LES and guinea pig colon myenteric plexus suggests that bombesin-containing neurons innervating those structures do so via substance P neurons (2). In contrast, the effect of bombesin on the canine stomach was not inhibited by substance P tachyphylaxis (12).

The observation that the putative substance P inhibitor, [D-Pro^2, D-Trp^{7,9}]substance P, also antagonizes the effect of exogenous bombesin further suggests that substance P mediates the effect of bombesin at the LES. Several studies have indicated that this substance P analogue is a partial agonist and weak antagonist of substance P (1, 20, 21). We were unable to show an inhibitory effect by [D-Arg^1, D-Trp^{7,9}, Leu^{11}]substance P on the feline LES response to either substance P or bombesin. This analogue has been shown to have antagonist properties in other organ systems when given in comparatively large doses. The lack of an inhibitory effect may be due to the relative low potency of this antagonist or to differences in substance P receptor subtypes in different species (4, 14, 21).

These studies do not indicate whether the interactions between substance P and bombesin nerves are direct or mediated by intermediate neurotransmitters. Tetrodotoxin eliminated the LES response to neural pathways activated by electrical vagal stimulation, direct field stimulation, and by balloon distension of the esophagus. While the effect of substance P was unaltered in most animals, it was variably increased or inhibited in others. It is possible that this mixed response of the LES to substance P after tetrodotoxin represents an incomplete inhibition of intrinsic inhibitory or excitatory nerves. The partial inhibition of substance P by tetrodotoxin raises the possibility that its effect on LES muscle may be via interneurons and not at the neuromuscular junction. The observation that the LES response to substance P or bombesin was unaffected by vagotomy or atropine suggests that neurons mediating these actions are located in the intrinsic enteric plexus.

Several immunohistochemical studies in different species have demonstrated bombesin-like immunoreactivity throughout the wall of the gastrointestinal tract with particular density in the myenteric plexus and the sphincters (13, 14, 15). The distribution is similar to that of substance P in the central nervous system and myenteric plexus (14, 17). This relationship provides anatomical support for these physiologic observations. The distribution of bombesin at the lower esophageal sphincter has not been investigated.

Distal esophageal acidification increased lower esophageal sphincter pressure in normal volunteers but not in patients with reflux esophagitis (22). In the cat, this response was mediated by nonvagal neural reflexes that are not inhibited by antagonists to several other types of neurotransmitters that are known to increase sphincter pressure (1). The demonstration that tachyphylaxis to two neuropeptides inhibits this response suggests that a polysynaptic reflex is involved and that peptidergic neurotransmitters may play an important role in the physiologic control of sphincter function.

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assessed and controlled; second, to define the effects of this intervention on myocardial energetics in terms of any changes dependent on or independent of alterations of these determinants of MVO_2; third, to define any change in myocardial sensitivity to catecholamine action after the withdrawal of long-term β-adrenoceptor blockade; fourth, to determine whether chronic β-adrenoceptor blockade alters the number, affinity, or biochemical activity of this autonomic receptor.

Methods

Four separate series of experiments, each employing paired sets of control and chronically β-blocked cats, comprise the data base for this study. In the first series, mechanical and metabolic behavior of myocardium isolated from these two sets of cats was measured. In the second series, the specific relationship of any differences found between these two sets of animals to changes in the activity of the cardiac β-adrenoceptor was examined. In the third series, the effect of chronic β-adrenoceptor blockade on β-adrenoceptor number, affinity, and affinity state was assayed. In the fourth series, the effect of chronic β-adrenoceptor blockade on the major immediate β-mediated biochemical response, cyclic AMP generation, was determined.

Experimental animals

The animals used were 1.7–3.6-kg adult cats of random sex. The cats in the control groups received normal saline (1 cm^3/kg i.p.) twice daily for 14 d prior to study. The chronically β-blocked cats were prepared and characterized as follows. First, their baseline responsiveness to β-adrenoceptor stimulation was established. For this purpose, the heart rate response to intravenous isoproterenol was measured electrocardiographically during sedation with ketamine hydrochloride (15 mg/kg i.m.). Isoproterenol then was infused at rates of 0.5, 1.0, and 2.0 μg/kg · min; heart rate was recorded after it had reached a plateau value at each infusion level. Immediately thereafter, administration of DL-propranolol hydrochloride, 5 mg/cm^3 in normal saline, was begun on a schedule of 5 mg/kg i.p. twice daily and continued for 14 d. The propranolol dosage was based on its approximation to the largest doses ordinarily employed clinically (9); the choice of twice daily drug administration was based on the demonstrated (10) effectiveness of this regimen; the 2–3-wk administration period was based on the clinically (11) and experimentally (12) demonstrated full effectiveness of propranolol administration after this approximate period of time. Second, on day 13 of this schedule, 12 h after the previous dose of propranolol, the heart rate response to isoproterenol again was measured in the same way. Propranolol administration then was continued through the final dose, which was given 12 h before the hearts were removed for study on the morning of day 14.

Mechanical and energetic measurements

Experimental apparatus. This first series of experiments utilized cat right ventricular papillary muscles. Their contractile and metabolic behavior was characterized in the flow respirometer shown in Fig. 1. This flow respirometer, the associated equipment, and the attendant methods have been described fully in previous articles (13, 14); only the most important

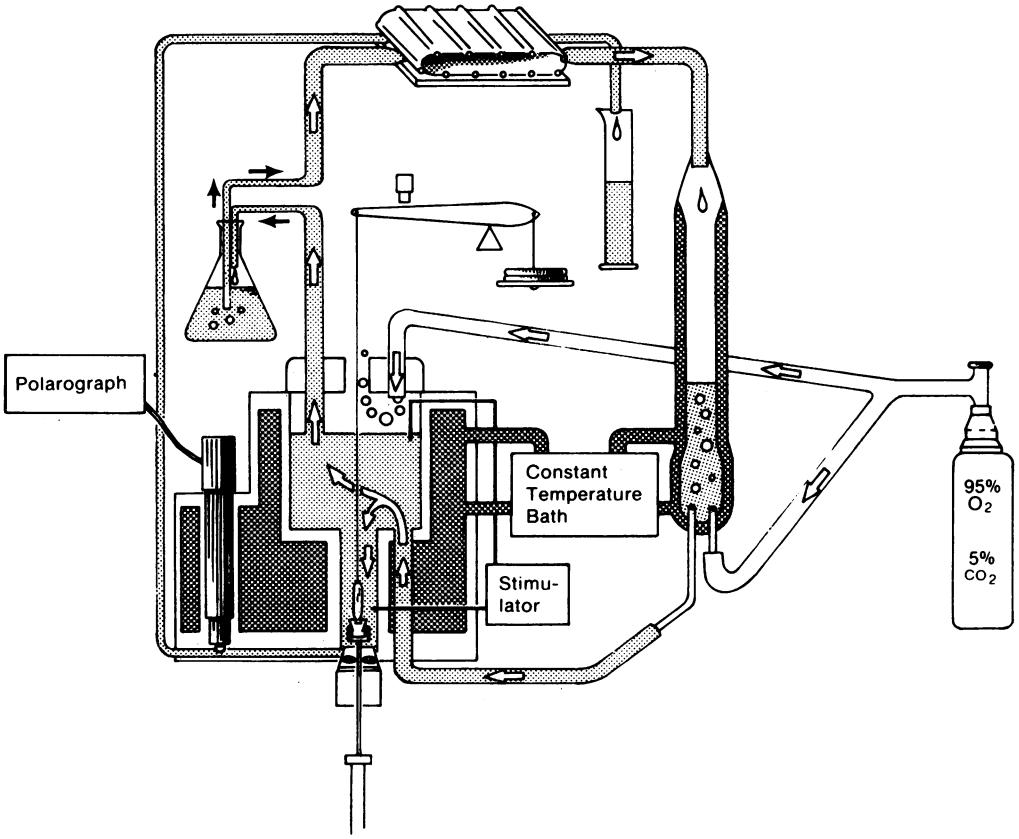

Figure 1. The polarographic myograph. The superfusate is held in a 1-liter Erlenmeyer flask; it is brought to 29°C and saturated with 95% O_2–5% CO_2 in a column to the right of the myograph. It is then brought to a chamber above the muscle in which back-diffusion is prevented by a second gas line. The majority of the superfusate is recirculated to the flask, and a smaller quantity is drawn past the muscle and then past the polarographic oxygen cathode to measure P_{O_2}. Flow rates are determined by the proportioning pump shown at the top of the diagram. The papillary muscle is shown mounted in a clip affixed to a rod; this rod passes through a mercury seal and is screwed onto the tension transducer shown below the muscle. The top of the muscle is attached to the lever of a displacement measurement and control system. This figure has been revised from one showing an earlier version of this polarographic myograph (19).

features are noted here and in the legend to Fig. 1. The principal feature of this apparatus is that it allows the simultaneous determination of mechanical behavior and oxygen consumption for the isolated, superfused cat papillary muscle preparation.

Muscle force and length were the two basic mechanical variables measured. Muscle force was measured from beneath the respirometer. The ventricular end of an excised papillary muscle was placed within a *C*-shaped metal clip sintered to the upper end of a steel rod; the bottom end of this rod was screwed directly onto a semiconductor strain gauge. This clip then was tightened and enclosed by screwing a cylindrical metal sleeve up over it. In this way, the muscle was held rigidly, and the enclosed clip produced only discrete end-segment damage and excluded any damaged tissue from the circulating superfusate used to measure oxygen tension. With the chordal end of the muscle fixed to the lever above the muscle by a tie at the chorda–muscle junction, force generated by the muscle was measured with very little stray compliance (<0.7 μM/mN) over the range of force studied. The damped and undamped natural frequency of this force measurement system, including both the signal conditioners and the optical and magnetic tape recorders, was >1 kHz. The area under the force curve, expressed as $\int$ active force ($\int$AF), was obtained from a digital summing circuit with a frequency response of direct current to 500 Hz, +1 dB, −3 dB, and a reset time of 5 ms. The rate of change of force (dF/dt) was obtained from a single-order high-pass filter with a −3 dB low-frequency cutoff of 50 Hz. Force was calibrated after each study by imposing known stresses spanning the experimental range. The digital summing circuit then was calibrated by square waves from a function generator; the high-pass filter was calibrated by triangular ramps. In each case, the abscissa was time, and the ordinate was referenced to the force calibration. Muscle length was measured from above the respirometer. This measurement, much less critical than that of force and its derived signals for this particular study, is illustrated in Fig. 1 and described in previous reports (13, 14).

The energetic measurement was that of the partial pressure of oxygen (Po_2) obtained polarographically; changes in this signal integrated over time quantitated MVO_2. This measurement assumes that oxygen consumption measured over long periods of time is an accurate reflection of metabolism occurring during the contractions themselves. This assumption is based on the aerobic nature of myocardial respiration (15) and on the use of a properly designed flow respirometer to study a stable muscle preparation. This flow respirometer was designed from well-defined criteria (16); these criteria have been used in the study of papillary muscle respiration in several older (17–19) and more recent (13, 14) investigations. The characteristics of this respirometer are as follows: the central tubular muscle chamber is 41 mm long and 3.5 mm wide, and has a volume of 0.4 ml; the sampling capillary at the bottom of the muscle chamber is 0.8 mm in diameter, with the oxygen cathode 39 mm distal to its origin; the superfusate flow rate past the muscle and then through this capillary is 6.25 ml/h. These characteristics prevent retrograde diffusion of oxygen from the muscle and assure a uniform concentration of oxygen across the diameter of the sampling capillary at the site of the oxygen cathode (Eq. 17 in Reference 16). The stability of the mechanical and energetic behavior of the muscle preparation over a 24-h period in this respirometer has been demonstrated before (13). Further documentation of the stability and sensitivity of the Po_2 recording system in this respirometer (see Fig. 2 in Reference 14), as well as original experimental MVO_2 records (see Figs. 2, 6, and 9 in Reference 13), are also available.

Experimental conditions. Rapid cardiectomy was performed after anesthesia was induced with sodium pentobarbital (30 mg/kg i.p.). The dimensions of the 19 right ventricular papillary muscles from the control cats, identified as group I, are given in the first two columns of Table I. The dimensions of the seven muscles from the chronically β-blocked cats used in this series, group II, are also given in Table I. The most important point with respect to muscle size is that the MVO_2 of superfused muscles of the dimensions listed in Table I is not limited by diffusion (19, 20).

Each muscle was superfused at 29°C by a Krebs–Henseleit buffer of the following millimolar composition: $CaCl_2$, 2.5; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.1; $NaHCO_3$, 24.0; Na acetate, 20.0; NaCl, 98.0; and glucose, 10.0, with 10 U of zinc insulin added per liter. This solution was equilibrated with 95% O_2–5% CO_2, with a resultant pH of 7.4, and circulated past the muscle from a 1-liter reservoir (Fig. 1).

Table I. Papillary Muscle Characteristics

	Length at L_{max}	Cross-sectional area	Resting force at L_{max}	Active force at L_{max}	Resting/total force at L_{max}
	mm	*mm²*	*mN/mm²*	*mN/mm²*	
Group I (n = 19)					
Mean±SE	6.97±0.46	0.79±0.05	4.71±0.26	43.84±2.30	0.097±0.003
Range	4.33–12.11	0.39–1.10	3.15–6.42	30.48–70.98	0.074–0.112
Group II (n = 7)					
Mean±SE	8.02±0.85	0.87±0.14	4.59±0.42	40.46±2.01	0.102±0.009
Range	5.33–11.94	0.17–1.10	3.20–6.37	31.99–46.91	0.087–0.148
Group III (n = 8)					
Mean±SE	7.05±0.72	0.99±0.07	4.22±0.21	45.04±3.29	0.085±0.005
Range	4.37–9.73	0.58–1.10	3.18–4.88	41.96–52.28	0.065–0.100
Group IV (n = 8)					
Mean±SE	7.54±1.04	0.64±0.09	4.56±0.17	45.38±1.78	0.089±0.004
Range	4.52–11.86	0.30–0.85	3.92–5.17	42.54–49.96	0.076–0.103

Group I consisted of normal cats; group II consisted of cats treated previously with propranolol; group III consisted of cats treated previously with normal saline; group IV consisted on cats treated previously with propranolol. There was no significant difference among the four groups by one-way analysis of variance (76) in any of these characteristics.

After each muscle was mounted in the respirometer, it was lightly preloaded and stimulated at 0.1 Hz until a stable mechanical response was obtained, a period of about 1 h. Field stimuli 5% above threshold and of alternating polarity with zero voltage offset between stimuli were employed; these conditions minimized electrolytic contamination of the superfusate and prevented any effect of stimulation itself on the polarograph system (13). The preload then was removed, and during isometric contractions the muscle was brought to L_{max}, that muscle length at which active (total minus resting) force is greatest. The mechanical characteristics of each group of muscles at this length are given in the last three columns of Table I. These characteristics have been used as indexes of the mechanical suitability of any particular cat papillary muscle preparation (21); the characteristics of the present muscles conform to these indexes.

After the experimental protocol described below was completed, muscle length was measured by a micrometer while a known preload was attached to the muscle. This length, along with the resting force portion of the length-vs.-force relationship used to obtain L_{max}, allowed calculation of absolute muscle length at L_{max}. Assuming a wet-to-dry weight ratio of 4 (22), a cylindrical muscle shape, and a specific gravity of 1, muscle cross-sectional area was calculated from the length at L_{max} and the dry weight: Area (square millimeters) = dry weight × 4 (cubic millimeters)/length (millimeters). Mechanical and metabolic results then were normalized as follows: (*a*) force as mN/mm^2 muscle cross-sectional area, (*b*) rate of force change as $mN/mm^2/s$, (*c*) time integral of active force ($\int AF$) as $mN/mm^2 \times s$, (*d*) time to peak force ("up" time) and for relaxation ("down" time) as milliseconds, (*e*) length change as muscle lengths, and (*f*) oxygen consumption (MVO_2) as nl/mg dry wt/contraction.

Experimental protocol. An outline of the format for this first series of studies is provided in the legend to Fig. 3 (q.v.). This shows both the sequence of the nine experimental interventions and the critical features of each. The full protocol is given below.

After the determination of L_{max} described in the last section, the stimulator was turned off, and the muscles were left quiescent until the P_{O_2} record reached a stable baseline. The muscles then were left at L_{max} for the entire experimental protocol that followed; resting force and length did not change significantly during the course of any study.

During each step of the protocol there were 120 contractions within either 4 or 8 min, depending on stimulation frequency. Muscle mechanics were determined during the midportion of each of these interventions to avoid variable effects from the brief but consistent force treppe occurring at the beginning of each. MVO_2 was determined as the total for the 120 contractions comprising each intervention. After each intervention, the muscles again were left quiescent until the P_{O_2} record returned to baseline.

The experimental protocol was the same for both the group I muscles from the control cats and the group II muscles from the chronically β-blocked cats. In step 1, a baseline was established for the subsequent mechanical and metabolic data. For this purpose, a low stimulation frequency of 0.25 Hz and a current 5% above threshold were employed to avoid the release of significant amounts of endogenous catecholamines (23, 24). In step 2, the effects of doubling contraction frequency to 0.50 Hz were determined. Any changes observed here when compared to the first step were considered to be independent of a β-adrenoceptor mediated response (25, 26). The effect of endogenous catecholamine release was tested in step 3 by doubling stimulation current (23, 27) while holding stimulation frequency at 0.25 Hz. After returning the stimulation current to 5% above threshold, 10^{-7} M norepinephrine was added in step 4 to determine the effect of exogenous catecholamines. This concentration of norepinephrine and the increased stimulation frequency of 0.50 Hz were used in step 5 to quantitate the additive effects of catecholamines and the treppe phenomenon upon cardiac inotropism. The superfusate then was replaced several times with drug-free medium to remove exogenous catecholamines from the muscle. In step 6, the drug-free superfusate was replaced with one containing 10^{-5} M propranolol, and the first step was repeated. The presence of propranolol, which blocked β-adrenoceptors, allowed us to measure any contribution of β-adrenoceptor activation under the baseline conditions previously established. The second step was repeated in step 7 in the presence of 10^{-5} M propranolol to assess the treppe response without β-adrenoceptor participation. To assess the effectiveness of β-adrenoceptor blockade on endogenous catecholamine release, the third step was repeated in step 8 in the presence of 10^{-5} M propranolol. In step 9, the effectiveness of β-adrenoceptor blockade of exogenous catecholamines was assessed by repeating step 1 in the presence of 10^{-5} M propranolol and 10^{-7} M norepinephrine.

The choice of superfusate drug concentration was based, for norepinephrine, on data (28) demonstrating a near-maximal effect on MVO_2 and inotropism at this concentration and, for propranolol, on complete blockade of the inotropic effects of exogenous catecholamines found at this propranolol concentration in preliminary experiments. In addition, this concentration of propranolol approximates the highest extracellular concentration of this drug commonly found in clinical usage (9). The time chosen for equilibration after each superfusate alteration was based on preliminary experiments done to determine the time required for a new plateau of myocardial mechanics to occur after the substitution of a superfusate containing a higher (5.0 mM) concentration of calcium. This time, 0.5 h, was doubled after each superfusate alteration. In each instance where a drug was used, it was added to 1 liter of fresh superfusate; the prior superfusate was withdrawn as the new one was introduced in order to maintain a constant system volume and drug concentration (Fig. 1).

After step 5 of the protocol, fresh control superfusate was substituted during 4 of the 19 studies under the conditions of step 1 to determine whether there was a return to the original mechanical and energetic behavior. After each change in superfusate composition, the P_{O_2} record was examined for any change in the MVO_2 of the resting, noncontracting muscle.

Adrenoceptor activity after chronic β-blockade

Experimental conditions. As before, rapid cardiectomy was performed after anesthesia was induced with sodium pentobarbital (30 mg/kg i.p.). The six right ventricular papillary muscles from control cats used in this second series of experiments are identified as group III; the six muscles from the chronically β-blocked cats are identified as group IV. The dimensions and baseline mechanical characteristics at L_{max} are given for both groups of muscles in Table I. The experimental apparatus and conditions were identical to those used before for the experiments on groups I and II, with the exception that the time required to measure MVO_2 in vitro during each intervention did not allow this measurement to be made during these complex and lengthy pharmacologic studies. Thus, the data in this series of experiments are confined to measurements of muscle mechanics at L_{max}.

Experimental protocol. This second series of experiments was designed both to evaluate the significance of any differences found between the two sets of muscles in the first series of experiments and to determine, through the use of adrenoceptor agonist dose-response curves combined with specific antagonists, whether any effect of chronic β-blockade is mediated by changes in the β-adrenoceptor.

The following protocol was the same for muscles from the group III and group IV cats. Step 1 established the initial baseline mechanical response at L_{max} to a low stimulation frequency of 0.25 Hz at 5% above threshold. The stimulus current then was doubled for 1 h to deplete endogenous catecholamines (23, 24). The initial positive inotropic effect disappeared during this hour in all cases. Both here and in all following steps, 20 μM cocaine was used to prevent tissue catecholamine uptake. In step 2, the dose-related response of both groups of muscles to α-adrenoceptor stimulation with 10^{-7} M to 3×10^{-6} M phenylephrine was examined. In step 3, the α-specificity of any observed phenylephrine effect was examined in terms of the dose-related response to 10^{-7} M to 3×10^{-6} M phenylephrine after α-adrenoceptor blockade with both 1 μM prazosin (29) and 1 μM yohimbine (30). Specific β-adrenoceptor responsiveness to norepinephrine was tested in step 4 by exposing the muscles to 10^{-9} M to 3×10^{-7} M norepinephrine in the presence of these same concentrations of prazosin and yohimbine. In addition, the maximum inotropic response to β-adrenoceptor stimulation was evaluated in step 5 by using 10^{-7} M and 10^{-6} isoproterenol. In step 6, any

differential response of the two groups of muscles to 10^{-9} M to 3×10^{-6} M norepinephrine during β-blockade with 10^{-5} M propranolol was sought. In step 7, the β-specificity of any response observed in the last step was tested by using 10^{-7} and 10^{-6} M isoproterenol in the presence of 10^{-5} M propranolol.

β-Adrenoceptor assays

Experimental conditions. In this third series of experiments, rapid cardiectomy again was performed after anesthesia was induced with sodium pentobarbital (30 mg/kg i.p.). The entire heart exclusive of atria was used; there were five hearts from saline-pretreated control cats, identified as group V, and five hearts from propranolol-pretreated cats, identified as group VI. Each heart then was divided into three specimens consisting of the right ventricular free wall exclusive of interventricular septum, a right ventricular papillary muscle, and the left ventricle inclusive of the interventricular septum. The tissue from each of these three sources was pooled separately for groups V and VI for the assay. This initial assay of the three pooled tissue types employed [^{3}H](±)carazolol as the radioligand. Because the interpretation of these studies was critically dependent on the accuracy of this characterization of the β-adrenoceptors, a second assay was then made in further groups of animals, where the entire heart exclusive of atria of six saline-pretreated control cats and six propranolol-pretreated cats each was assayed separately using [^{3}H](−)dihydroalprenolol as the radioligand. Further, both because noncardiocyte cells comprise up to 75% of the cellular population of cardiac tissue (31) and because the β-adrenoceptors of these noncardiocyte cells differ from those of the muscle cells (32), a third assay, again using [^{3}H](−)dihydroalprenolol as the radioligand, was made on cardiocytes isolated from saline-pretreated and propranolol-pretreated cats. Finally, in order to determine whether chronic β-blockade caused a change in the proportion of the β-adrenoceptors in the high-affinity state, a fourth assay was made to define the agonist-affinity states of the β-adrenoceptors in the entire heart exclusive of atria from six saline-pretreated control cats and six propranolol-pretreated cats.

Cardiac membrane vesicle and cardiocyte preparation. For the initial assay, the tissue homogenates and membrane vesicle fractions were prepared as described previously (33, 34). The three tissue specimens from each heart had been frozen very quickly in tongs cooled in liquid nitrogen immediately after cardiectomy; they were collected into pools of five specimens for each of the three tissue types from groups V and VI. Each set of pooled specimens was homogenized in 4 vol of ice-cold 0.25 M sucrose and 30 mM histidine during three 30-s passes through a tissue homogenizer. Tissue fragments, nuclei, and mitochondria were removed during several centrifugations at 11,600–15,000 *g* for 30 min each. The membrane vesicle fraction then was sedimented at 43,600 *g* for 30 min. In both this and the following assays, the protein content of the membrane vesicle fractions being compared was very similar.

For the second assay, using [^{3}H](−)dihydroalprenolol, the cardiac membrane vesicle fraction was prepared from each fresh heart by a standard method (35). Briefly, the heart was weighed, minced, suspended in 4 vol of ice-cold 0.25 M sucrose, 5 mM Tris-HCl, and 1 mM $MgCl_2$ at pH 7.4, and homogenized during three 20-s passes through a tissue homogenizer. After initial centrifugations to remove unwanted material, the cardiac membrane fraction was sedimented at 43,000 *g* for 30 min. The yield of membrane protein was 1.2–1.5 mg/g of cardiac wet weight (36).

For the third assay, quiescent, calcium-tolerant cardiocytes were prepared via enzymatic perfusion dissociation of the heart using a technique recently described in detail (37). This technique produced large numbers ($\sim 3 \times 10^6$ cells/g wet weight) of viable ventricular cardiocytes, which were washed free of the dissociation medium and resuspended in the same Krebs–Henseleit buffer described earlier.

Radioligand binding assay. In the first assay, the β-adrenoceptors in the membrane vesicle preparations were labeled with [^{3}H](±)carazolol. Membrane vesicles containing 50–200 μg of protein (38) were incubated at 37°C for 1 h in 1–5 ml of medium containing 50 mM Tris-HCl, 9 mM $MgCl_2$, 1 mM ascorbic acid, and 270 pM [^{3}H](±)carazolol at pH 7.5. During this hour, complete equilibration of [^{3}H](±)carazolol binding occurred. Reactions were terminated by filtration on glass fiber filters; quantitative recovery of labeled membrane proteins by this technique was demonstrated in an initial control experiment. The filters then were rinsed four times with 5-ml aliquots of ice-cold 50 mM Tris buffer containing 9 mM $MgCl_2$. The filters were dried, and the entrapped membrane proteins were solubilized by a strong alkali; after neutralization, the mixture was counted by liquid scintillation at 56% efficiency using an internal standard. Total and nonspecific binding were measured in triplicate. Specific binding of [^{3}H](±)carazolol, that displaceable by 20 μM (±)propranolol, was linear over the range of protein concentrations used and was >70% of total binding.

In the second assay, the β-adrenoceptors in the membrane vesicle fraction were labeled with [^{3}H](−)dihydroalprenolol and assayed in accordance with a recently described technique (35). Duplicate portions of membrane vesicles containing 100–300 μg of protein were incubated at 37°C for 15 min in 2.0 ml of 75 mM Tris-HCl, 25 mM $MgCl_2$, and 15 nM[^{3}H](−)dihydroalprenolol at pH 7.4. The reactions were terminated by filtration, and the radioligand was counted as in the first assay. Specific binding of [^{3}H](−)dihydroalprenolol, that displaceable by 100 μM (±)propranolol, was linear over the range of protein concentrations used and was >75% of total binding.

In the third assay, 1.5×10^6 rod-shaped cardiocytes were centrifuged gently and resuspended for 15 min at 37°C in 2.0 ml of 75 mM Tris-HCl, 25 mM $MgCl_2$, and 15 nM [^{3}H](−)dihydroalprenolol at pH 7.4. The assay techniques were duplicative of those used in the second assay.

Determination of β-adrenoceptor number and affinity. In a manner that we have described before in detail (34), saturability of specific [^{3}H](±)carazolol-binding sites was demonstrated by plotting the concentration of this ligand against the specific ligand bound. Scatchard analysis of specific [^{3}H](±)carazolol bound vs. the ratio of bound to free ligand then was performed. The intercept of the abscissa represents the maximum number of ligand-binding sites, B_{max}, and the negative reciprocal of the slope of the regression line yields an estimate of receptor affinity in terms of the equilibrium dissociation constant, K_D. For the second and third assays, the number and affinity of β-adrenoceptors as determined by [^{3}H](−)dihydroalprenolol were calculated in the same way.

Determination of agonist affinity states of the β-adrenoceptor. For this analysis, each of six hearts exclusive of atria was separately prepared from saline-pretreated control cats, identified as group VII, and propranolol-pretreated cats, identified as group VIII. Each heart was excised, minced, and homogenized in 4 vol of ice-cold 20 mM Tris-HCl at pH 7.5 during two 30-s passes through a tissue homogenizer. A crude membrane fraction was prepared by centrifugation at 20,000 *g* for 10 min in the same buffer. The pellet was resuspended in 20 mM Tris-HCl and 1 mM EDTA and centrifuged again. This final pellet was resuspended at a membrane protein concentration of 20–100 μg/ml in 20 mM Tris-HCl and 5 mM $MgCl_2$. The properties of the β-adrenoceptors were assessed by adding 100 μl of the membrane suspension to the following mixture: 40–50 pM of [^{125}I]iodocyanopindolol in a volume of 50 μl, a range of 0.01 nM to 100 μM (−)isoproterenol in a volume of 50 μl, and 300 μl of a buffer consisting of 20 mM Tris-HCl, 5 mM $MgCl_2$, and 0.0004% bovine serum albumin. Nonspecific binding was defined with 50 μM (−)isoproterenol. This mixture was incubated for 70 min at 37°C, and the samples then were filtered and washed (39). Each competition curve for (−)isoproterenol was subjected to a nonlinear regression analysis which we have described before (40). The data were fitted to a one-site model to determine the Hill coefficient and then to a two-site model to estimate the proportion and affinities of the high- and low-affinity states of the β-adrenoceptor (41).

Cyclic AMP assays

Stimulation of cyclic AMP. Isolated cardiocytes, $0.6–1.0 \times 10^6$ rod-shaped cells per heart, were obtained from saline-pretreated control cats, identified as group IX, and from propranolol-pretreated cats, identified as group X. These cells were incubated at 37°C in multiple 1-ml aliquots of the Krebs–Henseleit buffer described earlier; this medium contained 2 mM theophylline, a phosphodiesterase inhibitor used to prevent degradation of cyclic nucleotides. The cardiocytes then were exposed either to a range

of 3×10^{-8} M to 6×10^{-7} M isoproterenol HCl, a β-adrenoceptor-dependent stimulator of cyclic AMP generation, or to a range of 1×10^{-8} M to 3×10^{-7} M glucagon HCl, an adrenoceptor-independent stimulator of cyclic AMP generation (42). An incubation period of 5 min was used in each case; at this time, significant cyclic AMP has accumulated in isolated cardiocytes under these conditions, but the rate of cyclic AMP accumulation is still linear (43). In order to halt the cardiocyte response to either isoproterenol or glucagon, ice-cold perchloric acid was added to the cardiocyte isolation medium to a final concentration of 0.1 N; this mixture was placed on ice for 10 min and then sonicated for 1 min. After the pH was neutralized by adding ice-cold KOH, each sample was centrifuged to separate the precipitated cell debris and insoluble potassium perchlorate from the supernatant containing the nucleotides.

Determination of cyclic AMP. Cyclic AMP was measured by acetylation radioimmunoassay (44), with minor modifications as described below. Two 250-μl portions of each supernatant were used as sample duplicates in the radioimmunoassay. Each duplicate was mixed with triethylamine and then with acetic anhydride to acetylate the cyclic nucleotides. A 50-μl portion of each acetylated sample duplicate then was mixed with 50 μl of a cyclic AMP tracer consisting of succinyl 3-[^{125}I]iodotyrosine methyl ester, 200 μl of cyclic AMP antiserum diluted with 50 mM sodium acetate buffer, and 400 μl of rabbit γ-globulin as a blank. This reaction mixture was allowed to incubate overnight at 4°C. To each reaction mixture, ice-cold 60% ammonium sulfate was added and the suspension centrifuged to precipitate all antibody proteins. The supernatant was decanted, and the cyclic AMP–antibody precipitate counted in a gamma counter and compared to precipitates of cyclic AMP standards. Because competition for the limited antibody sites occurs between the radioactive tracer and the nonradioactive cyclic nucleotide, the amount of radioactivity bound to the antibody varied inversely with the concentration of the competing cyclic AMP ligand. The results were expressed as picograms of cyclic AMP per cardiocyte.

Results

The adequacy of β-adrenoceptor blockade in the groups of cats treated with proproanolol before study was established by their heart rate response to isoproterenol; these data are shown in Fig. 2. The upper panel shows that prior to propranolol treatment, there were significant increases in heart rate with each successive increase in isoproterenol dosage. The lower panel shows that on day 13 of propranolol treatment, 12 h after the previous drug dose, the initial heart rate was significantly less than that before β-adrenergic blockade, and there were no increases in heart rate with any level of isoproterenol infusion.

Mechanical and energetic measurements

Group I. For this saline-pretreated group, refer to the interventions indicated by the numbers on the abscissas both of Figs. 3 and 5 (*upper panel*). The interventions, which were the same for Figs. 3–5, are identified by number and briefly defined in the legend to Fig. 3. Intervention 1 is the control used for subsequent comparisons. There is a small but significant positive inotropic response to the treppe step (a doubling in stimulation frequency in intervention 2) and to endogenous catecholamine release (a doubling of stimulation current in intervention 3). This is reflected in each contractile index indicated on the ordinate of Fig. 3, with increases in active force, the time integral of active force, and the rates of force development and relaxation, as well as decreases in the time for peak force development and for complete relaxation. Interventions 2 and 3 in the upper panel of Fig. 5 show both the expected energetic result of increasing the absolute oxygen cost of each contraction, as well as the further energetic result of increasing oxygen consumption normalized

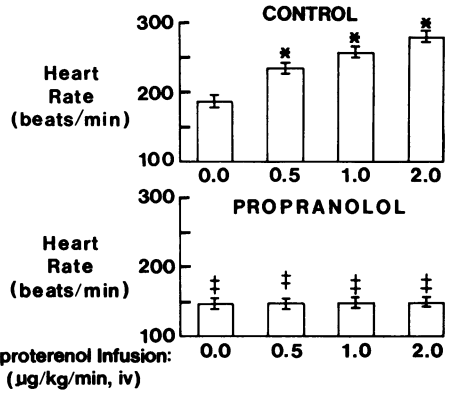

Figure 2. Heart rate responses to isoproterenol. The heart rates of eight cats were recorded during isoproterenol infusion at the levels given below each panel. The upper panel gives the data during the control state; the lower panel gives the data for the same cats on day 13 of β-adrenoceptor blockade with parenteral DL-propranolol (5 mg/kg i.p. twice daily). *Value with a significant difference from the preceding value shown to its left at the $P < 0.05$ level by Student's paired t test. ‡Value with a significant difference from the initial value in the control state at the $P < 0.05$ level by Student's unpaired t test. Heart rate did not change with any level of isoproterenol infusion during propranolol administration (76).

as MVO_2 per unit amount of force development or per unit of the time integral of active force. The increased normalized MVO_2 presumably reflects the metabolic cost of the inotropic effects of the second and third interventions, in addition to that resulting from the increased active force output associated with each. Intervention 4 shows that pharmacologic β-adrenoceptor activation with norepinephrine under conditions otherwise identical to those used for the first intervention produces mechanical and energetic changes similar in kind to those observed in the second and third interventions, although each change is greater in degree. Doubling contraction frequency after norepinephrine addition in intervention 5 produces only slight but directionally consistent further changes in contractile and energetic responses. Intervention 6 shows that pharmacologic β-adrenoceptor blockade under conditions otherwise identical to those used for the first control intervention produces no mechanical or energetic alterations. After propranolol addition, the treppe effect (intervention 7) is preserved, but the effects of endogenous (intervention 8) and exogenous (intervention 9) catecholamines are nullified.

Because no mechanical or energetic alterations under basal conditions were found with a fully β-adrenoceptor-blocking concentration of 10 μM propranolol in the sixth intervention, lower concentrations of this drug would not be expected to produce an effect and therefore were not examined. Instead, the experimental protocol focused on the interaction of a single effective dose of this drug with inotropic interventions independent of and dependent on the β-adrenergic system.

In the four muscles in which this was examined, return to the control superfusate after the fifth intervention produced a return to the same mechanical and energetic behavior observed during the first intervention in each instance. No change in resting MVO_2 was found after any of the changes in superfusate composition.

Group II. For this propranolol-pretreated group, refer to the interventions indicated by the numbers on the abscissas both of Figs. 4 and 5 (*lower panel*). The interventions are identical to

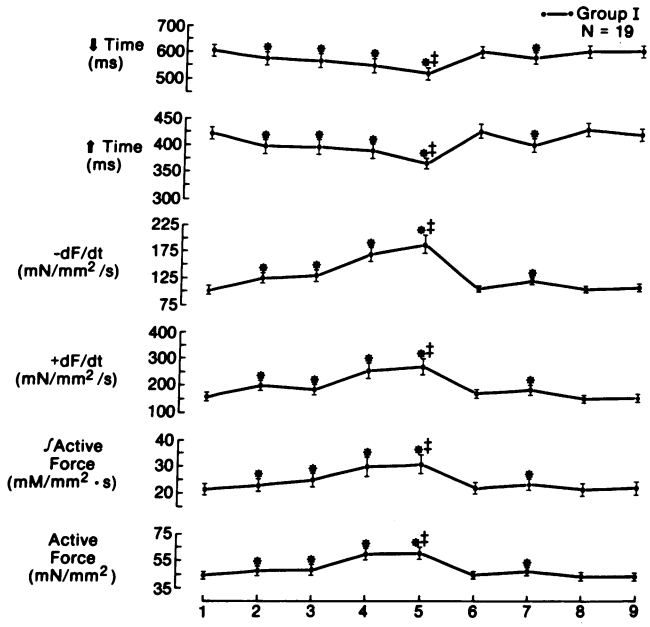

Figure 3. Myocardial mechanics at L_{max} for the normal group I muscles. The numerals on the abscissa indicate the following experimental conditions: *1.* Stimulation, 0.25 Hz; 5%> threshold; drug, none. *2.* Stimulation, 0.50 Hz; 5%> threshold; drug, none. *3.* Stimulation, 0.25 Hz; two times threshold; drug; none. *4.* Stimulation, 0.25 Hz; 5%> threshold; drug, 10^{-7} M norepinephrine bitartrate. *5.* Stimulation, 0.50 Hz; 5%> threshold; drug, 10^{-7} M norepinephrine bitartrate. *6.* Stimulation, 0.25 Hz; 5%> threshold; drug, 10^{-5} M DL-propranolol hydrochloride. *7.* Stimulation, 0.50 Hz; 5%> threshold; drug, 10^{-5} M DL-propranolol hydrochloride. *8.* Stimulation, 0.25 Hz; two times threshold; drug, 10^{-5} M DL-propranolol hydrochloride. *9.* Stimulation, 0.25 Hz; 5%> threshold; drugs, 10^{-5} M DL-propranolol hydrochloride + 10^{-7} M norepinephrine bitartrate. The notations on the ordinate indicate the following experimental measurements: *Active Force,* the peak force generated by the muscle during contraction; *∫ Active Force,* the product of active force and time summed continuously during contraction; *+dF/dt,* the maximum rate of force generation; *−dF/dt,* the maximum rate of relaxation; *"Up" Time,* the time from the end of the latent period to peak force generation; *"Down" Time,* the time from peak force generation to complete relaxation. *Value with a significant difference from the corresponding initial control value (intervention 1) at the $P < 0.05$ level. ‡Value with a significant difference from the corresponding second control value (intervention 2) at the $P < 0.05$ level. The Bonferroni multiple comparison method as applied to Student's paired *t* test was used in each case (76).

those used for the group I muscles and are identified and defined in the legend to Fig. 3. Chronic propranolol treatment with pharmacologically verified, complete β-adrenergic blockade produces no significant change in the baseline contractile (Fig. 4) or energetic (Fig. 5, *lower panel*) behavior shown for the first, control intervention in group II when compared to that observed during the first, control intervention for group I. Further, no progressive change in myocardial behavior after 2 up through 3 wk of propranolol administration was noted in individual group II muscles.

Interventions 2, 5, and 7 show that the responses to the treppe steps are similar to those observed for the group I muscles. In addition, as might be expected for a competitive antagonist, no persistence of β-adrenoceptor blockade was apparent after these muscles from propranolol-treated cats were equilibrated for an hour in the myograph before beginning the protocol. The group

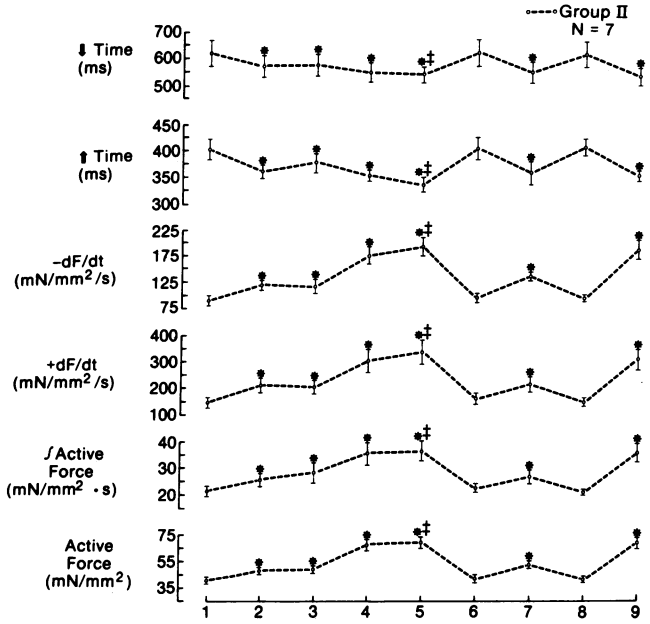

Figure 4. Myocardial mechanics at L_{max} for the previously β-blocked group II muscles. The numerals on the abscissa and the notations on the ordinate indicate the same experimental conditions and measurements, respectively, defined in the legend to Fig. 3. The symbols indicate the same statistical comparisons.

II muscles responded both to endogenous catecholamines (intervention 3) and to exogenous catecholamines (interventions 4 and 5).

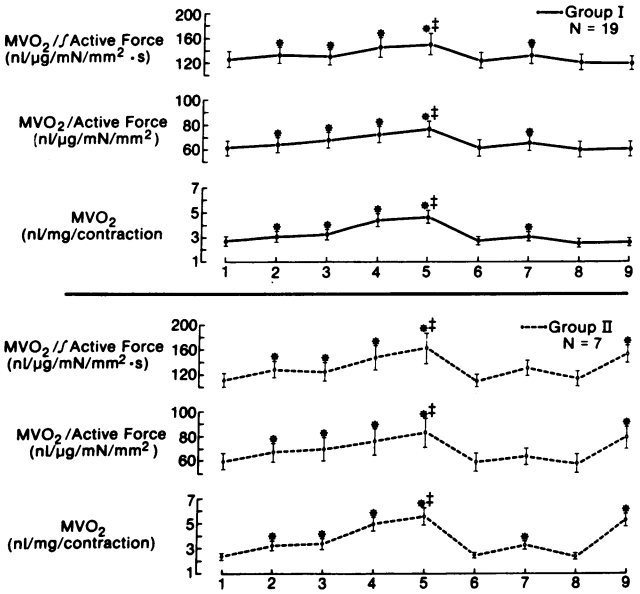

Figure 5. Myocardial energetics at L_{max} for the group I (*upper panel*) and group II (*lower panel*) muscles. The numerals on the abscissa of each panel indicate the same experimental conditions defined in the legend to Fig. 3, and the symbols indicate the same statistical comparisons. The notations on the ordinate of each panel indicate the following experimental measurements: *MVO_2,* myocardial oxygen consumption associated with each contraction; *MVO_2/Active Force,* myocardial oxygen consumption normalized for the peak force generated during contraction; *MVO_2/∫ Active Force,* myocardial oxygen consumption normalized for the time integral of active force during contraction.

The major difference between groups I and II is that the sensitivity of the group II muscles to catecholamine action is much greater than that of the group I muscles. For the group I muscles, the fourth intervention produced a 36% increase over its control in active force, a 60% increase in the maximum rate of force development, and a 62% increase in MVO_2 per contraction. No effect of exogenous catecholamines is apparent for these muscles in the ninth intervention in the presence of DL-propranolol. For the group II muscles, each measure of the response to catecholamine addition before propranolol addition was approximately doubled: the fourth intervention produced a 66% increase over control in active force, a 113% increase in the maximum rate of force development, and a 112% increase in MVO_2 per contraction. Of particular interest, the ninth intervention shows marked contractile and metabolic responses to exogenous catecholamines in the group II muscles, despite the presence of the same concentration of DL-propranolol that completely blocked this response in the group I muscles.

Adrenoceptor activity after chronic β-blockade

Groups III and IV. During step 1 of this protocol, papillary muscles from both the saline-pretreated group III cats and the propranolol-pretreated group IV cats exhibited baseline mechanical behavior at L_{max} that was very similar to that observed during the previous series of experiments. These comparative data are shown in Table I.

The α-agonist dose-response curves of step 2 showed a 38.7±19.9% increase in active force for group III and a 53.9±9.2% increase in active force for group IV at the highest dose of phenylephrine employed, 3×10^{-6} M. There was no significant difference in response between the two groups at any of the four phenylephrine doses used in terms of active force or the maximum rates of either force generation or relaxation. Step 3 examined the question of whether the positive inotropy seen in the last step was entirely an α-response or instead partially β-mediated, in that very little inotropic response was seen at phenylephrine concentrations below 3×10^{-7} M, where the α effect would clearly predominate. Using levels of prazosin and yohimbine sufficient to block substantially the α_1- and α_2-responses, respectively (45, 46), the phenylephrine dose-response curve was repeated, with the maximum dose again 3×10^{-6} M. At this dose, there were insignificant increases in active force of only 3.2±3.6% for group III and 6.2±3.1% for group IV, indicating that most of the response observed in step 2 was α-mediated. Together, steps 2 and 3 indicate that there is a modest and similar α-mediated inotropic response of both the group III and IV muscles.

Step 4 examined the responses of both groups of muscles to increasing doses of norepinephrine in the presence of the same α_1- and α_2-blockade just described. As shown in the three panels of Fig. 6, there was a significant decrement for group IV in the A_{50}, the norepinephrine concentration that produces half of the maximum response, with respect to each index of mechanical performance. Fig. 7 shows the results of step 6 of the protocol: when 10^{-5} M propranolol was added to superfusate containing the α_1- and α_2-blockers, the group IV muscles were again more sensitive to norepinephrine, with a clear leftward shift in the A_{50} for the three contractile indices. Thus, Fig. 6 shows that propranolol pretreatment of the group IV cats caused an increased myocardial sensitivity to the β-mediated mechanical effects of norepinephrine, as was suggested by the data in Figs. 4 and 5. Fig. 7 shows a symmetrical shift to the right for both groups IV and V in the norepinephrine dose-response relationship in the presence of propranolol, but with the difference between the two groups being preserved, suggesting that chronic propranolol treatment induces an increased myocardial sensitivity to catecholamine action which may become apparent at relatively low β-agonist levels when β-blockade is withdrawn.

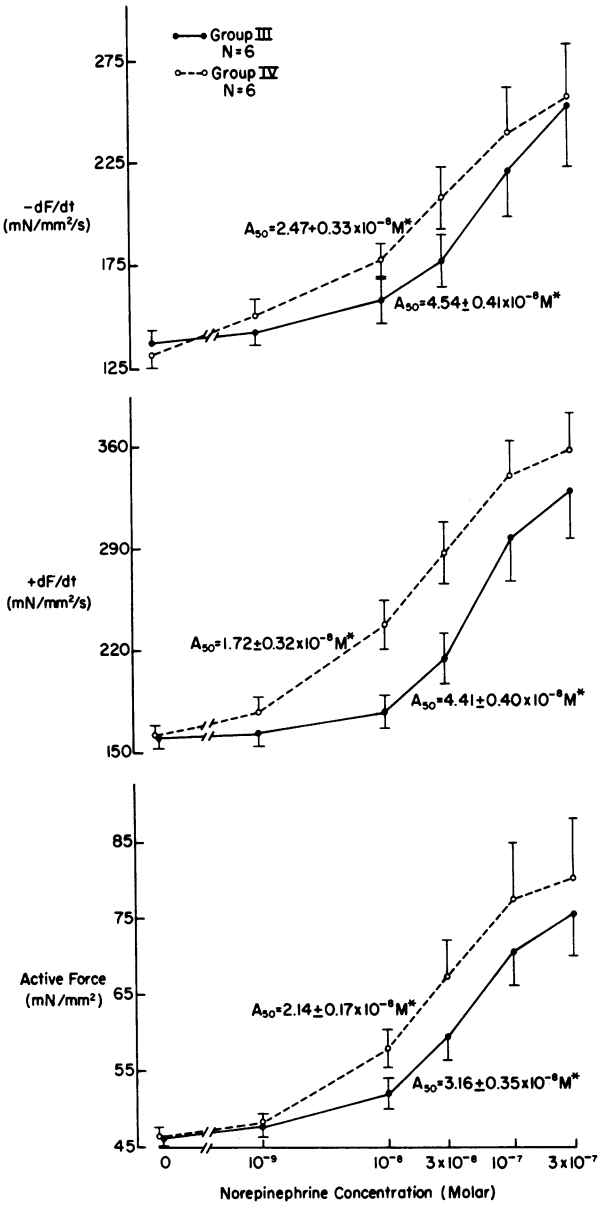

Figure 6. Norepinephrine dose-response curves for control (group III: •) and previously β-blocked (group IV: ○) muscles. The three panels show the mechanical behavior of the muscles in terms of the three contractile indices shown on the ordinate in response to the concentrations of norepinephrine shown on the abscissa when no other drug was present in the bath. The A_{50} values were compared by Student's unpaired *t* test. There was no significant difference by Student's unpaired *t* test in any of the three mechanical indexes between groups III and IV with respect to the maximal responses shown in the three panels.

The β-specificity of the changes seen in the group IV muscles was further examined in steps 5 and 7. In step 5, the β-agonist isoproterenol was used, again with the same levels of prazosin

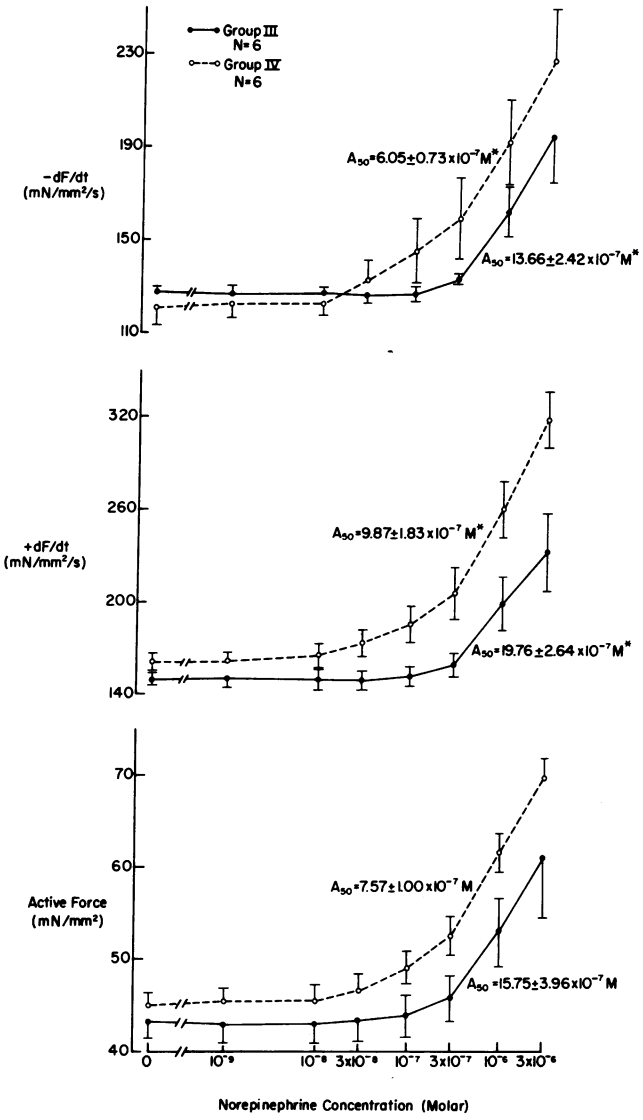

Figure 7. Norepinephrine dose-response curves for control (group III: ●) and previously β-blocked (group IV: ○) muscles when 10^{-5} M DL-propranolol was also present in the bath. The three panels show the mechanical behavior of the same muscles for which dose-response curves are shown in Fig. 6. The A_{50} values were compared by Student's unpaired *t* test. There was no significant difference by Student's paired *t* test within either group between the initial values in the three panels of Fig. 6, with no norepinephrine present, and subsequent values, obtained when all drugs had been removed from the bath, for the same three indexes following the second set of dose-response curves shown in this figure.

and yohimbine, to show that the maximum inotropic response was similar to that obtained with norepinephrine. The response to 10^{-6} M isoproterenol, with respect to the maximum norepinephrine response for each of the contractile indexes shown in the three panels of Fig. 6, did not vary by >5% for either group of muscles. In step 7, it was found that the decrement in response to a given dose of isoproterenol in the presence of 10^{-5} M propranolol was similar for both groups to that seen in comparing the norepinephrine responses in Fig. 6 to those in Fig. 7. Thus, steps 5 and 7 suggest that the effect of propranolol pretreatment on cardiac adrenoceptor activity is largely a β-mediated effect.

β-Adrenoceptor assays

Groups V and VI. The results of the assay of β-adrenoceptor number and affinity for the pooled right ventricles from five saline-pretreated group V cats and five propranolol-pretreated group VI cats are shown in Fig. 8. The Scatchard plots for these two data sets were tested for coincidence and found not to be different (47). That is, the abscissal intercepts and the slopes of the Scatchard lines shown in the inset of Fig. 8, the β-adrenoceptor number and the negative reciprocal of β-adrenoceptor affinity, respectively, were similar in groups V and VI. Specifically, the number of β-adrenoceptors was 519 fM/mg of protein in the control tissue and 452 fM/mg of protein in the tissue from the propranolol-pretreated cats. The receptor affinity was 87 pM for the control tissue and 103 pM for the tissue from the propranolol-pretreated cats. In addition, no difference between the two groups was found with respect to the number or affinity of left ventricular β-adrenoceptors or with respect to receptor number in right ventricular papillary muscles.

When a second assay was done in which each heart from six saline pretreated and 6 propranolol-pretreated cats was measured separately using as the radioligand [^{3}H](−)dihydroalprenolol, the number of β-adrenoceptors in the control group was 518 fM/mg of protein, a value virtually identical to the pooled right ventricular value obtained in the first assay using [^{3}H](±)carazolol. In addition, there was no difference between the saline-pretreated and the propranolol-pretreated groups with respect to β-adrenoceptor number or affinity.

In the third assay, using cardiocytes isolated from saline-pretreated and propranolol-pretreated cats, there was again no apparent effect of β-blockade. There were 4.4×10^5 β-adrenoceptors per cell in the first group, and there were 3.9×10^5 β-adrenoceptors per cell in the second group. There was also no difference in β-adrenoceptor affinity in these two groups of cardiocytes. Thus, none of these three assays demonstrated a change in β-adrenoceptor number or affinity as the result of prolonged β-adrenoceptor blockade.

Groups VII and VIII. The results of the assay of agonist affinity states for ventricular tissue from the saline-pretreated group VII cats and the propranolol-pretreated group VIII cats are illustrated in Fig. 9. The Hill coefficient, n_H, was derived from each isoproterenol competition curve; it was 0.65 for the group VII cats and 0.63 for the group VIII cats. Because n_H was significantly <1 in each case, the existence of multiple affinity states of the β-adrenoceptor in the myocardium from each group was indicated (41, 48). Estimates of the proportion, B_H, and of the relative affinity of the high-affinity state, K_H, and of the low-affinity state, K_L, of the β-adrenoceptor were obtained by fitting the competition data to a two-site model. The pooled data from the group VII and VIII cats were virtually superimposable, as were the two-site curves generated by nonlinear regression analysis. Individual isoproterenol competition curves for each cat also were analyzed; as shown in Table II, no significant difference was found with respect to any of the parameters defined above. Thus, no difference in the agonist affinity state of the β-adrenoceptor was found to result from prolonged β-adrenoceptor blockade.

At the same time that these isoproterenol competition experiments were done on the Group VII and VIII cats, Scatchard analysis of [^{125}I]iodocyanopindolol saturation curves determined for each heart were done on the same membrane preparations. Using this very high-affinity radioligand (49) on this relatively

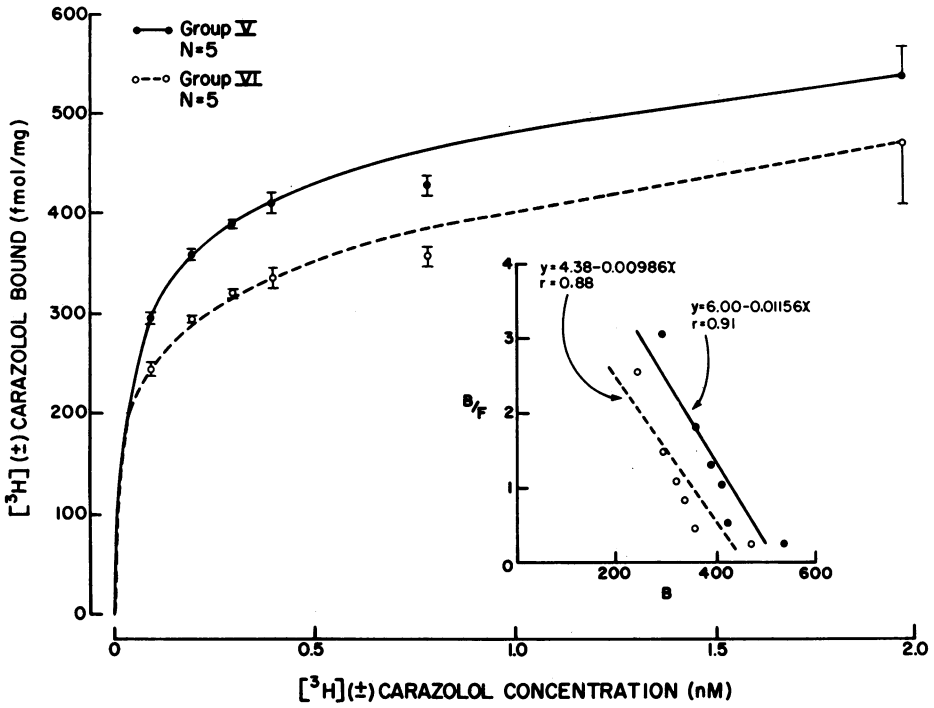

Figure 8. [^{3}H](±)carazolol saturation-binding curves in right ventricular tissue from control (group V: ●) and previously β-blocked (group VI: ○) cats. Each point is the mean±standard error for a triplicate value obtained by subtracting nonspecific binding from total binding at a particular concentration of [^{3}H](±)carazolol. The inset shows the Scatchard plots of these points: similar receptor numbers (abscissal intercepts) and similar receptor affinities (negative reciprocals of slopes) for [^{3}H](±)carazolol in right ventricles from the two groups are apparent.

crude membrane preparation, we obtained a lower value for the number of β-adrenoceptors than we had obtained with our previous assays using [^{3}H](±)carazolol and [^{3}H](−)dihydroalprenolol on a somewhat more purified (33) membrane preparation. Specifically, the number of ligand binding sites, B_{max}, was 139±19 fM/mg of protein for group VII and 128±20 fM/mg of protein for group VIII; the equilibrium dissociation constant, K_D, was 19.2±1.5 pM for group VII and 17.7±1.4 pM for group VIII. Again, there was no significant difference between the two groups with respect to these values.

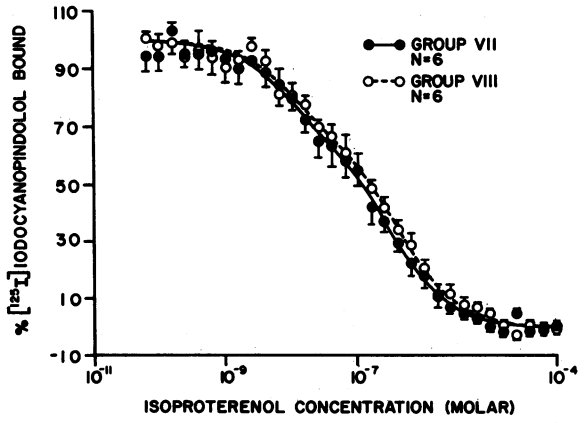

Figure 9. Isoproterenol competition curves in ventricular tissue from control (group VII: ●) and previously β-blocked (group VIII: ○) cats. Nonlinear regression analysis (40) was used to fit a two-site model to the data in order to estimate the proportions and relative affinities of the high-affinity and the low-affinity states of the receptor. No significant difference was found either in the proportion or in the affinity of the receptors in the high-affinity state after propranolol treatment. Each point is the mean±standard error for six experiments, where each experimental value was measured in triplicate.

Cyclic AMP assays

Groups IX and X. The results of the assays of cyclic AMP accumulation by ventricular cardiocytes isolated from the saline-pretreated group IX cats and the propranolol-pretreated group X cats are shown in Fig. 10. In the presence of theophylline and with a 5-min incubation period in each case, the maximal cyclic AMP accumulation induced by isoproterenol in the group IX cardiocytes was 2.53±0.23 pM/10^6 cells; a similar value of 2.49±0.12 pM/10^6 cells was found for the group X cardiocytes. However, as shown by the top two points in the upper panel of Fig. 10, this peak accumulation of cyclic AMP occurred in the group IX cardiocytes at an isoproterenol concentration that was twice that required for the same peak accumulation in the group X cardiocytes. Under the same experimental conditions, the maximal cyclic AMP accumulation induced by glucagon in the

Table II. Comparison of Parameters from Isoproterenol Competition Experiments

	n_H	K_H	K_L	B_H
		nM	*nM*	%
Group VII (n = 6)				
Mean±SE	0.69±0.05	27±14	677±313	38±6
Group VIII (n = 6)				
Mean±SE	0.67±0.06	17±8	596±77	33±9

n_H, Hill coefficient; K_H and K_L, concentration of competing drug necessary to inhibit 50% of the binding of the radioligand to the high (H) and low (L) affinity sites; B_H, proportion of high affinity sites. Group VII consisted of normal cats; group VIII consisted of cats treated previously with propranolol. There was no significant difference between the two groups with respect to any parameter.

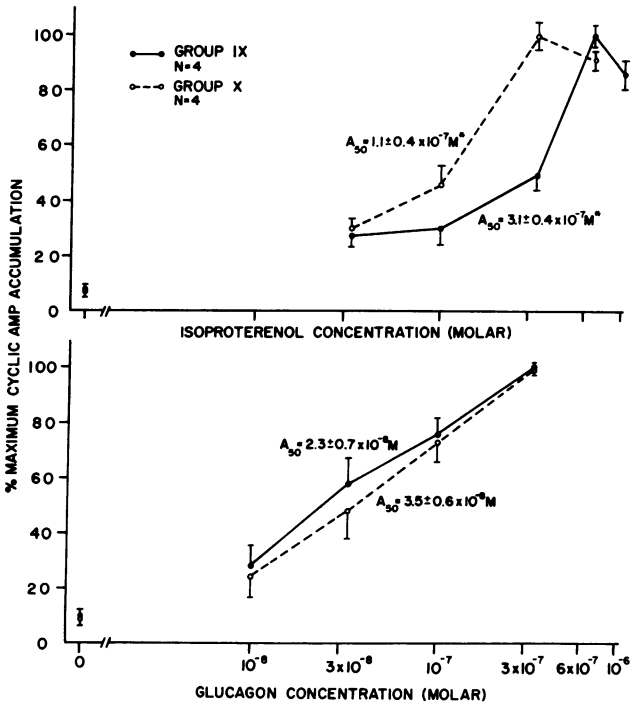

Figure 10. Isoproterenol (*upper panel*) and glucagon (*lower panel*) dose-response curves for cardiocytes from control (group IX: •) and previously β-blocked (group X: ○) ventricular myocardium. Each panel shows, as a percent of the maximum response for that group, cyclic AMP accumulation in response to the indicated concentrations of the two stimulatory agents. The A_{50} values were compared by Student's unpaired *t* test.

group IX cardiocytes was 1.74±0.10 pM/10^6 cells; a similar value of 1.77±0.62 pM/10^6 cells was found for the group X cardiocytes. Here, in contrast to the case for isoproterenol stimulation, this same peak accumulation of cyclic AMP occurred in the two groups of cardiocytes at the same maximal concentration of glucagon, as indicated by the top two points in the lower panel of Fig. 10.

The dose-response relationships shown in the upper panel of Fig. 10 demonstrate a significant shift to the left, or potentiation of cyclic AMP accumulation in response to graded isoproterenol concentrations, for the cardiocytes from propranolol-pretreated cats. This difference is exemplified by the threefold decrement in the A_{50} value for the group X cardiocytes when compared to that for the group IX cardiocytes. In contrast, neither the dose-response relationships nor the A_{50} values in the lower panel of Fig. 10 show a difference between the group IX and X cardiocytes. Thus, whereas cyclic AMP generation that is not adrenoceptor-mediated is equivalent in the two groups of cells, substantially enhanced β-adrenoceptor-mediated cyclic AMP generation is seen in cardiocytes isolated from the hearts of cats treated chronically with propranolol.

Discussion

The principal findings of this study may be considered in terms of the effects of both acute in vitro β-blockade and chronic in vivo β-blockade. Acute in vitro β-blockade, under well-defined conditions for isolated myocardium, (*a*) has no effect on myocardial mechanics, either at rest or during contraction, (*b*) is without influence on a basic inotropic function not mediated through the β-adrenergic system, the treppe effect, and (*c*) has no effect on MVO_2 independent of its blockade of endogenous and exogenous catecholamine-induced mechanical changes. Chronic in vivo β-blockade (*a*) results in increased myocardial sensitivity to catecholamine action upon its withdrawal, (*b*) does so without any effect on the number, affinity, or affinity state of myocardial β-adrenoceptors, but (*c*) does lead to more efficient coupling of β-adrenoceptor activation to cyclic AMP generation.

Mechanical and energetic effects of β-blockade

The first goal of these studies was to elucidate the contractile and metabolic actions of β-adrenoceptor blockade on the myocardium itself, without having to account simultaneously for interrelated effects on the rest of the organism. In the intact organism, the mechanisms responsible for the effectiveness of β-adrenoceptor-blocking agents in the treatment of angina pectoris usually have been thought to be the reductions of heart rate, arterial pressure, and contraction velocity (50, 51) observed both at rest and with exercise after their use. However, there is also evidence that these agents may have beneficial effects on the myocardium in addition to those related to improvements in these three basic hemodynamic determinants of MVO_2. These postulated myocardial effects include (*a*) selective redistribution of blood flow to ischemic myocardium, (*b*) morphologic effects related to chronic β-adrenoceptor blockade, and (*c*) direct metabolic effects not necessarily mediated through the β-adrenergic system.

The first postulated effect, redistribution of myocardial blood flow, is seen in the ischemic hearts of conscious dogs after propranolol administration and results in flow decreasing in normal areas and increasing in ischemic ones; there is a selective redistribution towards the most ischemic myocardial areas (10, 52). Several other concurring studies as well as further supporting data demonstrating blood flow redistribution are described in a recent review (53).

The mechanisms for blood flow redistribution towards ischemic myocardium are probably complex. They might involve direct effects of β-adrenoceptor blockade on the coronary vasculature (52, 54), indirect effects on myocardial blood flow dependent on changing ventricular wall stress (10), and rate-dependent alterations in both relative and absolute diastolic time intervals. Nevertheless, the basis for the expectation that flow distribution towards ischemic myocardium should have beneficial metabolic effects additive to those dependent on hemodynamic factors alone seems straightforward.

The second postulated effect consists of several morphologic changes that might be expected to result in a reduction in myocardial ischemia. These changes, which have been reported after prolonged β-adrenoceptor blockade in experimental animals (12, 55), include a reduction in relative mitochondrial volume, a reduction in myocardial mass, an increased intravascular volume, and a reduced mean path length from capillary to cardiocyte. Each of these changes might be expected to result in either decreased myocardial oxygen demand or increased oxygen delivery; taken together, they would be expected to reduce myocardial ischemia in vivo in a manner largely independent of the more obvious and immediate β-adrenergic influences on hemodynamic factors. However, no functional effect of possible morphologic changes during chronic β-adrenoceptor blockade was found in the present study when the muscles from pro-

pranolol-pretreated cats were studied in vitro in the absence of catecholamines.

The third postulated effect concerns the more basic question of whether either the immediate or the chronic myocardial metabolic effects of β-adrenoceptor blockade can be understood solely in terms of the prevention of the positive inotropic effects of increased adrenergic tone or whether there are instead direct metabolic effects not necessarily related to the β-adrenergic system. The initial phase of this study, summarized in Figs. 3–5, was directed in large part towards an analysis of this question. For both groups of muscles, the first three interventions established a control for comparison of later interventions, a response to a rate increase, and a response to the release of endogenous catecholamines. The next two interventions established the response to exogenous catecholamines and any additional response to a superimposed rate increase. The final four interventions assessed the effect of β-adrenoceptor blockade first on baseline behavior and then on behavior in response to the positive inotropic interventions previously characterized.

The steady-state mechanical response to the rate increase used in the second intervention is of the sort to be expected on the basis of extensive previous data reviewed elsewhere (56). The metabolic responses to this rate increase included both the greater absolute MVO_2 per contraction associated with greater active stress and the greater normalized MVO_2 associated with augmented inotropism. Although the metabolic response of isolated myocardium to the treppe effect has not been characterized as completely as the mechanical response, there are data showing that the positive-force staircase is associated with increases both in the amount of time during which membrane depolarization permits calcium entry into the cardiocyte (57) and in the number of myosin heads interacting with the actin filaments during contraction (58). Taken together, these data provide a reasonable mechanism for the enhanced MVO_2 observed in this study during the rate change in the second intervention.

The absolute and relative increases in active force were less after a rate increase in the presence of catecholamines than was the case after the same rate increase in the absence of catecholamines. This diminished force–frequency ratio in the presence of catecholamines probably reflects a partial exhaustion of the frequency-dependent inotropic reserves of the myocardium (59). The fact that a frequency-dependent effect was observed to any extent in the presence of a near-maximal inotropic concentration of exogenous norepinephrine (28) is consistent both with evidence that the treppe effect is calcium-dependent (60) and with data suggesting that norepinephrine acts in part by a mechanism that is independent of the intracellular calcium concentration (61).

The absolute and relative increases in both active force and MVO_2 after a rate increase in the presence of propranolol were very similar to those found after the same rate increase in the absence of propranolol. This finding first confirms that frequency-dependent mechanical effects are not mediated through the β-adrenergic system (62, 63) and, second, shows clearly for the first time that β-adrenoceptor blockade affects neither the MVO_2 associated with each contraction showing a frequency-dependent increase in active force nor that associated with the enhanced inotropic state itself.

The final three points to note in discussing the in vitro contractile and energetic effects of β-adrenoceptor blockade of the control muscles are first, that it had no effect on baseline contractile or energetic function, second, that it was without effect on the MVO_2 of the quiescent muscle, and third, that this drug prevented any deviation from baseline conditions in response both to a maneuver shown to release endogenous catecholamines and to exogenous catecholamine administration. There is good evidence that propranolol blocks catecholamine-induced cyclic AMP generation (64) and that the formation of cyclic AMP stimulates glycogenolysis and lipolysis (65). Therefore, in a setting of myocardial ischemia in the intact organism with significant adrenergic tone, it is not surprising that propranolol is capable of preserving high energy phosphate stores (66). In contrast, no primary effect of propranolol on the oxygen consumption of normal isolated myocardium is apparent in these data. Thus, although it has been postulated that β-adrenoceptor blockade has an effect on myocardial metabolism that is independent of its effect on either cardiac autonomic nervous control (53) or hemodynamic variables (67, 68), no support for these concepts is found either in these data or in other recent data (69) derived in a somewhat less direct manner.

Regulation of the β-adrenergic receptor

The second goal of these studies was to define any long-term heterologous "up-regulation" of the cardiac β-adrenoceptors during their blockade. Clinical interest in this postulate stems both from the possibility that an increase in β-adrenoceptor number during administration of a pharmacological antagonist might lead to decreased effectiveness of a fixed dose of the antagonist with time and from the possibility that withdrawal of the β-adrenergic blocking agent might produce a "hyperadrenergic state" with potentially serious consequences. Indeed, this latter effect is a well-described, if somewhat controversial, clinical entity (70). However, the mechanisms that might produce such an effect are not yet established (8).

Either an increased number of β-adrenoceptors or hypersensitivity of the β-adrenoceptors to adrenergic stimulation after withdrawal of β-adrenoceptor blockade would seem to be the most straightforward mechanisms for this effect, but two recent reviews (2, 8) point out that neither has been found uniformly in animal and clinical studies. An animal study (4) has shown that chronic β-adrenoceptor blockade results in a doubling of the number of cardiac β-adrenoceptors, and a clinical study (5) has shown that propranolol administration is associated with an increase in the density of lymphocytic β-adrenoceptors and hypersensitivity of sympathetic reflexes after drug withdrawal. However, several other recent studies have failed to uniformly demonstrate either up-regulation of the β-adrenoceptor during its blockade (6–8) or a hyperadrenergic withdrawal state (11, 71, 72).

These disparate results probably reflect in large part the complex pharmacodynamics of propranolol and the multitude of variable effects produced by β-adrenoceptor blockade in the intact organism. There are data from isolated cardiac tissue (73) studied after chronic in vivo β-blockade that show consistent electrophysiologic changes and some changes in muscle mechanics suggestive of an enhanced inotropic state. However, the mechanical performance of both the control and the drug-treated muscles was so profoundly depressed that no useful conclusions can be drawn from the mechanical data.

In the present study, the data shown in Figs. 3–5 for the papillary muscles from control and propranolol-pretreated cats demonstrate an augmented physiologic response of the myocardium to adrenergic stimulation after the withdrawal of chronic β-blockade. This statement is based both on the increased con-

tractile and energetic responses of these muscles to catecholamines and, most strikingly, on the failure of a concentration of DL-propranolol which abolished the effect of catecholamines on the control muscles to do so with the muscles from propranolol-pretreated cats. This finding is both confirmed and shown to be β-adrenoceptor-specific by the data in Figs. 6 and 7. Thus, an increased sensitivity of cardiac muscle to catecholamine action after the withdrawal of chronic β-blockade, a reasonable basis for a hyperadrenergic withdrawal state, is clearly demonstrated by this study.

Despite a diligent effort, we could find no basis for this increased cardiac catecholamine sensitivity in terms of a change in either the number or the affinity of the cardiac β-adrenoceptors after chronic β-adrenoceptor blockade. That is, three separate assays of cardiac tissue using three different radioligands, as well as a further assay of isolated cardiocytes, failed to demonstrate an effect of chronic propranolol treatment on the B_{max} and K_D of the β-adrenoceptors of either the heart as a whole or of the right ventricle, from which the mechanical and metabolic data were derived, specifically. In addition, there was no effect of chronic propranolol treatment on the proportion of β-adrenoceptors in the high-affinity state. These findings necessitated an alternative explanation for the observed cardiac catecholamine hypersensitivity after chronic β-blockade. A reasonable possibility, for which there is the basis developed below, would be an increased biological effect of a given level of β-adrenoceptor activation.

As summarized in several recent reviews (8, 74, 75), there is considerable evidence that not only β-adrenoceptor number and affinity but also the degree of coupling between the activated β-adrenoceptor and the effector enzyme, adenylate cyclase, is subject to dynamic regulation. If such a mechanism were operative here, the seeming paradox in the present study of catecholamine hypersensitivity without a change in the cardiac β-adrenoceptor after propranolol treatment might be resolved. That is, increased cyclic AMP generation at a given level of catecholamine-induced β-adrenoceptor activation in propranolol-pretreated cardiac tissue would not only suggest a tighter coupling between the receptor and effector molecules in this tissue but would also provide a reasonable explanation for the catecholamine hypersensitivity seen after cardiac β-blockade in this study. The data shown in Fig. 10 do, in fact, show an increase in β-adrenoceptor-specific cyclic AMP generation in propranolol-pretreated myocardium.

Conclusion

Acute β-adrenoceptor blockade in vitro, either of normal myocardium or of myocardium previously subjected to chronic β-adrenoceptor blockade in vivo, has no effect on myocardial mechanics or energetics independent of its effect on catecholamine action. Chronic β-adrenoceptor blockade in vivo increases myocardial sensitivity to catecholamine action upon its withdrawal, not by up-regulation of β-adrenoceptor number, affinity, or affinity state, but instead by causing more effective coupling of the activated cardiac β-adrenoceptor to cyclic AMP generation.

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