, and that the slopes of subsequent curves are similar (Broadley and Lumley, 1977).

Antagonist drugs, when necessary, were added to the buffer prior to generating the third dose-response curve. timolol maleate was added 40 minutes before, and was present throughout the generation of the third dose-response curve. Ro 03-7894 (3.2×10^{-4} M) was added to the bath for 30 minutes, then washed out with drug-free buffer for four hours, prior to the generation of the third dose-response curve. Parallel controls were exposed to an equivalent volume of the solution used to dissolve the antagonist, but were otherwise treated iden-

tically. Stock solutions of isoproterenol, metaproterenol, and timolol maleate were dissolved in 0.1 N HCl, and Ro 03-7894 in ascorbic acid solution (1 mg/ml). All drug dilutions were prepared fresh daily, and kept on ice.

Resources

<u>Drugs:</u>	Timolol Maleate	from Merck, Sharpe & Dohme
	Ro 03-7894	from Roche Products, Ltd.
	dl-Isoproterenol	from Sigma Chemical Co.
	dl-Metaproterenol	from Boehringer Ingelheim
	Streptozotocin	from Sigma Chemical Co.

Instruments: E & M Type Six Physiograph
Narco Type A Tension Transducer
Narco Type B Tension Transducer
Narco Stimulator

Thermomix Circulation Pump and Heater
E & M (MK III) Pneumatic Pulse Transducer
Narco (7211) Electrosphygmograph Coupler
E & M Type Four Physiograph

Serum

<u>Analysis Kits:</u>	Serum Glucose 510	from Sigma Chemical Co.
	Amerlex T-4 RIA Kit	from Amersham Corp.

DATA ANALYSIS

Plotting Dose-Response Curves

Responses were measured as the total rate or contraction frequency (bpm) and total developed tension (g) of the right and left atria, respectively, at each agonist concentration. Possible changes in the sensitivity of right or left atria to the agonist between the second and third curves which might occur in the absence of antagonist drugs were accounted for using parallel control atria which were not treated with the antagonist (Broadley and Nicholson, 1979). Mean

responses of untreated atria to each agonist concentration during the generation of the third dose-response curve were expressed as a fraction of the mean responses to the equivalent agonist concentration occurring during the generation of the second dose-response curve. These fractions were then applied as correction factors for antagonist treated atria. The response to each agonist concentration during the second dose-response curve was multiplied by the appropriate correction factor, to yield a corrected dose-response curve. The effect of the antagonist on the third curve was then determined by comparison with the corrected second curve.

The responses were standardized to a percentage maximum response scale. Increases in rate or tension above the resting levels were expressed as a percentage of the maximum increase. This was calculated by dividing the individual increase in rate or tension by the maximum rate or tension increase and multiplying the resulting quotient by 100%.

Estimations of agonist potency (EC_{50} values) were calculated as the negative log of the agonist concentration which produced half the maximum response. Arithmetic mean values of the EC_{50} are not normally distributed (Fleming *et al.*, 1972), but the logarithmic values are. Therefore, pD_2 values ($-\log EC_{50}$) were compared by the student's *t*-test for normal and diabetic atria.

Calculation of Antagonist Affinity by Using a Competitive Beta-Adrenoceptor Antagonist:

pA_2 values for the antagonism of isoproterenol-induced positive

inotropic and positive chronotropic response by timolol maleate in non-diabetic (CT) and diabetic (STZ) rat atria were calculated by the method of Arunlakshana and Schild (1959). Dose-response curves were obtained before and in the presence of one of four timolol maleate concentrations (10^{-9} , $10^{-8.5}$, 10^{-8} , and $10^{-7.5}$ M). Uncorrected responses, measured as the increase in tension or rate above resting levels at each isoproterenol concentration, were expressed as a percentage of their own maximum increase for both the second and third curves. The EC_{50} of each individual curve was then determined.

As described above, sensitivity changes not due to the antagonist were corrected for by comparing the results obtained from control experiments receiving no antagonist. Any shift between the second and third curve was expressed as a mean ($n=6$) correction factor ($\overline{CF} = \{ (EC_{50, \text{curve 3}}/EC_{50, \text{curve 2}})^n$). The individual EC_{50} values of the second curves were corrected by multiplication by the mean correction factor to yield a corrected EC_{50} value. The corrected EC_{50} values were used to obtain individual dose ratios ($DR = EC_{50, \text{curve 3}} / \text{corrected } EC_{50, \text{curve 2}}$). Each dose ratio was converted to $\log (DR-1)$ values. The mean $\log (DR-1)$ values (\pm S.E) were plotted against the negative \log molar concentration of timolol maleate ($-\log B$) for both rate and tension responses. Individual $\log (DR-1)$ and $-\log B$ points were used to calculate a regression line (least squares analysis). The values of slope, y-intercept, and x-intercept (pA_2), with 95% confidence limits were calculated (Tallarida, 1979). The pA_2 values for both non-diabetic and diabetic rats were compared using an unpaired

Student's t-test.

Estimation of Agonist Affinity Using a Partial Beta-Adrenoceptor Agonist:

Dissociation constants (K_p) for metaproterenol were calculated for control (CT) and diabetic (STZ) rat atria by the method of partial agonists described by Waud (1969). The atria were exposed to cumulative concentrations of isoproterenol twice with a washing in drug-free buffer after each exposure to yield two dose-response curves. A third dose-response curve was constructed using cumulative concentrations of metaproterenol. The second dose-response curve was corrected as described earlier (see "plotting dose-response curve"). The increases in rate or tension in response to each isoproterenol concentration were plotted as a percentage of the maximum increase. The increases in rate or tension in response to each metaproterenol concentration were plotted as a percentage of the maximum possible increase, which was calculated by subtracting the resting level prior to the third dose-response curve from the corrected second dose-response curve maximum total rate or tension.

Equiactive concentrations of isoproterenol (A) and metaproterenol (P) obtained from the linear portion (Thron, 1970) of each curve were determined. The reciprocal values for each atrium were plotted as $1/A$ versus $1/p$ to yield the following line:

$$1/A = \frac{e_A \cdot K_p}{K_A \cdot e_p} \cdot 1/p + \frac{e_A}{e_p} \cdot \frac{1}{K_A}$$

where e_A and e_p correspond to relative efficacy values for

isoproterenol and metaproterenol, respectively.

The dissociation constant (K_p) for metaproterenol equals the slope divided by the intercept, which are obtained from linear regression analysis. Dissociation constants for diabetic and normal atria were compared to an unpaired Student's t-test.

Estimation of Agonist Affinity Using An Irreversible Beta-Adrenoceptor Antagonist:

Dissociation constants (K_A) for isoproterenol were calculated for normal (CT), diabetic (STZ) and food restricted (FR) rat atria by the method of irreversible antagonism derived by Furchgott and Bursztyn (1967). Individual pre- (2nd curve) and post-antagonist (3rd curve) dose-response curves were plotted as described earlier (see "plotting dose-response curves") for total responses. The increase in rate and tension above the resting levels prior to each dose-response curve were plotted as a percentage of the corrected second curve maximum increase (the maximum possible increase). This method avoids possible misinterpretation arising from any change in the resting levels induced by the antagonist.

Equiactive molar concentrations of isoproterenol obtained before [A] and after washout of antagonist [A'] were determined from the linear portion of the plot of % corrected second curve maximum response versus the negative log concentration (M) of isoproterenol. The mean reciprocal (\pm S.E.) values were plotted as $1/A$ versus $1/A'$. The following equation was used to determine dissociation constants:

$$1/A = \frac{1-q}{q} \cdot \frac{1}{K_A} + \frac{1}{q} \cdot 1/A'$$

where q is the fraction of remaining receptors unoccupied by R_0 03-7894.

Using linear regression analysis, the mean dissociation constants (K_A) and fraction of active receptors remaining (q) with 95% confidence limits were calculated as follows:

$$K_A = \frac{[\text{Slope}-1]}{\text{intercept}}, \quad q = \frac{1}{\text{slope}}$$

Because constants for normal, diabetic, and food restricted rat atria were compared using one way analysis of variance (Daniel, 1978).

RESULTS

The Streptozotocin Diabetic Model*

Differences between streptozotocin-induced diabetic food-restricted, and control animals are shown in Table 2. The STZ-induced diabetic rats exhibited reduced body weight, as did the food-restricted rats though to a lesser extent, when compared to age-matched control rats. Polyuria, hyperglycemia, and glycosuria accompanied the diabetic state, as well as, polydipsia and polyphagia (data not shown). The diabetic state significantly lowered serum thyroxine levels compared to the food-restricted group. Normal thyroxine levels range from 3-4 ug/dl (Fein et al., 1980). Diabetes produced a slight hypertension and bradycardia. There were no significant differences between the potencies (pD_2 values) for isoproterenol from control, food-restricted, and diabetic atria.

Figures 1-3 depict results from weekly monitoring of heart rate, blood pressure, and body weight following the injection of streptozotocin or citrate vehicle. Diabetes produces significant changes in all three (bradycardia, hypertension, and reduced body weight) within three weeks after injection of streptozotocin and these changes persist throughout the ten week experimental period.

* "Diabetes" will be used to describe the diabetic condition induced by streptozotocin injection, with the implicit recognition that chemically induced diabetes may differ in some respects from the true diabetic state.

Table 2: Characteristics of Diabetic and Control Groups

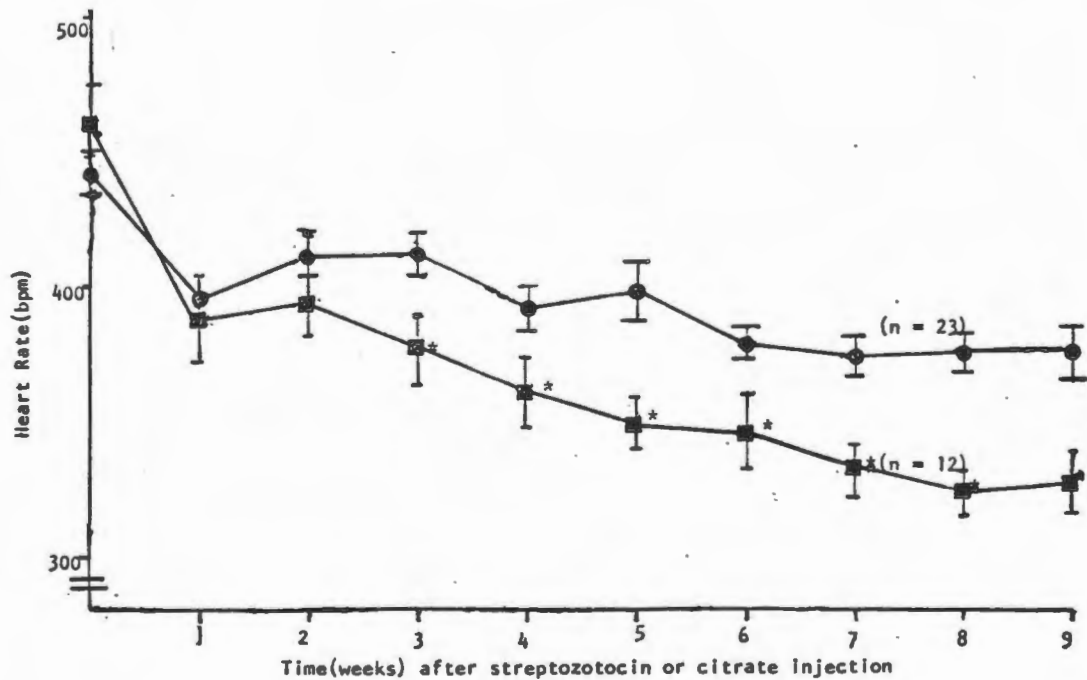
	CT	STZ	FR
BW	526 ± 16 (n=43)	413 ± 10* (n=37)	464 ± 10* (n=9)
Glu	239 ± 14 (n=20)	651 ± 37* (n=21)	279 ± 48* (n=5)
T-4	-	1.22 ± 0.17** (n=38)	2.41 ± 0.47 (n=8)
Urine	0.03 ± 0.01 (n=23)	0.53 ± 0.05* (n=12)	0.05 ± 0.01 (n=9)
Urine Glu	0 (n=23)	2 +* (n=12)	0 (n=9)
BP	136 ± 3 (n=23)	148 ± 2* (n=12)	-
HR	376 ± 9 (n=23)	327 ± 11* (n=12)	-
RA	8.37 ± 0.92 (n=43)	8.28 ± 0.58 (n=37)	8.37 ± 0.26 (n=9)
pd ₂			
Iso LA	8.51 ± 0.53 (n=43)	8.62 ± 0.44 (n=37)	8.15 ± 0.22 (n=9)

Values represent mean ± 95% confidence intervals. Numbers in parentheses represent the sample size. Asterisk (*) means that the value is significantly different (P < 0.05) than the control value; the double asterisks means that the value is significantly different than the food restricted value.

Abbreviations

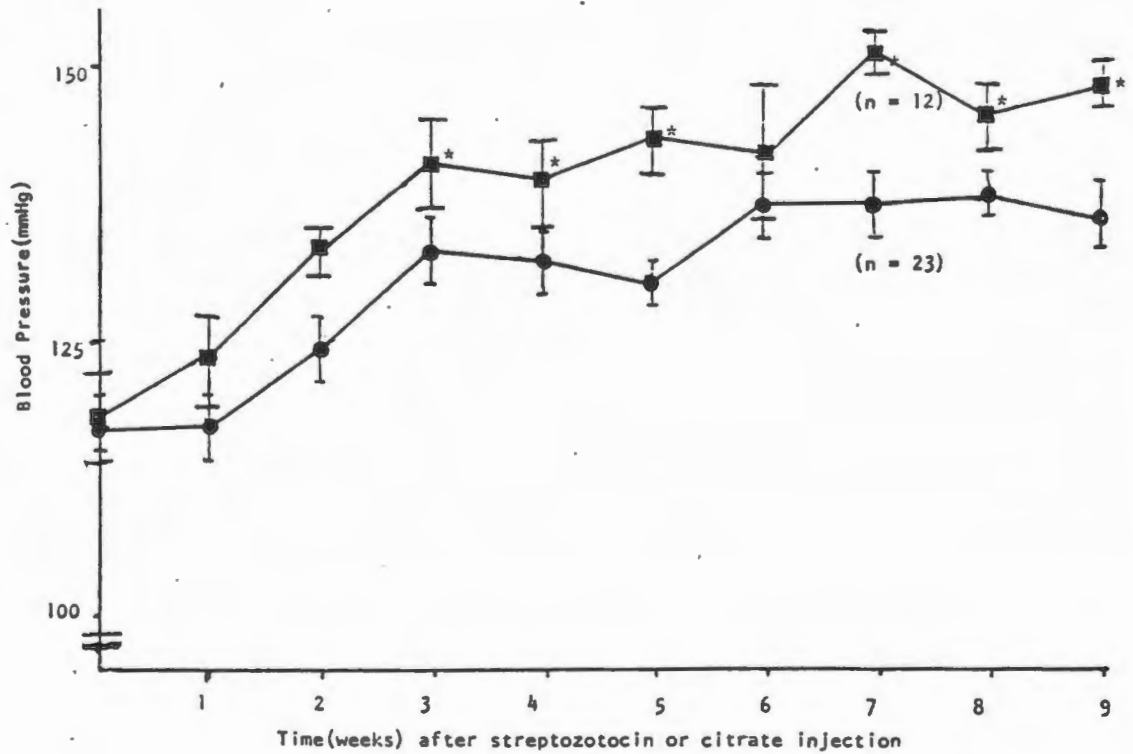
CT = Age-matched controls fed ad libitum; STZ = Streptozotocin-induced diabetes, 10 weeks after the induction of diabetes; FR = Age-matched controls on a food-restricted diet; BW = Body weight (grams); Glu = Serum glucose levels (mg/ml); T-4 = Serum thyroxine levels (µg/dl); Urine = Urine output (ml/g BW); Urine Glu = Urine glucose levels (%); BP = Systolic blood pressure (mm Hg); HR = Heart rate (beats per minute); pd₂ = -log EC₅₀ of the molar concentration of isoproterenol producing half the maximal response; RA = Right atria; LA = Left atria.

Figure 1: Effect of STZ-Induced Diabetes on Heart Rate

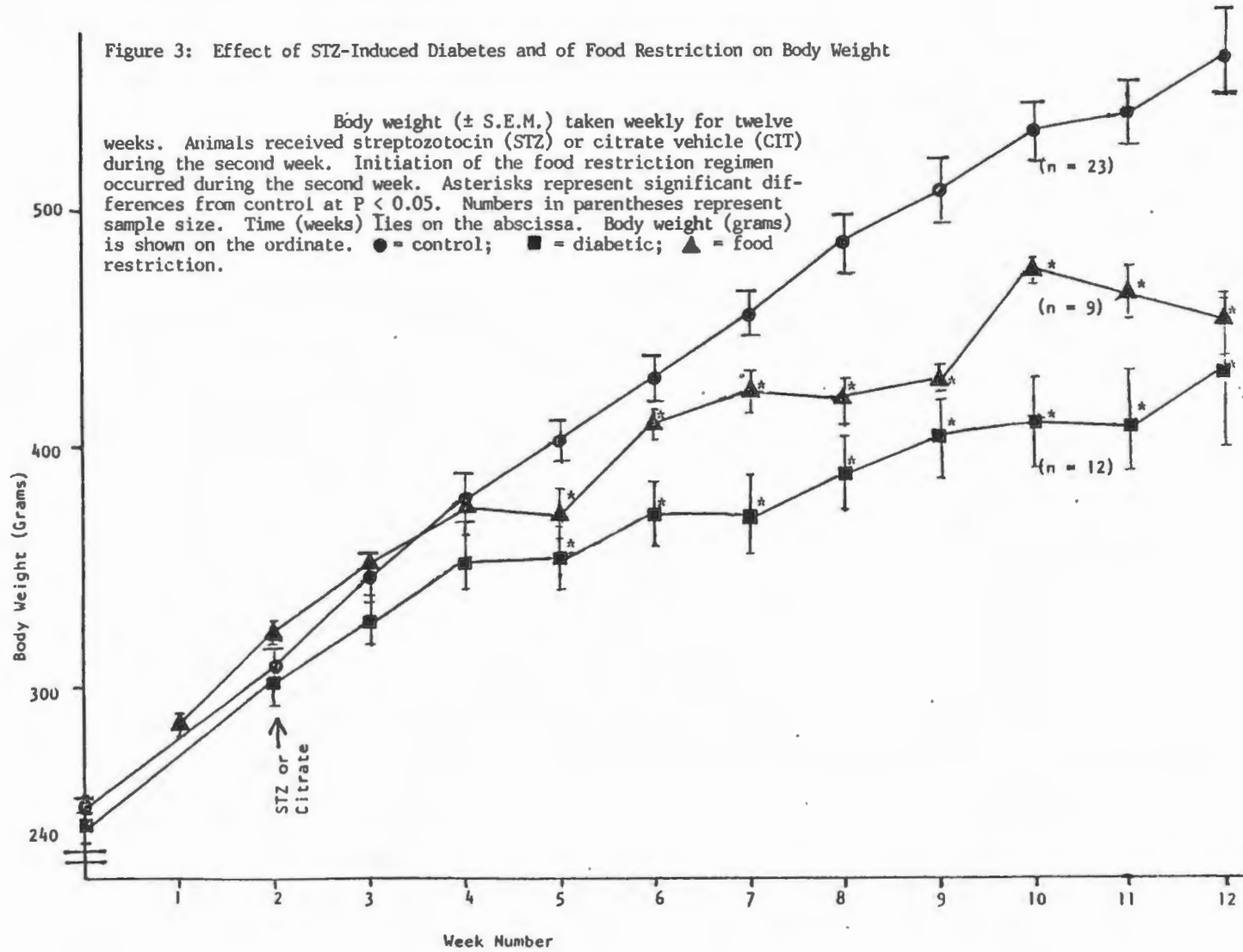


Heart rates (\pm S.E.M.) taken weekly during the 10 week experimental period from control (\bullet) and diabetic (\blacksquare) rats. A typical age-dependent bradycardia development occurs in both groups with the diabetic rats showing significant reductions within three weeks after injection of streptozotocin. Asterisks represents significant differences from control at $P < 0.05$. Numbers in parentheses represent sample size. Time (weeks) after streptozotocin or citrate vehicle lies on the abscissa. Heart rate expressed as beats per minute (bpm) is shown on the ordinate.

Figure 2: Effect of STZ-Induced Diabetes on Systolic Blood Pressure



The mean systolic blood pressure (\pm S.E.M) taken weekly during the 10 week experimental period from control (●) and diabetic (■) rat. A typical age-dependent elevation in blood pressure occurs in both groups with the diabetic rats showing significant elevations above control within two weeks after the injection of streptozotocin. Asterisks represent significant differences from control at $P < 0.05$. Numbers in parentheses represent sample size. Time (weeks) after streptozotocin or citrate vehicle injection lies on the abscissa. Systolic blood pressure (mm Hg) is shown on the ordinate.



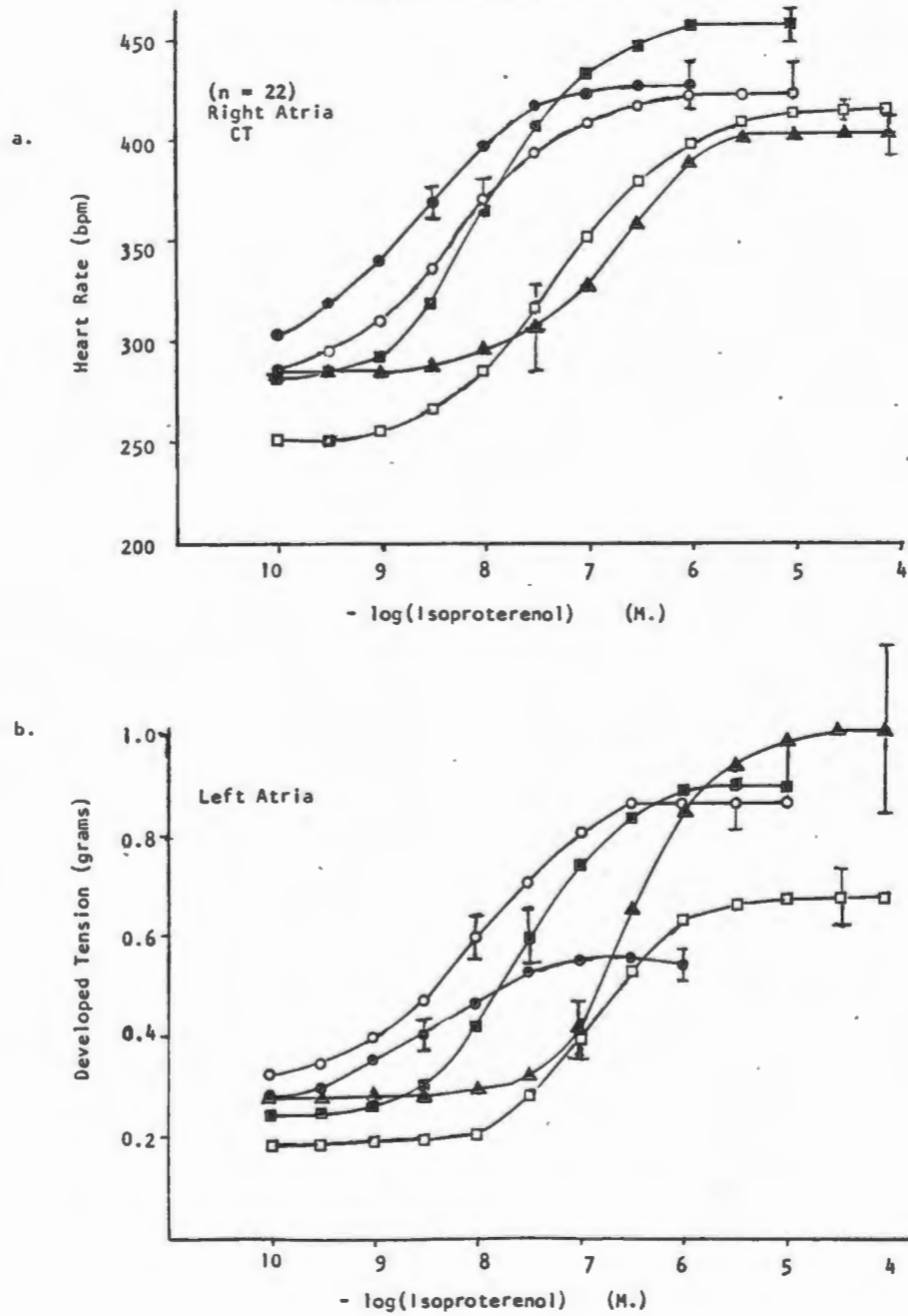
Competitive Antagonism of the Responses to Isoproterenol by Timolol Maleate

Figures 4 a-d depict the mean rate and tension dose-response curves to isoproterenol in the presence of increasing concentrations of timolol maleate. Increasing concentrations of timolol maleate (10^{-9} , $10^{-8.5}$, 10^{-8} , and $10^{-7.5}$ M) displaced the cumulative dose-response curves (control and diabetic) to isoproterenol to the right in parallel fashion without significantly ($P \leq 0.05$) depressing the maximum response. Diabetic right atria (figure 4c) had lower resting rates ($P \leq 0.001$) than control right atria (figure 4a), but the maximum rates were not different. Diabetes did not significantly affect the resting or maximum developed tension of left atria (figures 4b and d). Figures 5 a-d show the mean increase in rate and tension above resting levels, expressed as a percentage of the maximum possible increase (determined by subtracting the resting level from the maximum response obtained during the pre-antagonist dose-response curve), for control and diabetic atria. The dose-response curves for isoproterenol exhibit parallel shifts to the right of the curve generated in the absence of timolol maleate in a timolol maleate concentration-dependent manner.

Figures 6a and b depict Schild plots constructed from the mean log (DR-1) values shown in Table 9 (see appendix) for right and left atria, respectively. Regression analysis of the individual log (DR-1) values for the appropriate timolol maleate concentration yields the pA_2 value and slope for each line. Table 3 shows the mean pA_2 values

(\pm 95% confidence interval) and slopes (\pm 95% confidence interval) for each Schild plot shown in figures 6a and b. Diabetes had no effect on either the slope of the Schild plot or the estimate of antagonist binding affinity (pA_2) in either right or left atria. The slopes of the individual Schild plots do not vary significantly from unity.

Figure 4 a-d: Effect of Timolol Maleate on the Absolute Response of Non-Diabetic and Diabetic Rat Atria to Isoproterenol



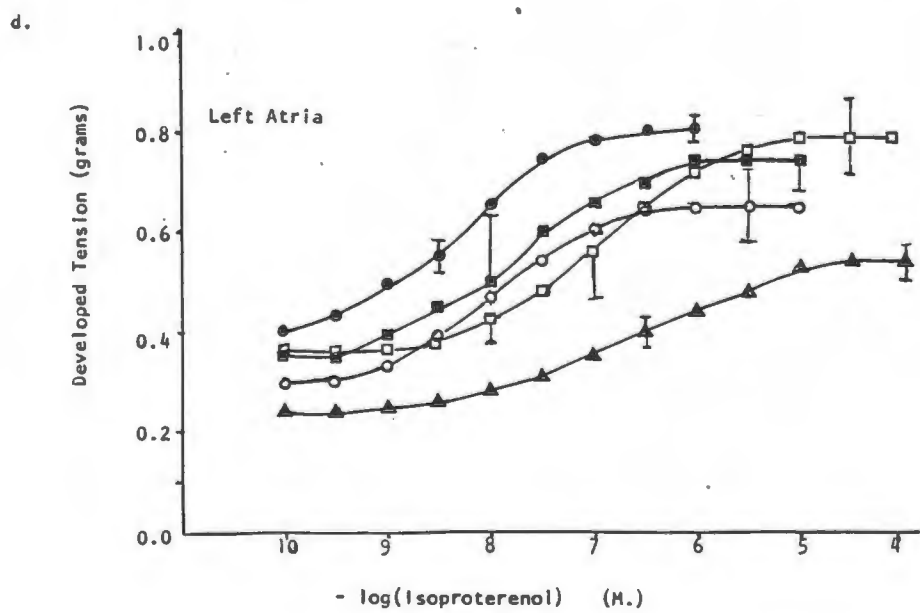
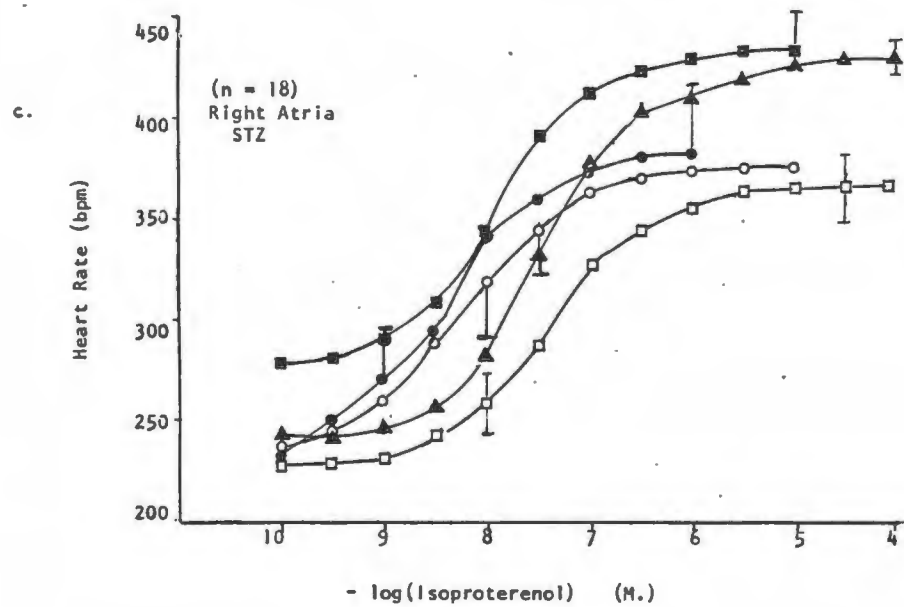


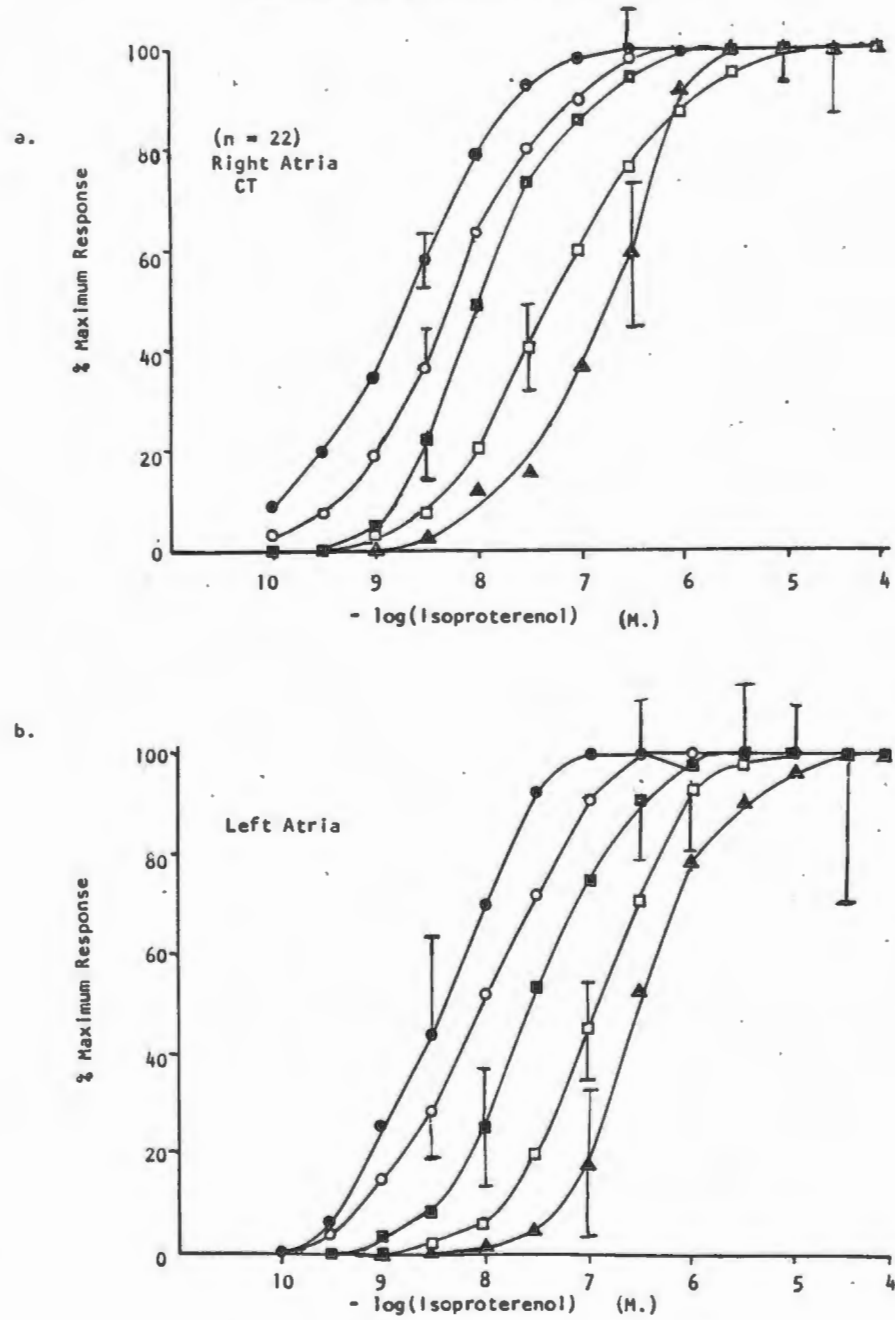
Figure 4 a-d: Effect of Timolol Maleate on the Absolute Response of Non-Diabetic and Diabetic Rat Atria to Isoproterenol

Mean cumulative dose-response curves (\pm S.E.M.) to dl-isoproterenol in the presence of various concentrations of timolol maleate from control (a and b) and diabetic (c and d) rat atria. The curves represent the mean rate (\pm S.E.M.) and tension (\pm S.E.M.) response at each isoproterenol concentration. The mean diabetic resting rate (c) is significantly ($P < 0.001$) lower than the mean control resting rate (a). A thirty minute incubation period without or with timolol maleate preceded the generation of each curve. The concentrations of timolol maleate: (●) no timolol maleate present, (○) 10^{-9} M, (■) $10^{-8.5}$ M, (□) 10^{-8} M, and (▲) $10^{-7.5}$ M. Numbers in parentheses represent the sample size.

Figure 5 a-d: Effect of Timolol Maleate on the Relative Response of Non-Diabetic and Diabetic Rat Atria to Isoproterenol

Mean cumulative dose-response curves (\pm S.E.M.) to dl-isoproterenol in the presence of various concentrations of timolol maleate from control (a and b) and diabetic (c and d) rat atria. The curves represent the mean increase in rate (a and c) and tension (b and d) above the resting levels plotted as a percentage of the maximum increase for each isoproterenol concentration. Increasing timolol maleate concentrations shift the dose-response curve to isoproterenol further to the right without depressing the maximum response. The concentrations of timolol maleate are: (●) no timolol maleate present, (○) 10^{-9} M, (■) $10^{-8.5}$ M, (□) 10^{-8} M, and (▲) $10^{-7.5}$ M. Numbers in parentheses represent the sample size.

Figure 5 a-d: Effect of Timolol Maleate on the Relative Response of Non-Diabetic and Diabetic Rat Atria to Isoproterenol



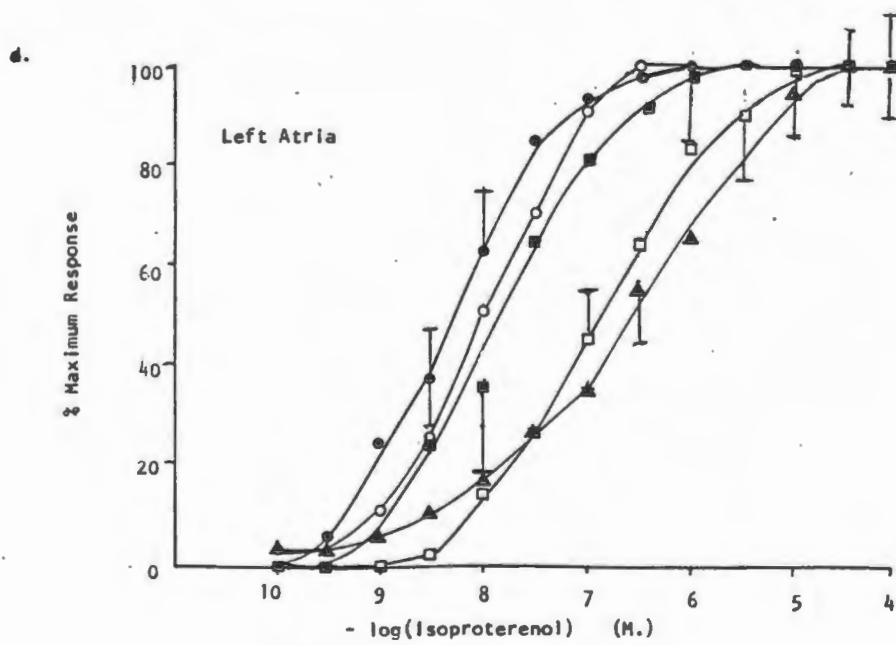
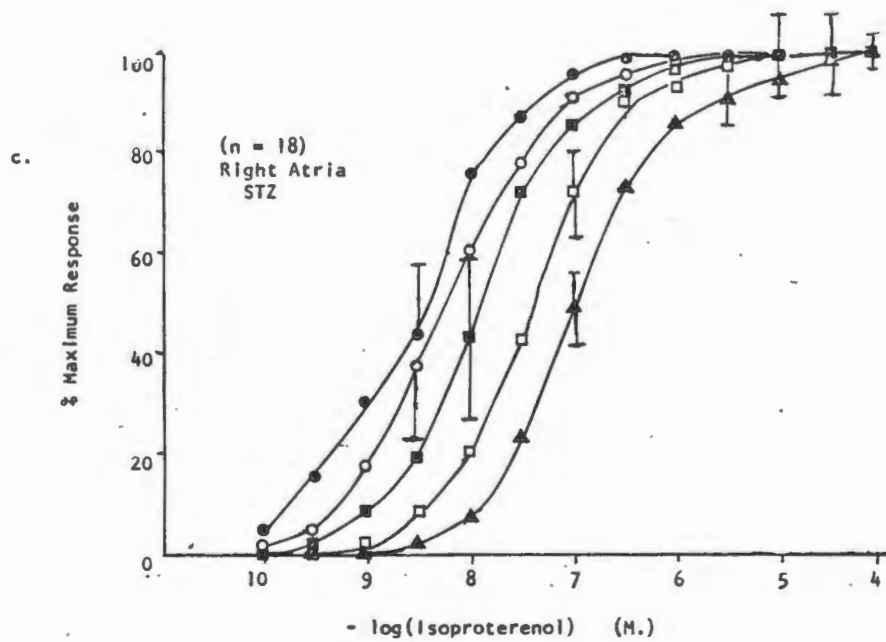
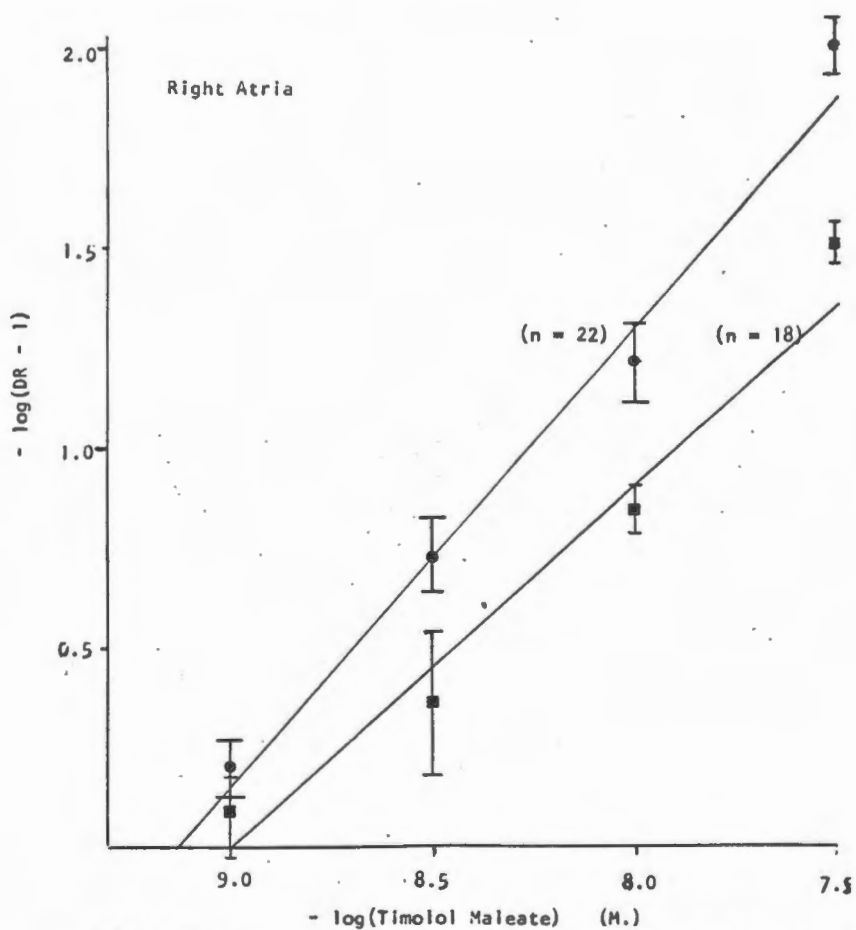
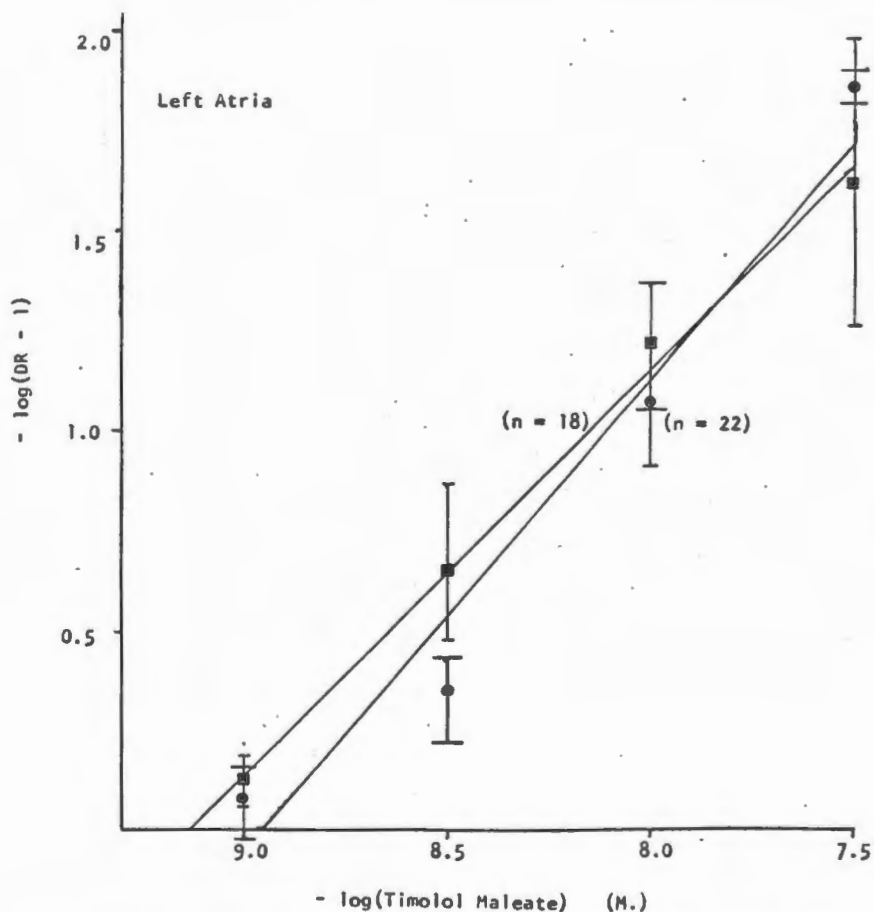


Figure 6 a-b: Effect of Diabetes on the Schild Plots of Rat Atrial Responses to Isoproterenol in the Presence of Various Concentrations of Timolol Maleate



Schild plots from control (●) and diabetic (■) rat atria, obtained by plotting the mean $\log(\text{DR}-1)$ values (\pm S.E.M.) from tables 3a and 3b versus the negative logarithm of the timolol maleate concentration (M). Figure 6a represents the right atria and figure 6b represents the left atria. Regression analysis of the Schild plots is depicted in table 4. Numbers in parentheses represent the sample size.

Figure 6 a- b: Effect of Diabetes on the Schild Plots of Rat Atrial Responses to Isoproterenol in the Presence of Various Concentrations of Timolol Maleate



Schild plots from control (●) and diabetic (■) rat atria, obtained by plotting the mean $\log(\text{DR}-1)$ values (\pm S.E.M.) from tables 3a and 3b versus the negative logarithm of the timolol maleate concentration(M). Figure 6a represents the right atria and figure 6b represents the left atria. Regression analysis of the Schild plots is depicted in table 4. Numbers in parentheses represent the sample size.

Table 3: Schild Plot Analysis

	Right Atria		Left Atria	
	<u>pA₂</u>	<u>m</u>	<u>pA₂</u>	<u>m</u>
CT (n=22)	9.13 ± 0.09	1.16 ± 0.18	8.95 ± 0.03	1.18 ± 0.25
STZ (n=18)	8.98 ± 0.12	0.94 ± 0.21	9.14 ± 0.21	1.02 ± 0.40

Mean pA₂ values (± 95% confidence interval) of right and left atria from control and diabetic rat atria. No significant differences (P > 0.05) exist between the pA₂ values from control and diabetic atria. The mean slopes (± 95% confidence interval) do not differ from unity. The number in parentheses represent the sample size.

Irreversible Antagonism of the Responses to Isoproterenol by Ro 03-7894

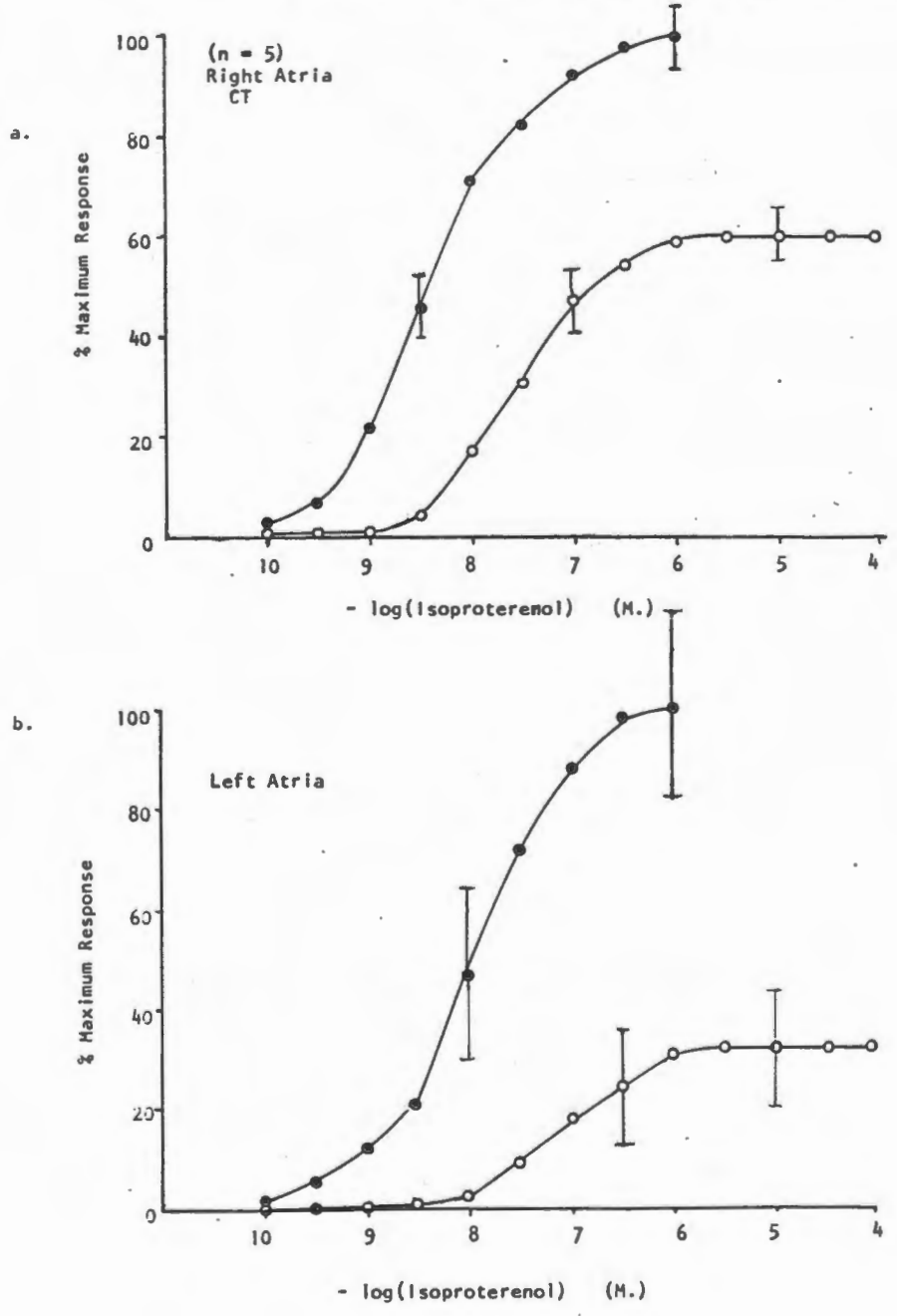
Ro 03-7894 (3.24×10^{-4} M) is used as an irreversible beta-adrenoceptor antagonist, and displaces the dose-response curves to isoproterenol to the right and reduces the maximum response (Nicholson and Broadley, 1977). Figures 7 a-f depict the mean dose-response curves, expressed as a percentage of the maximum possible increase, for rate (a,c,e) and tension (b,d,f) from control (a and b), food restricted (c and d), and diabetic (e and f) atria. Ro 03-7894 significantly reduced the resting rates and tensions (P < 0.05) and reduced the maximum rate and tension responses of rat atria to isoproterenol (Table 4). Neither diabetes nor food restriction significantly altered the effects of Ro 03-7894 rat atrial dissociation constants for isoproterenol (Table 4). Therefore, neither diabetes nor food restriction alters beta-adrenoceptor agonist binding affinity. The fraction of unoccupied receptors (q) were not significantly different (P > 0.05) between experimental groups, but exhibited a high degree of variability (Table 4).

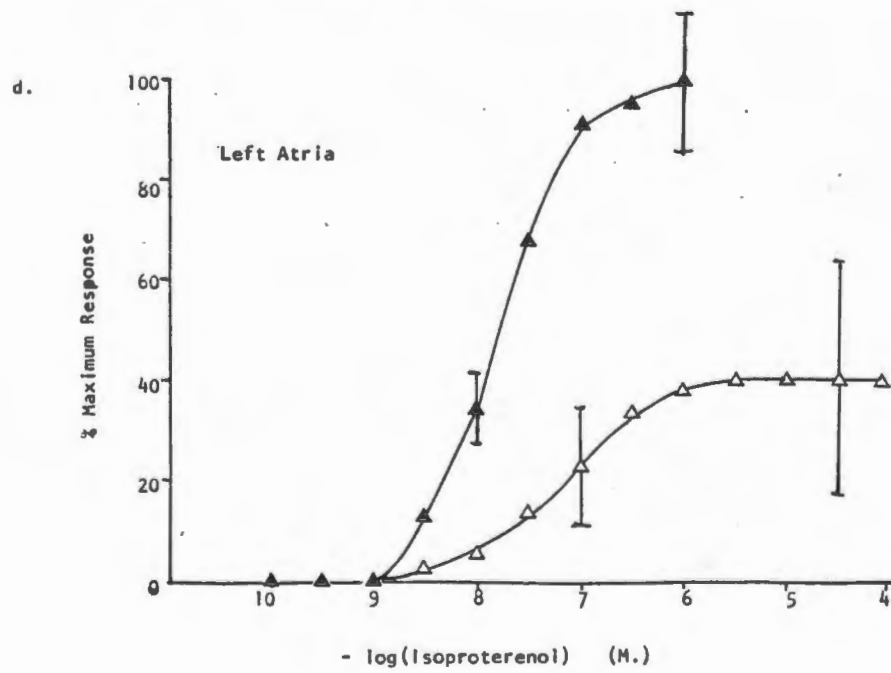
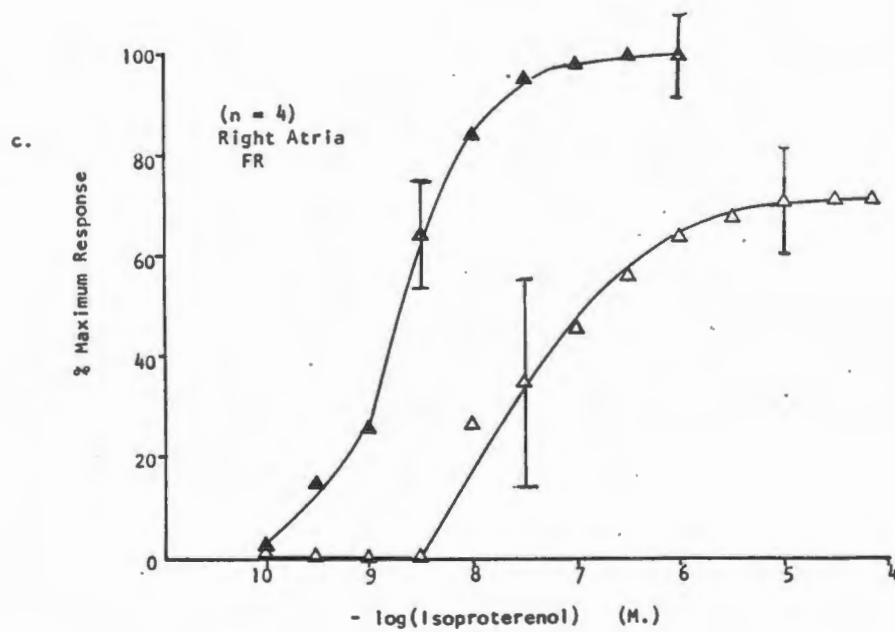
Table 4: Antagonism by Ro 03-7894 and the Effect of Diabetes on the Dissociation Constants for Isoproterenol

<u>Group</u>	<u>% Max</u>	<u>K_A (nM)</u>	<u>q</u>
<u>Right Atria</u>			
CT (n=6)	60.8 ± 5.0	18.2 ± 15.4	.095 ± .030
STZ (n=4)	67.5 ± 7.6	15.3 ± 7.8	.114 ± .051
FR (n=4)	56.0 ± 5.5	17.3 ± 5.4	.021 ± .004
<u>Left Atria</u>			
CT (n=6)	33.8 ± 7.4	16.9 ± 5.3	.057 ± .026
STZ (n=4)	70.5 ± 12.0	26.8 ± 12.2	.202 ± .090
FR (n=4)	40.0 ± 4.0	10.7 ± 3.5	.117 ± .023

Mean dissociation constants (\pm S.E.M.) for isoproterenol of cardiac beta-adrenoceptors. No significant differences ($P > 0.05$) exist between dissociation constants from control, food-restricted, and diabetic atria. The fraction of unoccupied receptors (q) within experimental groups are not significantly different ($P > 0.05$). The post-antagonist maximum responses (% max) are significantly ($P < 0.05$) reduced from the maximum possible response. Numbers in parentheses represent sample size.

Figure 7 a-f: Effect of Ro 03-7894 on the Relative Response of Non-Diabetic, Food Restricted, and Diabetic Rat Atria to Isoproterenol.





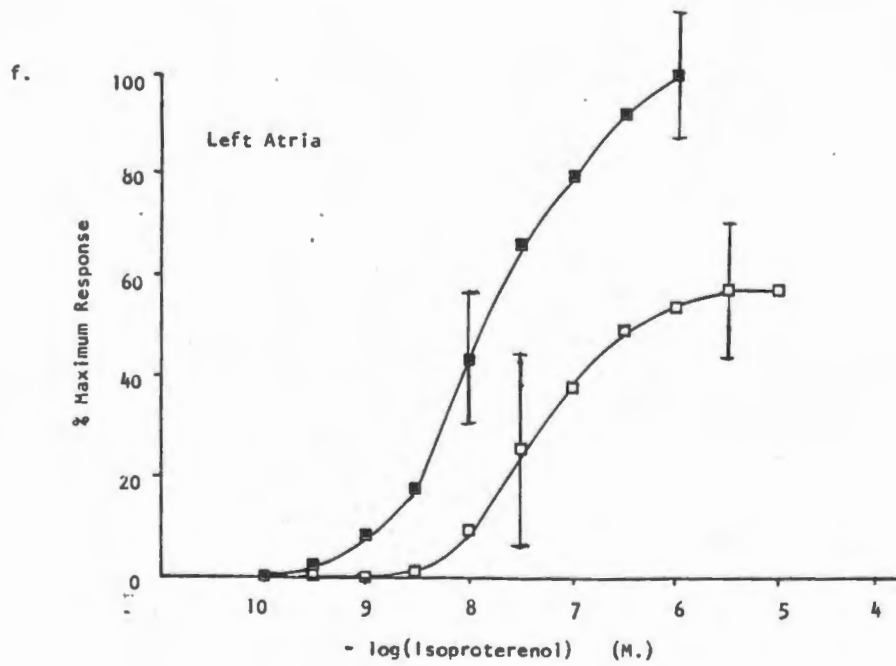
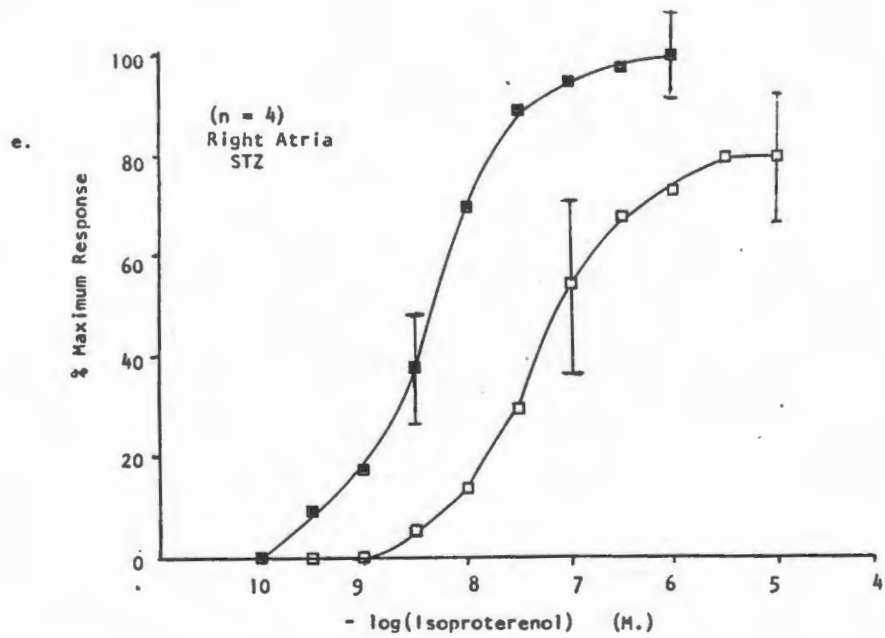


Figure 7 a-f: Effect of Ro 03-7894 on the Relative Response of Non-Diabetic, Food-Restricted, and Diabetic Rat Atria to Isoproterenol

Mean dose-response curves (\pm S.E.M.) to isoproterenol in the absence (solid points) and presence (open points) of 3.24×10^{-4} M Ro 03-7894, after a 30 minute incubation period followed by a 4 hour washout period. The presence of Ro 03-7894 displaces the dose-response curve to the right and reduces the maximum response. Figures a,c,e, represent the positive chronotropic response and figures b,d,f represent the positive inotropic response to isoproterenol. Pre-antagonist curves were corrected from controls as described in text. ● = control animals, ■ = diabetic animals, ▲ = food-restricted animals. Numbers in parentheses represent the sample size.

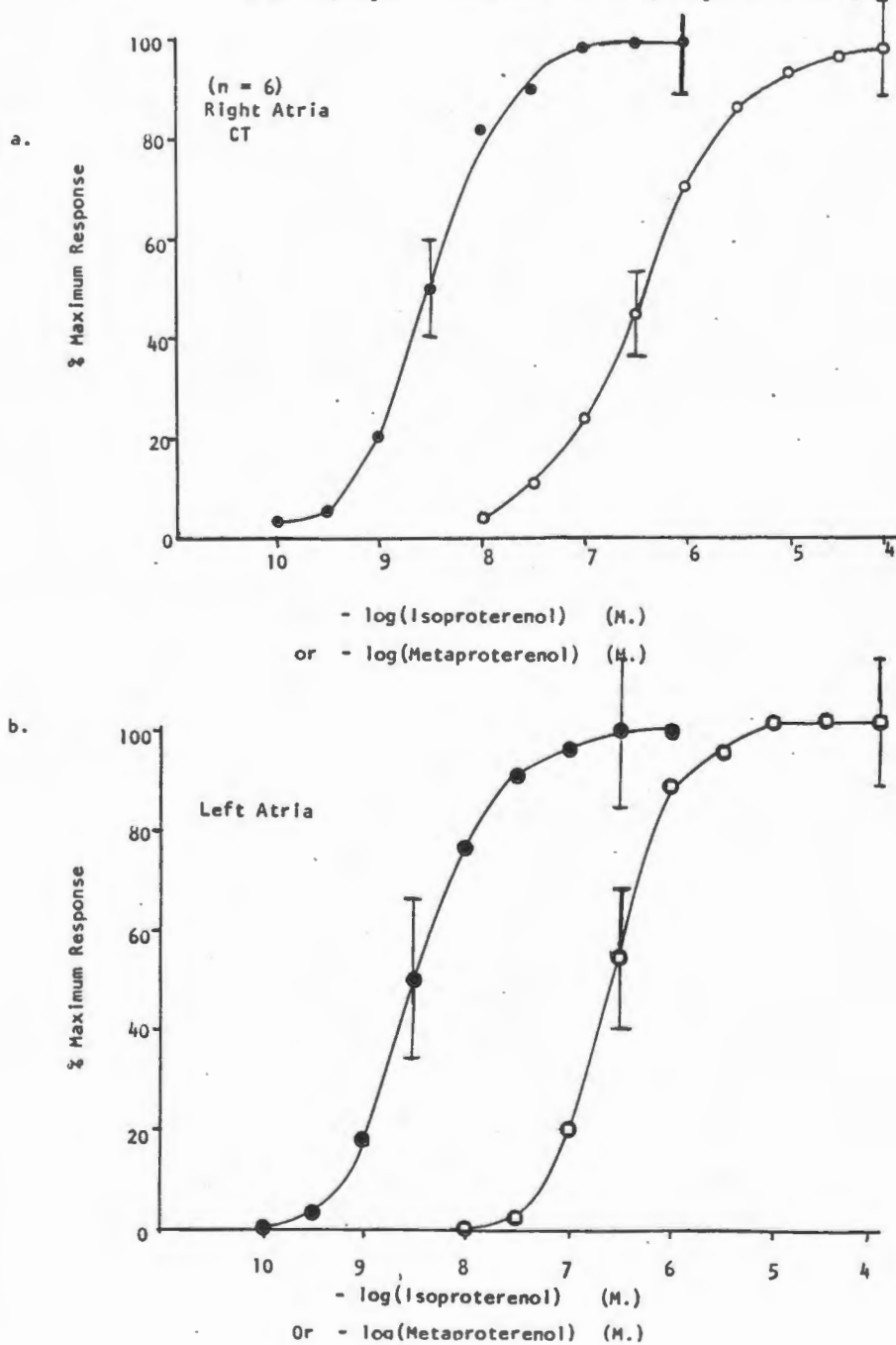
Diabetic and Non-Diabetic Atrial Responsiveness to Isoproterenol and to Metaproterenol

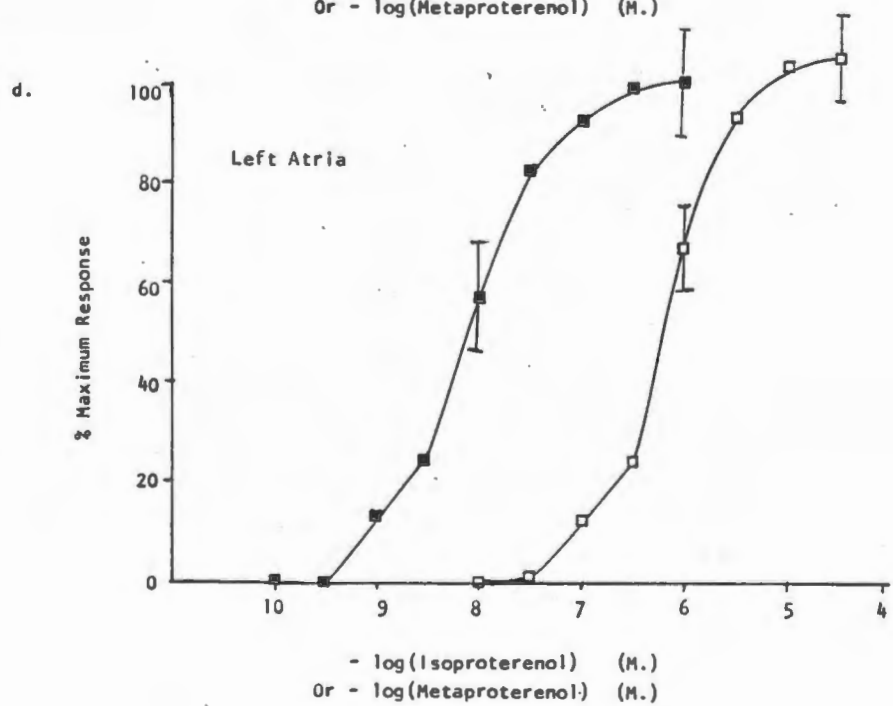
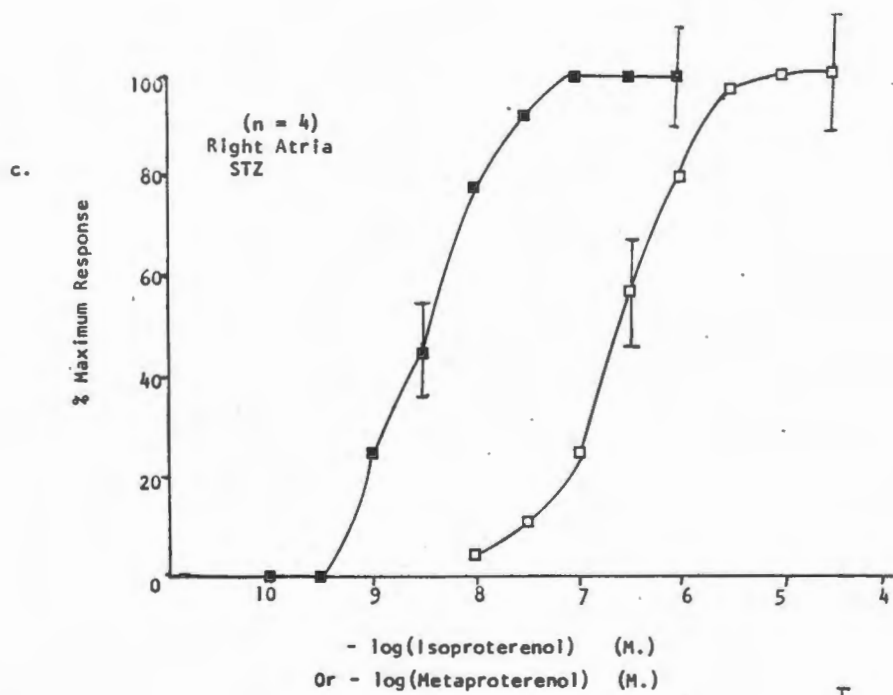
Metaproterenol produces parallel dose-response curves shifted to the right of dose-response curves produced with isoproterenol. Figures 8 a-d depict the mean dose-response curves obtained with isoproterenol and then with metaproterenol producing the same maximum response ($P \leq 0.05$), but requiring higher concentrations to produce the maximum response.

Figure 8 a-d: Relative Response of Non-Diabetic and Diabetic Rat Atria to a Full (Isoproterenol) and Partial (Metaproterenol) Agonist.

Mean cumulative dose-response curves (\pm S.E.M.) to isoproterenol (solid symbols) and metaproterenol (open symbols) from control (a and b) and diabetic (c and d) atria. The lines represent the mean increase in rate (a and c) and tension (b and d), plotted as a percentage of the maximum possible increase. The mean resting levels are the same ($P > 0.05$) for the isoproterenol and metaproterenol. No significant reduction ($P > 0.05$) in the maximum responses occur when metaproterenol is the agonist. Table 6 contains the EC_{50} value for each curve. Numbers in parentheses denote sample size.

Figure 8 a-d: Relative Response of Non-Diabetic and Diabetic Rat Atria to a Full (Isoproterenol) and Partial (Metaproterenol) Agonist.





The mean EC₅₀ values of isoproterenol and metaproterenol for left and right atria are compared in Table 5. The EC₅₀ values of metaproterenol are one hundred-fold higher than those of isoproterenol.

Table 5: Comparison of Agonist Potencies (EC₅₀ Values) between Isoproterenol and Metaproterenol for the Positive Chronotropic and Positive Inotropic Response for Non-Diabetic and Diabetic Rat Atria

<u>Right Atria</u>		
	<u>Isoproterenol</u>	<u>Metaproterenol</u>
CT (n=5)	$[3.37 \pm 0.61] \times 10^{-9}$	$[2.72 \pm 0.06] \times 10^{-7}$
STZ (n=4)	$[3.77 \pm 0.70] \times 10^{-9}$	$[2.70 \pm 0.50] \times 10^{-7}$

<u>Left Atria</u>		
	<u>Isoproterenol</u>	<u>Metaproterenol</u>
CT (n=5)	$[3.25 \pm 0.57] \times 10^{-9}$	$[4.36 \pm 0.43] \times 10^{-7}$
STZ (n=4)	$[8.20 \pm 1.21] \times 10^{-9}$	$[5.33 \pm 1.00] \times 10^{-7}$

Mean EC₅₀ values (\pm S.E.M.) of isoproterenol and metaproterenol, obtained from control and diabetic atria. Isoproterenol is about one hundred times more potent than metaproterenol. EC₅₀ values from diabetic atria do not differ significantly ($P > 0.05$) from control atria. Numbers in parentheses denote sample size.

Individual experiments yielded pairs of agonist concentrations which produce the same rate or tension response. Plotting the reciprocals of each pair as $1/[\text{isoproterenol}]$ versus $1/[\text{metaproterenol}]$ for each experiment yields straight-lines. Regression analysis of these lines provides values for the slope, y-intercept, and correlation coefficient. The quotient of the slope divided by the y-intercept equals the dissociation constant for metaproterenol. Table 6 contains dissociation constants for metaproterenol from control and diabetic atria. No significant differences

($P > 0.05$) were observed between control and diabetic atria. Diabetes does not alter metaproterenol binding affinity.

Table 6: Effect of Diabetes on the Dissociation Constants for Metaproterenol

	K_{Meta} (μM)	
	<u>Right Atria</u>	<u>Left Atria</u>
CT (n=5)	5.06 \pm 3.24	0.50 \pm 0.14
STZ (n=4)	5.20 \pm 2.55	1.55 \pm 1.49

Mean dissociation constants (\pm S.E.M.) for metaproterenol binding to beta-adrenoceptors from control and diabetic atria. No significant differences ($P > 0.05$) exist between dissociation constants from right or left atria. Numbers in parentheses denote sample size.

DISCUSSION

The results of the present study did not support the original hypothesis that diabetes reduces beta-adrenoceptor agonist binding affinity in rat atria. These findings are in agreement with radioligand binding studies recently reported by Ingebretson et al. (1983) and Williams et al. (1983), showing that neither acute nor chronic diabetes affected cardiac beta-adrenoceptor binding curves. STZ-induced diabetes did not alter the estimated dissociation constants for isoproterenol or metaproterenol in the present study. The values for the dissociation constants for isoproterenol obtained in the present study agree closely with those reported for guinea pig atria (Table 7) and those of Williams et al. (1983), using control (51 ± 15 nM), food restricted (70 ± 11 nM), and STZ-induced diabetic (78 ± 24 nM) rat hearts. The value for the dissociation constants for metaproterenol closely correspond with published values using guinea pig atria and different pharmacological techniques (Table 8).

Table 7: Dissociation Constants for Isoproterenol (Guinea Pig Atria)

	K_{Iso} (nM)		<u>Source</u>
	<u>Right Atria</u>	<u>Left Atria</u>	
	37.7 ± 14.5 (n=5)	28.0 ± 6.9 (n=8)	Broadley and Williams, 1982, 1983
a.	90.7 ± 36.0 (n=9)	84.7 ± 14.0 (n=9)	Broadley and McNeill, 1983
b.	29.0 ± 13.0 (n=4)	23.0 ± 7.0 (n=4)	
c.	38.0 ± 18.0 (n=4)	24.0 ± 23.0 (n=4)	Broadley and Nicholson, 1980

K_{iso} values are the means ± S.E. mean calculated through the use of irreversible receptor blockade by Ro 03-7894, (a) 7.6×10^{-4} M, (b) 6.4×10^{-4} M, or (c) 3.24×10^{-4} M with a 3 hour washout period. Numbers in parentheses denote sample size.

Table 8: Dissociation Constants for Metaproterenol (Guinea Pig Atria)

	K_{Meta} (μ M)		<u>Source</u>
	<u>Right Atria</u>	<u>Left Atria</u>	
	8.4 ± 2.5 (n=6)	4.3 ± 1.3 (n=4)	Broadley and Williams, 1982, 1983
	5.4 ± 2.1 (n=6)	7.6 ± 2.9 (n=8)	Williams and Broadley, 1983
	4.2 ± 2.0* (n=4)	14.7 ± 8.3* (n=4)	
	10.0 ± 2.2 (n=4)	2.4 ± 1.3 (n=4)	Broadley and Nicholson, 1980

K_{Meta} values are the means ± S.E. mean calculated through the use of irreversible receptor blockade by Ro 03-7894 (7.6×10^{-4} M) with a 3 hour washout period. Asterisks signify the use of functional antagonism with carbachol to calculate the dissociation constant. Numbers in parentheses denote sample size.

Values for dissociation constants have a large variability (Broadley and Nicholson, 1978). This variability reduces the sensitivity of statistical tests in distinguishing differences between dissociation constants. The magnitude of the variability in the present study is similar to those reported in the studies cited in Table 8.

The estimations of the fraction of unoccupied receptors (q) varied, which limits the ability to detect changes in beta-adrenoceptor number. There were no significant differences between any of the estimates of the fraction of unoccupied receptors. Furthermore, no specific trend in the estimates occurs: for right atria, the food restricted group had the lowest estimates (0.02), but for left atria, the control group had the lowest estimates (0.057). The irreversible antagonist, theoretically, inactivates the same number of beta-adrenoceptors and a reduction in beta-adrenoceptor number would be reflected by lower estimates of the fraction of unoccupied receptors. If diabetes does reduce beta-adrenoceptor number, then the estimations of the fraction of unoccupied receptors in diabetic atria should be lower than those in control atria. However, atrial size can influence the estimation of the fraction of unoccupied beta-adrenoceptors, since a larger tissue will contain greater amounts of beta-adrenoceptors, and atrial size was an uncontrolled variable. The variations in atrial size limits the usefulness of the estimation of the fraction of unoccupied receptors.

Diabetes does not alter antagonist binding affinity of cardiac

beta-adrenoceptors. Several radioligand binding studies (Savarese and Berkowitz, 1980; Ingebretson et al., 1981; Heyliger et al., 1982) demonstrate that chronic diabetes had no effect of [H^3]-DHA binding affinity in rat ventricular tissues. The present study confirms by alternative methodology that antagonist binding affinity of cardiac beta-adrenoceptors is not affected by diabetes. The pA_2 values for timolol maleate from control and diabetic atria were not significantly different from each other (Table 3). The estimates of the pA_2 values are consistent with those found by other investigators (Dreyer and Offermeier, 1980). The pA_2 value equals the negative logarithm of the antagonist's dissociation constant when the slope of the Schild plot is unity (Tallarida et al., 1979). None of the slopes of the Schild plots differed significantly from each other. Thus, the antagonist binding affinities are the same in control and diabetic atria.

Diabetes is a heterogenous disease state; other complications such as hypothyroidism (Fein et al., 1980; Pittman et al., 1981; Penpargkul et al., 1981; Malhorta et al., 1981), hypertension (Christlieb, 1973; Igarashi et al., 1978; Kawashima et al., 1978; Factor et al., 1981; Sasaki and Bunag, 1982; Hayashi et al., 1983) or hypotension (Pfaffman, 1980; Jackson and Carrier, 1981; 1983), bradycardia (Savarese and Berkowitz, 1980) may accompany the diabetic state. In the present study, streptozotocin produced a diabetic state characterized by glycosuria, polyuria, polyphagia, polydipsia, and reduced body weight. Bradycardia and a slight hypertensive effect accompanied the diabetic condition. The diabetic rats exhibited

decreased serum thyroxine levels (hypothyroidism) and increased serum glucose levels (hyperglycemia).

The presence of other disease states suggests that alterations in the diabetic myocardium may not have a single underlying cause-and-effect relationship, but instead may be due to a multiplicity of factors. It appears highly unlikely that streptozotocin itself could have produced the reductions in beta-adrenoceptor number. Streptozotocin is void of cardiotoxic effects at a tissue and sub-cellular level (Fein et al., 1980; 1981). However, hypothyroid animals have been shown to contain a decreased number of cardiac beta-adrenoceptors with alterations in antagonist (DHA) binding affinity (Ciaraldi et al., 1977; McConnaughey et al., 1979; Chang et al., 1982). Ischac et al. (1983) found that hypothyroidism had no effect on agonist potency (pA_2), produced bradycardia, and had no effect on maximum responses to isoproterenol. Furthermore, in general, hypertension can cause a reduction in beta-adrenoceptor number without altering [H^3]-DHA binding affinity (Williams et al., 1977; Woodcock et al., 1979). Because diabetes reduces both alpha- and beta-adrenoceptor density and hypothyroidism increases alpha-adrenoceptor density, Williams et al. (1983) discounts hypothyroidism as the primary cause of the reduction in beta-adrenoceptor density seen in diabetes. Furthermore, Williams et al. (1983) found that diabetes had no effect on muscarinic receptor number which suggests that alterations in adrenergic receptors are specific. Fein et al. (1980) considers it unlikely that hypothyroidism produced the altered mecha-

nics exhibited by diabetic papillary muscle because of the lack of correlation between the free T_4 index and the altered mechanical properties. Bhalla et al. (1980) found no difference in beta-adrenoceptor number between control and spontaneously hypertensive rats, instead they found a reduced affinity of beta-adrenoceptors for isoproterenol. The altered metabolic status of diabetic rats may also produce changes in beta-adrenoceptor sensitivity. Increased plasma lipid content can reduce cardiac beta-adrenoceptor number without affecting antagonist binding affinity (Wince and Rutledge, 1981).

Certain cardiac disease states such as ischaemia (Feuvray et al., 1979) and heart failure (Bristow et al., 1982) have been associated with a reduction in beta-adrenoceptor number without alterations in [H^3]-DHA binding affinity. It has already been noted that diabetics have a higher incidence of mortality from heart disease than the non-diabetic population (Kannel, 1979). Ischaemia and heart failure are often the end results of heart disease. Thus, diabetes may predispose the heart to congestive heart failure in part by reducing beta-adrenoceptor number without altering affinity.

Food restriction does not reproduce the cardiac alterations which occur with diabetes (Fein et al., 1980; Penpargkul et al., 1981; Malhorta et al., 1981). The present study shows that caloric deprivation induced by restricting food intake did not alter the dissociation constants for isoproterenol. Williams et al. (1983) also demonstrated that caloric deprivation did not produce the alterations in beta-adrenoceptors that occur in the diabetic state.

In summary, the present study confirms that chronic diabetes does not affect cardiac beta-adrenoceptor binding affinities for agonists or antagonists. Diabetes lowered the basal heart rate, but had no affect on the maximum chronotropic responses to isoproterenol or metaproterenol. In addition, diabetes had no affect on agonist potencies (EC_{50}) for positive chronotropic and positive inotropic responses. The methodology examined the maximum response and receptor binding characteristics and did not quantitate the time course of the response. The reduced responsiveness to catecholamines in vivo may result from a slower formation of agonist-receptor complexes due to the reduction in beta-adrenoceptor number which has been demonstrated by radioligand techniques (pharmacological techniques used in the present study cannot quantitate receptor number). Thus, chronic diabetes does not alter beta-adrenoceptor-drug binding characteristics, but may influence the effect of drug-receptor binding.

APPENDIX

Table 9 contains the mean EC₅₀ values, obtained from corrected pre-antagonist and post-antagonist dose-response curves to isoproterenol in the presence of different timolol maleate concentrations, and the mean values of the logarithm (dose ratio minus one (\pm S.E.M.)), obtained from the individual dose ratios from each set of atria. The table also includes the timolol maleate concentration (M) present during the generation of the post-antagonist curve.

Table 9: Schild Plot Points from STZ-induced Diabetic and Normal Rat Atria

Mean EC_{50} values from corrected pre-antagonist dose-response curves to isoproterenol (see "Plotting Dose-Response Curves") and from post-antagonist dose-response curves to isoproterenol for each concentration of timolol maleate (M) for right and left atria. Corrected pre-antagonist and post-antagonist EC_{50} values yield a dose ratio (DR) for each timolol maleate concentration, which is converted to the $\log(DR-1)$ value. The table shows the mean $\log(DR-1)$ values ($\pm S.E.M.$), which represent the ordinate values for the Schild plot. The values for both control (CT) and diabetic (STZ) atria are shown. Numbers in parentheses denotes the sample size.

Table 9: Schild Plot Points from STZ-induced Diabetic and Normal Rat Atria

Right Atria

	Timolol Maleate Concentration (M)	Mean EC ₅₀ (nM) Corr. Pre-Antag. Curve	Mean EC ₅₀ (nM) Post-antag. Curve	Mean log (DR-1)
CT	10 ⁻⁹ (n=6)	2.88	5.89	0.20 ± 0.07
	10 ^{-8.5} (n=6)	1.79	10.6	0.73 ± 0.09
	10 ⁻⁸ (n=6)	2.93	57.4	1.21 ± 0.10
	10 ^{-7.5} (n=6)	1.61	152	2.00 ± 0.07
STZ	10 ⁻⁹ (n=4)	3.48	6.55	0.09 ± 0.08
	10 ^{-8.5} (n=4)	2.85	9.04	0.36 ± 0.18
	10 ⁻⁸ (n=4)	5.23	40.3	0.84 ± 0.06
	10 ^{-7.5} (n=4)	2.12	74.3	1.51 ± 0.05

Left Atria

CT	10 ⁻⁹ (n=6)	8.36	11.3	0.08 ± 0.08
	10 ^{-8.5} (n=6)	10.3	36.1	0.35 ± 0.13
	10 ⁻⁸ (n=6)	9.64	137	1.07 ± 0.06
	10 ^{-7.5} (n=6)	4.94	377	1.86 ± 0.04
STZ	10 ⁻⁹ (n=4)	7.00	8.52	0.13 ± 0.07
	10 ^{-8.5} (n=4)	4.36	32.4	0.65 ± 0.22
	10 ⁻⁸ (n=4)	6.46	151	1.22 ± 0.15
	10 ^{-7.5} (n=4)	5.12	576	1.62 ± 0.36

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