Contractile and Biochemical Responses of Cardiac and Skeletal Muscle to Isoproterenol Covalently Linked to Glass Beads

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SUMMARY The effects of (-)-isoproterenol covalently linked to glass beads on inotropic state, cyclic AMP concentration, and phosphorylase a to b conversion was studied in cat and guinea pig papillary muscles. Biochemical responses also were measured in mouse diaphragm sections. In cat papillary muscles under normal conditions and in guinea pig muscles partly depolarized with 22 mM K+ an increased inotropic state and phosphorylase activation could be dissociated from the formation of cyclic AMP. This contrasts with close correlation reported between isometric tension and cyclic AMP concentration in guinea pig papillary muscles exposed to varying concentrations of isoproterenol dissolved in the bath medium. The cyclic nucleotide did increase in guinea pig papillary muscles exposed to a freshly prepared batch of isoproterenol beads and in the mouse diaphragms. The type and age of the bead preparation had marked effects on all responses that were measured and on the rate of leakage of biologically active products from the beads. Nevertheless, experimental conditions could be obtained under which the initiation of the contractile and biochemical responses was probably limited to intense stimulation of receptors at or near the points of contact between beads and muscle and not due to gross leakage into the bath. The results indicate that cyclic AMP is probably involved in the initiation but not the propagation of the inotropic response of papillary muscles exposed to isoproterenol glass beads.

THE APPLICATION of microscopic glass beads containing covalently bound catecholamine to various myocardial preparations produces positive inotropic and chronotropic responses. The beads have the following properties: they are 20-300 μM in diameter and contain 1-10 pmol of catecholamine per bead bound to the glass through a silicon-propylaminophenylidazole side chain to the 6 position of the catechol ring. The addition of as few as 1-10 beads to the surface of cardiac muscle was sufficient to elicit a response, whereas unsubstituted aryl glass beads were inactive. The magnitude of the contractile responses were comparable to those elicited by 10-1,000 nM concentrations of catecholamines in solution. A positive inotropic response was seen within 30 seconds after placement of the beads on the muscle. This diminished rapidly after the beads were removed or after the addition of a β-blocker to the bath medium. The effect of the beads was not altered in papillary muscles obtained from animals that had been treated with reserpine, indicating that the action of the beads was direct and not mediated by release of endogenous norepinephrine. However, the assertion that the activity of these beads represent immobilized, covalently bound catecholamine has been challenged. Evidence has been presented that pharmacologically effective concentrations of soluble products were released from beads, and that contact between beads and the responsive preparation was not required.

Recently Venter et al. have reported that addition of three isoproterenol glass beads to the surface of isolated cat papillary muscles caused an inotropic response but not an elevation of cyclic AMP concentration in these muscles. A cyclic AMP increase was, however, observed with heart cells in tissue culture. Previous experiments from our laboratory on guinea pig papillary muscles have shown cyclic AMP formation to precede the activation both of contraction and of phosphorylase with low concentrations of free catecholamine. Formation of phosphorylase a was delayed and occurred only with concentrations of catecholamine 10 times the threshold for the positive inotropic and cyclic AMP responses. We also have given preliminary reports that after depolarization of guinea pig papillary muscles with 22 mM K+, isoproterenol bound to glass beads restored mechanical activity and activation of phosphorylase without necessarily increasing cyclic AMP. Watanabe and Besch, however, have found that soluble isoproterenol restored contractility in association with elevation of cyclic AMP in isolated, perfused guinea pig hearts. Thus there are contradictions between results obtained with immobilized catecholamines and the effects of these agents in solution in terms of the role of cyclic AMP in the inotropic response of heart muscle. The postulate that the active agent in catecholamine glass beads remains immobilized has been questioned.

Our purpose then has been to investigate the properties

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of isoproterenol covalently linked to glass beads, with special attention to the problem of leakage of biologically active material from the beads and to examine the relationship between cyclic AMP formation, phosphorylase activation, and the inotropic response of papillary muscles exposed to beads in normal bathing medium and medium containing depolarizing concentrations of K⁺.

**Methods**

**PAPILLARY MUSCLES**

Hearts were excised from male Hartley guinea pigs (350-450 g) and male or female cats (2.5-5.0 kg). Right (cat) or left (guinea pig) ventricular papillary muscles were prepared as previously described except that the muscles were mounted horizontally, rather than vertically, in baths containing 30-35 ml of Krebs' bicarbonate medium at 22-24°C continuously gassed with 95% O₂ and 5% CO₂. The base of the muscle was placed in a plastic clip attached to a Statham strain gauge (gold cell UC-3) while the tendinous end was tied with 5-0 silk suture to an adjustable micrometer. A preload tension of 0.5-1.0 g was applied and the muscle was stimulated at 0.2 Hz by square wave pulses 4 msec in duration. The pulse was delivered by a Grass S4KR stimulator to two platinum field electrodes placed parallel to the long axis of the muscle. The voltage was adjusted to 10-15% above threshold.

After a 30-minutes equilibration, muscle length was adjusted to obtain peak isometric tension. The muscle then was equilibrated for an additional 30 minutes, when the maximal inotropic response to paired electrical stimulation (PES) was determined for each muscle. The stimulus voltage and rate were maintained but a second stimulus was introduced with a delay which produced a maximal increase in isometric tension (750-900 msec for cat and 450-650 msec for guinea pig papillary muscles).

After each cat papillary muscle returned to control tension and equilibrated for 20 minutes it was exposed to isoproterenol (1.0 μM). After attainment of a maximal contractile response the bath fluid was changed three times. The muscle then was exposed to either soluble isoproterenol (1.0 μM) or to isoproterenol glass beads. At the peak contractile response (2-3 minutes) the muscle was rapidly frozen with stainless steel tongs cooled in liquid nitrogen. The muscles were stored at −20°C until assayed. Control cat papillary muscles were treated exactly as experimental muscles except that they were not exposed again to soluble isoproterenol or to isoproterenol glass beads.

Because the positive inotropic response to isoproterenol (1.0 μM) persisted for up to 60 minutes in some guinea pig muscles they were not challenged with an initial test dose of isoproterenol. Following PES some guinea pig papillary muscles were depolarized by increasing external K⁺ concentration from 3.6 to 22 mM. Ten minutes after changing the external K⁺ concentration the effectiveness of the potassium block was tested by increasing the stimulus voltage in a stepwise manner from 10 V to 60 V. Approximately 90% of the muscles tested in this manner showed no mechanical activity at any voltage used. The stimulus voltage was then set at 25 V for the remainder of the experiment. After an additional 15-minutes equilibration period the muscles were challenged with either soluble isoproterenol or isoproterenol glass beads and were frozen at various times after the addition of drug.

All contractile responses were recorded on paper by a Gould Brush 440 recorder or a Grass model 7 polygraph. Isometric tension was measured from the base to the apex of the contraction curve. Time to peak tension was determined at a paper speed of 25 mm sec⁻¹. The rate of tension development was measured by determining the initial slope of the contraction curve. Rate of relaxation of guinea pig papillary muscles was measured by determining the slope of the contraction curve following attainment of peak tension. Active tension development and the rate of rise of tension development for cat papillary muscles were corrected for cross-sectional area, which was calculated (assuming the muscle to be a cylinder) from the frozen weight and length of the isometrically contracting muscle. A similar correction for these parameters for guinea pig papillary muscles was not made because of the asymmetric shape of these muscles.

**MOUSE DIAPHRAGMS**

Male Swiss-Webster mice (15-20 g) were killed by cervical dislocation, then decapitated, and the diaphragms were removed. Each diaphragm was cut in half. The hemidiaphragms were rinsed with 0.9% NaCl, placed into a bath containing 15 ml of Krebs' bicarbonate medium maintained at 37°C, and continuously gassed with 95% O₂ and 5% CO₂ through perforated polyethylene tubing. The tissue bath was constructed from a polystyrene Petri dish (5.4 cm in inside diameter and 1.3 cm deep) with a rubber disk 2.6 cm in diameter protruding 2.0 mm from the bottom of the dish. The hemidiaphragms were immobilized by being pinned to the rubber disk to ensure continuous glass bead to muscle contact. One half of each diaphragm was exposed either to soluble isoproterenol or to isoproterenol glass beads for 10 minutes. The other half of the diaphragm was placed in a separate chamber and was used as the control. At the end of 10 minutes of incubation, a time at which increases in cyclic AMP concentration or phosphorylase activation were maximal and stable, the muscles were trimmed free of tissue not in contact with the beads and frozen.

**BATHING MEDIUM**

The bathing medium contained, in millimoles per liter: NaCl, 119; KCl, 2.7; CaCl₂, 2.5; NaHCO₃, 25; MgSO₄, 1.18; KH₂PO₄, 1.18; and glucose, 5.5. K⁺ medium (22 mM) was identical to the above solution except for an equimolar reduction in Na⁺ concentration.

**ANALYTICAL PROCEDURES**

**Tissue Extraction**

Muscle samples were stored at −60°C until assayed. Manipulations prior to homogenization were carried out in a −20°C cold box. Frozen solution remaining on the surface of the muscle after freezing was removed and the muscle then was weighed. Each (cat and guinea pig papil-
lary) muscle was carefully trimmed of tissue that had not been compressed by the clamp freezing process. The papillary muscle was cut along the longitudinal axis and each segment reweighed. The larger portion (2.5–4 mg) was used to determine cyclic AMP concentration, and the smaller portion (2–3 mg) was used to measure phosphorylase activity. Only guinea pig papillary muscles that weighed less than 8 mg were used for biochemical or physiological studies reported here. Muscles with a mass greater than 8 mg appeared to be hypoxic, since these muscles had elevated phosphorylase activity ratios (>0.25). Cat papillary muscles (which averaged 15 mg in weight) did not show elevated phosphorylase ratios (<0.1), thus no weight limitation was imposed. Diaphragm muscles were weighed in a similar manner and 5- to 15-mg samples were used for biochemical determinations.

Phosphorylase Assays

For glycogen phosphorylase assays the weighed muscles were treated as previously described.9 Papillary muscles were homogenized in 50 vol (based on sample weight) of an ice-cold solution containing 4 mm ethylenediaminetetraacetate (EDTA), 20 mm β-glycerophosphate, 20 mm β-mercaptoethanol, and 20 mm KF, pH 6.8. Diaphragm muscle phosphorylase activity is more sensitive to elevation in temperature during homogenization, hence a modification of the method of Stull and Mayer13 for rabbit gracilis muscle was used. After warming to −35°C the muscle was placed in 0.3 ml of a 60% (vol/vol) aqueous glycerol solution containing 100 mm KF and 100 mm EDTA, pH 6.7. The homogenization was carried out at −35°C with a motor-driven pestle ( precooled to −35°C) for 1-second periods (12 rotations/sec) every 10–15 seconds for 4 minutes. After the addition of 0.5 ml of an aqueous solution of 20 mm β-glycerophosphate, pH 6.7, the material was allowed to thaw for 10 minutes and then mixed and centrifuged at 3,000 g for 15 minutes. The supernatant fluid was assayed immediately for phosphorylase activity by the fluorometric method of Hardman et al.14 Glycogen phosphorylase was assayed in duplicate in the direction of glucose 1-phosphate formation in the absence or presence of 2 mm AMP. The results are expressed as the ratio of activity. −AMP/+AMP. An increase in this ratio indicates an increase in the conversion of phosphorylase from the b form to the a form of the enzyme.

Cyclic AMP Assays

Muscle samples were homogenized and extracted in trichloroacetic acid and neutralized as described previously. The extract was assayed in duplicate for cyclic AMP by the method of Mayer et al.15 This assay is based on the activation by cyclic AMP of partially purified rabbit skeletal muscle protein kinase as the incorporation of [32p]-ATP into casein. Cyclic AMP concentration is expressed as picomoles per milligram wet weight.

MATERIALS

(−)-Isoproterenol glass beads were prepared according to the method of Venter and Dixon.16 The term glass bead is a misnomer and the glass support is in fact crushed glass. This makes quantification difficult because each microscopic chip is unique. Each application consisted of a heterogeneous population of chips in terms of size, shape, surface area, and amount of isoproterenol bound to the surface of the glass or sequestered in the porous glass. However, there is a dose-response relationship between the number of beads placed onto a muscle and the contractile response elicited. Two types of porous glass were used, both having a pore size of 550 Å. Batch GZO-3940 (40- to 80- or 80- to 120-mesh) was obtained from Corning Glass Co. and has been used and characterized previously by Venter and co-workers.9 The second preparation of isoproterenol (N-693) glass beads (40– to 80-mesh) was obtained from Conring in 1974. GZO-3940 beads contained approximately 2 μmol of isoproterenol/g of glass,3 and N-693 beads contained approximately 5 μmol of isoproterenol/g of glass. The catecholamine glass beads were protected from light and stored in 0.1 N HCl at 4°C until used. Each day a sample of glass beads (50 mg) was placed in a small sintered glass funnel and washed with 300 ml of 0.1 N HCl followed by 300 ml of 0.9% NaCl. Just prior to use the beads were washed with Krebs' bicarbonate buffer and then applied to the surface of the muscle with a Pasteur pipette. The orange color of the beads allowed their precise placement onto the muscle surface without accumulation of beads on the bottom of the bath.

(±)-Isoproterenol (Sigma) stock solution was made daily in 0.1% sodium metabisulfite in a concentration sufficient for a 0.1-ml addition to the bathing medium to result in the desired final concentration of the drug.

STATISTICAL METHODS

Statistical analyses were performed using Student's t-test for paired or unpaired observations. A probability of 0.05 was accepted as a significant difference. Results are reported as ±1 SE. The papillary muscles and diaphragms obtained from a given animal were assigned to a specific treatment in a random manner.

Results

Although a single bead elicited a contractile response in some papillary muscles, reproducible responses in all muscles were observed only when 10 or more beads were used. In all experiments reported here the muscles were covered with the maximal number of beads that could be placed onto the muscle surface. Care was taken to limit the beads to a single layer. With the beads used (20–100 μm in diameter) this amounted to 15–40 beads with guinea pig papillary muscles, 20–50 beads with cat papillary muscle and 100–200 beads with diaphragm muscles.

MECHANICAL RESPONSES TO GZO-3940 ISOPROTERENOL GLASS BEADS

In cat and guinea pig papillary muscles the contractile response after addition of isoproterenol (1.0 μM) or the GZO-3940 isoproterenol beads began within 30–45 seconds and reached a maximum within 2–3 minutes in cat muscles and within 4–6 minutes in guinea pig muscles. On removal of either form of isoproterenol from the bath the augmented tension in cat papillary muscles returned to
control within 5 minutes (isoproterenol beads) or 10 minutes (soluble isoproterenol). In contrast, augmented tension in guinea pig papillary muscles due to either form of isoproterenol did not return to control levels until 30-90 minutes after removal of catecholamine. The duration of action was proportional to the catecholamine concentration, the length of exposure, and the soluble catecholamine used (isoproterenol > epinephrine > norepinephrine), and was inversely related to temperature (unpublished observations).

The maximal inotropic response to isoproterenol glass beads was measured as an increase in isometric tension, rate of tension development, and decrease in time to peak tension. These were compared with peak responses elicited with PES and a supramaximal concentration of free isoproterenol (1 μM) (Fig. 1 and 2). With both species the maximal contractile response elicited by GZO-3940 isoproterenol beads, while substantial (35% increase in cat and 55% in guinea pig isometric tension), was always less than that observed with PES (80-150%) or 1 μM isoproterenol (90-190%). The application of either alyamine glass beads (no catecholamine substituent) or (+)-epinephrine glass beads to cat or guinea pig papillary muscles produced no inotropic response (data not shown).

An important consideration when working with catecholamine glass beads is the possibility that the observed responses are mediated by attainment of pharmacological concentrations of free catecholamine in the bathing medium. Positive inotropic responses were elicited only by isoproterenol beads in direct contact with the muscle. A large excess of beads placed at the bottom of the 30 to 35-ml bath had no effect on isometric tension of muscles obtained from either cat or guinea pig. On several occasions the medium that had been used to wash the beads was applied to the surface of a papillary muscle. A positive inotropic response was never observed even when a small volume (1-2 ml) of medium had been in contact with a large number of beads (500 mg) for several hours. The medium, obtained from a bath that contained a muscle at peak contractile response to a saturating amount of isoproterenol beads, was placed on the surface of a second papillary muscle. No contractile response was observed in the second muscle, although it was sensitive to the direct addition of the beads or soluble isoproterenol.

**BIOCHEMICAL RESPONSES TO GZO-3940 ISOPROTERENOL GLASS BEADS**

Cyclic AMP concentrations and glycogen phosphorylase activity were determined in papillary muscles frozen about 4 minutes after exposure to isoproterenol beads or free drug at the time of peak tension (Table 1). Previous experiments had shown that during 10-minute exposures to 0.01 or 1 μM isoproterenol cyclic AMP concentration increased to a maximum in 30 seconds and was then maintained constant; phosphorylase activity ratio rose slowly in the presence of 1 μM isoproterenol, reaching a maximum in 3-4 minutes. In the present experiments the administration of soluble isoproterenol (1 μM) to both cat or guinea pig papillary muscles resulted in increased cyclic AMP concentration and in the elevation of -AMP/AMP phosphorylase activity ratio. In contrast, the appli-
Although an elevation of myocardial cyclic AMP was not detected, this does not rule out the possibility that cyclic AMP was elevated in those cells in direct contact with or in close proximity to the glass beads. Therefore, mouse diaphragms, which have a larger surface to volume ratio than papillary muscle, were exposed to 100-200 beads as well as to soluble isoproterenol (Table 2). Soluble (±)-isoproterenol (10 nM) or (−)-isoproterenol glass beads caused an increase in cyclic AMP and activation of phosphorylase. The responses obtained with isoproterenol beads were similar to those observed with soluble isoproterenol.

MECHANICAL AND BIOCHEMICAL RESPONSES TO N-693 ISOPROTERENOL GLASS BEADS: EVIDENCE OF LEAKAGE

The contractile response elicited by the second preparation of isoproterenol glass (N-693) beads in guinea pig papillary muscles in normal (3.6 mM) and high (22 mM) potassium media are summarized in Table 3. A representative polygraph tracing illustrating the initial time course and magnitude of the contractile response to these beads. 1 μM isoproterenol, and PES in guinea pig papillary muscles is also presented (Fig. 3). The delay in onset and the time required to reach peak contractile response with these beads in 3.6 mM K⁺ was the same as observed with GZO-3940 glass beads on both cat and guinea pig papillary muscles. This preparation of isoproterenol glass beads increased isotropic tension by 150-250%, an increase as great as that elicited by PES or 1.0 μM isoproterenol and much larger than the 55% increase in tension produced by the GZO-3940 beads on guinea pig papillary muscles.

Cyclic AMP concentrations and −AMP/+AMP phosphorylase activity ratio were determined from muscles frozen at the time of peak contractile response about 4 minutes after the addition of drugs, as described in Methods. Values are mean ± se. Tension data obtained with these muscles are summarized in Figures 1 and 2. Numbers in parentheses denote number of experiments.

* Significant difference from control (P < 0.05) as determined by Student's t-test.

<table>
<thead>
<tr>
<th></th>
<th>Cat papillary muscle</th>
<th></th>
<th>Guinea pig papillary muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cyclic AMP (pmol·mg wet wt⁻¹)</td>
<td>Phosphorylase (−AMP/+AMP)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.54 ± 0.07</td>
<td>0.08 ± 0.001</td>
</tr>
<tr>
<td></td>
<td>(±)-Isoproterenol (1 μM)</td>
<td>0.92 ± 0.08*</td>
<td>0.16 ± 0.02*</td>
</tr>
<tr>
<td></td>
<td>(−)-Iso-glass beads (GZO-3940)</td>
<td>0.62 ± 0.08</td>
<td>0.12 ± 0.01*</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.68 ± 0.06</td>
<td>0.15 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>(±)-Isoproterenol</td>
<td>1.61 ± 0.18*</td>
<td>0.50 ± 0.09*</td>
</tr>
<tr>
<td></td>
<td>(−)-Iso-glass beads (GZO-3940)</td>
<td>0.74 ± 0.12</td>
<td>0.17 ± 0.07</td>
</tr>
</tbody>
</table>

Cyclic AMP concentrations and −AMP/+AMP phosphorylase activity ratio were determined from muscles frozen at the time of peak contractile response about 4 minutes after the addition of drugs, as described in Methods. Values are mean ± se. Tension data obtained with these muscles are summarized in Figures 1 and 2. Numbers in parentheses denote number of experiments.

* Significant difference from control (P < 0.05) as determined by Student's t-test.
The muscles were quickly removed from the chamber and frozen in liquid nitrogen. In the case of hemidiaphragms exposed to glass beads, only that portion of the muscle directly in contact with the beads was dissected and frozen for assay of cyclic AMP and phosphorylase activity.

Values are mean ± se. Numbers in parentheses denote number of experiments.

* Significant difference (P < 0.05) from control as determined by the Student’s t-test.

However, cat papillary muscles did not show a greater contractile response to these N-693 beads. The control tension and the peak contractile response of cat and guinea pig papillary muscles to PES or 1 μM isoproterenol were not different in the experiments with GZO-3940 and N-693 beads (data not shown).

Not only was the contractile response of guinea pig papillary muscles more sensitive to the N-693 beads, but this preparation of isoproterenol beads also increased cyclic AMP concentration and the −AMP/+AMP phosphorylase activity ratio (Table 4; 3.6 mM K⁺). These responses were nevertheless smaller than those elicited by 1 μM isoproterenol.

A possible explanation of the increased activity of these beads in guinea pig muscle is that pharmacologically effective concentrations of agonist leaked into the bath. The same set of experiments described earlier were repeated with these beads, i.e., a large excess of beads was placed at the bottom of the 30- to 35-ml bath; the buffer used to wash the beads was added; the bath fluid from a responding muscle was exchanged with that of another muscle known to be sensitive to free catecholamine. No positive inotropic response was elicited in any of these experiments. The possibility of leakage was further tested by placing a guinea pig papillary muscle into a bath with a small volume (8 ml). Approximately 250 isoproterenol glass beads were placed on a platform at the bottom of the bath. This platform was attached to a micromanipulator that allowed the platform containing the beads to be positioned at various distances from the muscle. Ten minutes after placement of the beads 4 mm from the muscle a positive inotropic response was observed (Fig. 4). This experiment was repeated four times with similar results. There was always an 8- to 15 minute delay in the onset of the contractile response. When these same beads were placed on the muscle surface there was a delay of only 30–60 seconds before the contractile response. If the beads were allowed to remain in the washing buffer 1 hour or longer before being placed on the muscle, both time of onset and maximal response were unchanged. However, when the beads were placed on the platform in the bath the inotropic response was always weaker but with no alteration in the delay of onset. The same experiment was repeated in the regular 30- to 35-ml bath. No inotropic response was observed with these beads when they were not in direct contact with the guinea pig papillary muscles, even after 60 minutes of exposure. These experiments indicate that sufficient leakage of material from glass support to produce a biological response in guinea pig papillary muscles.

### Table 2: Biochemical Responses of Mouse Diaphragm Exposed to Soluble Isoproterenol and to Isoproterenol Glass Beads (Batch GZO-3940)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cyclic AMP (pmol/mg wet wt⁻¹)</th>
<th>Phosphorylase (−AMP/+AMP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.18 ± 0.04</td>
<td>0.04 ± 0.002</td>
</tr>
<tr>
<td>(±)-Isoproterenol (10 nm)</td>
<td>0.59 ± 0.08</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>(–)-Iso-glass beads (GZO-3940)</td>
<td>0.36 ± 0.04</td>
<td>0.10 ± 0.01</td>
</tr>
</tbody>
</table>

### Table 3: Contractile Responses of Normal and Depolarized Guinea Pig Papillary Muscle Exposed to Soluble and Immobilized Isoproterenol Beads (Batch N-693)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Isometric tension (g)</th>
<th>Time to peak tension (msec)</th>
<th>dT/dt (g sec⁻¹)</th>
<th>−dT/dt (g sec⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.6 mM K⁺</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.87 ± 0.08</td>
<td>420 ± 11</td>
<td>2.3 ± 0.2</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>PES</td>
<td>2.24 ± 0.2</td>
<td>390 ± 11</td>
<td>7.9 ± 0.9</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>(–)-Iso-glass beads (N-693)</td>
<td>2.0 ± 0.03</td>
<td>340 ± 13</td>
<td>7.8 ± 1.4</td>
<td>4.0 ± 0.7</td>
</tr>
<tr>
<td>(±)-Isoproterenol (1 μM)</td>
<td>2.5 ± 0.4</td>
<td>270 ± 12</td>
<td>12.5 ± 3.0</td>
<td>5.8 ± 0.8</td>
</tr>
<tr>
<td>22 mM K⁺</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PES</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(–)-Iso-glass beads (N-693)</td>
<td>2.7 ± 0.4</td>
<td>320 ± 16</td>
<td>12.0 ± 2.3</td>
<td>6.3 ± 0.6</td>
</tr>
<tr>
<td>Isoproterenol (1 μM)</td>
<td>2.1 ± 0.4</td>
<td>293 ± 17</td>
<td>11.0 ± 2.3</td>
<td>5.6 ± 1.3</td>
</tr>
</tbody>
</table>

dT/dt = rate of tension development; −dT/dt = rate of relaxation. Values are mean ± se. Numbers in parentheses denote number of experiments.

Contractile data were obtained at the peak response induced by paired electrical stimulation (PES), isoproterenol glass beads (N-693), or soluble isoproterenol (1 μM) as described in Methods. All interventions induced significant changes (P < 0.05) in all parameters measured when compared to control by paired Student's t-test. There were no significant differences between the responses elicited in 3.6 mM K⁺ and the responses obtained in 22 mM K⁺ depolarized hearts.
Because of the relatively greater effect observed in guinea pig papillary muscles to the N-693 beads their actions on mouse diaphragm were also examined. The experimental procedure was modified to determine whether leakage could effect biochemical changes in diaphragm. The two halves of the diaphragms were placed into a single 15-ml bath, as illustrated in Figure 5. Ten minutes after the addition of isoproterenol glass beads to one half of a hemidiaphragm (segment A, Fig. 5) the hemidiaphragms were quickly cut in half to produce four tissue samples. A through D. and frozen in liquid nitrogen. Only the segment of muscle that was in direct contact with beads (A) responded with both an elevation in cyclic AMP concentration and an increase in the phosphorylase activity ratio. The other three segments (B, C, and D) did have elevated phosphorylase ratios as compared to control samples. This response to the beads was less than that observed with 2.5 mM isoproterenol (Fig. 5).

The contractile response to the N-693 isoproterenol glass beads gradually diminished over a period of several months. Although these (−) isoproterenol glass beads still induced a positive inotropic response in guinea pig papillary muscles (80–90% increase in isometric tension as compared to 150–250% increase observed initially with these beads), they no longer elicited a measurable change in cyclic AMP concentration or in phosphorylase activity ratio at any time after their application (data not shown). These responses were similar to those elicited by GZ0-3940 isoproterenol glass beads in guinea pig (Fig. 2 and Table 1) and cat papillary muscle (Fig. 1 and Table 1).

**RESPONSES IN DEPOLARIZED PAPILLARY MUSCLES (22 mM K+)**

Isoproterenol glass beads, as well as soluble isoproterenol (1 μM) restored contraction in response to phasic electrical stimulation after the papillary muscles had been made unresponsive to electrical stimulation by exposure to 22 mM K+. The rate of development of increased isometric tension was the same with soluble drug as with isoproterenol glass beads. There were no differences in maximal tension, time to peak tension, rate of tension development, and rate of relaxation when responses to beads and soluble drug were compared or when the responses obtained in depolarized preparations were compared with those obtained in normal media (3.6 mM K+).

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**Table 4: Biochemical Responses in Normal and Depolarized Guinea Pig Papillary Muscles Exposed to Soluble and Immobilized Isoproterenol Beads (Batch N-693)**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cyclic AMP (pmol·mg wet wt−1)</th>
<th>Phosphorylase Activity (–AMP/+AMP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.52 ± 0.04</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>(±)-Isoproterenol (1 μM)</td>
<td>1.50 ± 0.20*</td>
<td>0.49 ± 0.07*</td>
</tr>
<tr>
<td>(−)-Iso-glass beads (N-693)</td>
<td>0.95 ± 0.34*</td>
<td>0.24 ± 0.05*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cyclic AMP (pmol·mg wet wt−1)</th>
<th>Phosphorylase Activity (–AMP/+AMP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.62 ± 0.03</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>(±)-Isoproterenol (1 μM)</td>
<td>2.04 ± 0.30*</td>
<td>0.40 ± 0.03*</td>
</tr>
<tr>
<td>(−)-Iso-glass beads (N-693)</td>
<td>0.78 ± 0.20</td>
<td>0.25 ± 0.06*</td>
</tr>
</tbody>
</table>

Guinea pig papillary muscles were exposed to no drugs, soluble (±)-isoproterenol (1 μM), or to isoproterenol glass beads (batch N-693) in the presence or absence of a depolarizing concentration of potassium (22 mM) and then clamped at peak contractile response (4–6 minutes). Muscles were assayed for cyclic AMP concentration and phosphorylase activity as described in Methods. Tension data obtained with these muscles are shown in Figure 3 and Table 3.

Values are means ± SE. Numbers in parentheses denote number of experiments.

* Significant differences (P < 0.05) from respective control as determined by the Student's t-test.
Muscle responses to isoproterenol glass beads

Discussion

Contractile and biochemical responses

The qualitative biochemical responses to immobilized isoproterenol were more variable and differed considerably from the responses to soluble isoproterenol. The effects of the beads depended on the type of glass used, the age of the activated preparation, the species of animal, and the type of muscle.

Despite these variations, the biochemical actions of isoproterenol glass beads are different from those of isoproterenol solution. Saturating amounts of the beads elicited augmentation of contraction that must have propagated through a large fraction of the volume of the papillary muscles. This follows from the data that isoproterenol glass beads augmented the contractile responses of guinea pig muscles to nearly the same extent as did a maximally effective concentration of soluble isoproterenol and approximately equal to that produced by PES (Table 3, Fig. 2). However, in most of the experiments there was no

K+; Table 3. Soluble isoproterenol (1 μm) elicited increases in cyclic AMP concentration and phosphorylase activity ratio in depolarized muscles similar to those observed in nondepolarized controls (Table 4). However, while the application of isoproterenol glass beads to the muscle surface increased the phosphorylase activity ratio 2-fold and restored contractility in depolarized muscle, there was no detectable increase in cyclic AMP concentration at any time point examined (Table 4 and Fig. 6). This is in contrast to the results obtained with these beads in nondepolarized muscles, where increases in cyclic AMP concentration were observed. The application of unsubstituted arylamine glass beads or (+)-epinephrine glass beads to depolarized muscles did not produce any detectable biochemical or physiological changes.

Figure 4: Effect of (-)-isoproterenol glass beads (batch N-693) with and without contact to a guinea pig papillary muscle in a bath of 8-ml capacity. Either 14-20 beads were placed on the surface of the muscle, or 250 beads were placed on a small platform attached to a micromanipulator which was used to adjust the distance between the beads on the platform and the muscle. Distance measurements were made with a micrometer eyepiece of a dissecting microscope mounted perpendicular to the muscle bath. A: responses were elicited by beads just after the completion of the washing procedure described in Methods. B: responses to the same beads were determined after they had been allowed to equilibrate with Krebs' bicarbonate medium for 1 hour. In both A and B application of 14-20 beads to the surface of the muscle induced a response within 45 seconds. Placement of 250 beads at a distance of 4 mm also elicited a response but with an 8- to 15-minute delay. The magnitude of the latter response was much less after the beads had been equilibrated for 1 hour (B) than immediately after application (A).

Figure 5: Biochemical responses of mouse diaphragm exposed to soluble isoproterenol (ISO) or to isoproterenol glass beads (batch N-693). Diaphragms were removed and cut in half. Both halves were placed in a single 15-ml chamber (37°C) as described in Methods. Isoproterenol glass beads were placed onto segment A. After 10 minutes of incubation the two muscle strips were cut in half and each segment (A, B, C, and D) was frozen in liquid nitrogen. Control muscles and those exposed to soluble isoproterenol were included in each experiment. They were placed in separate chambers. (●) above a bar indicates a significant difference (P < 0.05) from control as determined by Student's t-test. Values are mean ± SE of six experiments.
measurable increase in cyclic AMP. This observation has also been made by Venter et al.\(^8\) The results are clearly different from the responses of papillary muscles to soluble catecholamines. Augmentation of isometric tension and elevation of cyclic AMP have been found to be closely coupled when examined as a function of isoproterenol concentration and time of exposure to the drug.\(^9\)\(^,\)\(^17\) Elevations of the phosphorylase activity ratio were less sensitive to drug concentration and was considerably delayed.\(^9\)

Interpretation of these results must take into account that two totally different types of responses are compared: mechanical events and those that represent the consequence of enzyme actions (cyclic AMP concentration) or enzyme activity measured directly (the phosphorylase activity ratio). Furthermore, measurements of contractility were continuous and offer the use of paired statistical comparisons. In contrast, biochemical data were derived from noncontinuous samples. This difficulty cannot be resolved until the increased inotropic state caused by catecholamines can be quantified at the molecular level. However, examination of the variance of the cyclic AMP data in this study on immobilized isoproterenol and our previous study on the drug in solution\(^1\) still lead to the following conclusion. Isoproterenol beads can produce a propagated inotropic response that is not associated with a comparable elevation of cyclic AMP, whereas there is no dissociation of mechanical events from cyclic AMP formation in response to isoproterenol in solution.

Two sets of results do suggest that cyclic AMP is involved in the initiation as opposed to the propagation of the inotropic response to catecholamines: the effects of the freshly prepared N-693 beads on guinea pig papillary muscle (Table 4) and the response of mouse diaphragm to the GZO-3940 beads (Table 2). In both experiments cyclic AMP concentrations were elevated by the beads. This could be due to a sufficiently extensive activation of receptors so that a detectable change was recorded for the entire muscle. This would be most likely in the diaphragms because they are only about 100 \(\mu\)m thick and only the area in contact with 200 beads was analyzed. This interpretation is consistent with the observation that isoproterenol glass beads increased cyclic AMP in monolayer cultures of heart cells but not in cat papillary muscles.\(^8\) The surface area of susceptible cells in contact with the beads may be the critical factor in determining whether an increase in cyclic AMP concentration can be detected.

**LEAKAGE OF BIOLOGICALLY ACTIVE MATERIAL FROM THE BEADS**

Venter et al.\(^3\) reported that glass beads substituted with \(^{14}\)C-labeled catecholamines leaked at the rate of 0.001% per hour. They calculated that, during the time of development of the contractile response after the beads had been placed on the muscle, the concentration of solubilized products would be in the femtomolar range, i.e., more than 3 orders of magnitude below an effective concentration. However, Yong\(^6\) and Yong and Richardson\(^7\) have disputed these results and have reported much higher rates of leakage. The controversy centers on the manner and duration of washing of the beads after the diazotization of the catecholamines to the arylamine glass.\(^4\)\(^,\)\(^6\) In the present studies, leakage sufficient to cause an inotropic response in the absence of bead-tissue contact was observed only when a large number of beads were placed into a small volume bath, in which case the observed contractile response was always delayed by 8–15 minutes after the beads were placed into the bath. This delay was unaffected by the distance between the beads and the muscle. Yong and Richardson\(^7\) and Venter and co-workers\(^1\)\(^,\)\(^2\)\(^,\)\(^8\) as well as ourselves have observed that removal of active beads from contractile tissue to the bottom of the bath caused a rapid decrement of the augmented contractile response. This suggests that the leakage material is not stable. In contrast, application of a small number of beads to the muscle surface induced an increase in isometric tension within 20–45 seconds and maximal biochemical changes within 3–6 minutes which persisted as long as the beads were in contact with the muscles.

Our results exclude the possibility that the contractile and biochemical responses that we measured were due to gross leakage of active components from the beads. However, they neither support nor deny the contention that the action of the beads is limited to receptors on cells in direct contact with the covalently bound catecholamine.\(^4\)\(^,\)\(^8\) The variation in biological activity between the two bead preparations used on papillary muscles and the results obtained with mouse diaphragms (Table 2 and Fig. 6) suggest that we need to determine whether cyclic AMP is synthesized only in cells in direct contact with the beads or in more distant cells as a result of limited diffusion (<100 \(\mu\)m) of an active
leakage product into the tissue. With regard to the latter possibility, Venter et al. have examined 6-(p-nitrophenyl-diazo)catecholamines, congeners of the product expected from peptide bond cleavage of the bead side chain. These substances were somewhat more potent than catecholamines in stimulating papillary muscle contractility.

**RESPONSES IN DEPOLARIZED PAPILLARY MUSCLES**

Increasing extracellular K+ to 22 mM partly depolarizes heart tissue and is known to inactivate fast Na+ currents. This blocks propagated depolarization and tension development in response to electrical stimulation. The addition of soluble catecholamine restores these events to depolarized muscles. The restored action potential is probably carried primarily by Ca2+ ions and to a lesser extent by Na+ ions. The application of isoproterenol glass beads restored contractility with changes in parameters, such as rate of tension development, similar to those produced by soluble isoproterenol in depolarized muscles and by both beads and soluble drug in nondepolarized preparations. We have also shown that action potentials were restored and propagated over the surface of the muscles in proportion to the number of beads applied at one end of the papillary muscle. The action potentials were measured with intracellular electrodes placed at various distances from the site of application of the beads (Becker, Ingebretsen, and Mayer, unpublished observations).

The addition of a maximally effective concentration of soluble isoproterenol (1 μM) elicited similar increases in cyclic AMP formation and in the activation of glycogen phosphorylase in both depolarized and nondepolarized guinea pig papillary muscles, confirming the results obtained by Watanabe and Besch with K+-depolarized perfused hearts. The application of isoproterenol glass beads elicited an increase in the activation state of phosphorylase in both depolarized and nondepolarized muscle, but cyclic AMP formation was increased significantly only in nondepolarized papillary muscles. A similar lack of effect of soluble catecholamines on cyclic AMP formation had been observed in perfused rat hearts depolarized by 56 mM K+. Although contractile events were not restored by catecholamines at this high concentration of K+, biochemical events subsequent to cyclic AMP formation, i.e., phosphorylase kinase or phosphorylase, could be partly activated. The mechanism by which an elevation in extracellular K+ concentration interferes with catecholamine-induced changes in formation of cyclic AMP is currently under investigation.

In conclusion, we wish to stress several aspects of the manner in which catecholamine glass beads should be used to produce physiological changes and to examine biochemical mechanisms of action of catecholamines. It is important that each preparation of catecholamine glass be well characterized to eliminate the possibility that the observed responses are due to gross leakage of biological material into the bathing medium. This was accomplished with the two preparations of beads used in our experiments by using well washed beads and by demonstrating that the rate of leakage, when it did occur, was too slow to account for the biochemical and physiological responses observed after direct application of the beads to muscle. It is also important that a set of experiments be performed with the same preparation of catecholamine glass and within a short period of time to avoid inconsistencies with different batches of beads and to reduce the effects of deterioration of biological activity.

The mode of action of catecholamine glass beads and the role of cyclic AMP cannot be resolved from the investigations presented here. It is necessary to consider two possibly distinct aspects of the responses to the beads: initiation and propagation. Isoproterenol glass beads probably interact with a small percentage of the total number of receptors at or near points of bead-tissue contact. The responses are then propagated to the rest of the tissue. If this assumption is correct, then cyclic AMP is likely to be involved in initiation, but not in propagation, of responses to the rest of the tissue. Propagation is probably carried by an inward transmembrane Ca2+ current. Isoproterenol glass beads restored contractility and caused phosphorylase b to a conversion in depolarized papillary muscles. This is consistent with the evidence that both of these events are regulated by Ca2+.

**Acknowledgments**

We thank Dr. J. C. Venter for preparing and making available to us the isoproterenol-glass beads used in this study.

**References**

16. Venter JC, Dixon JE: Production of glass bead immobilized catechol-
THE LEFT ventricle of patients with systemic hyperten-
sion is subjected to an intermittent pressure load for sev-
eral reasons. The inherent intensity of the disease process is variable;1 the antihypertensive effects of several com-
monly used drugs in the treatment of hypertension are partially dependent on posture;2 and the compliance of
patients to antihypertensive drug regimens frequently is inconsis-
tent.3 Although the effects of persistent pressure loads on the development of ventricular hypertrophy have been examined extensively4,5 the effects of an intermittent pressure load on the development of ventricular hypertrophy have not received much attention.

If the net rate at which hypertrophy progresses is equal to the net rate at which it regresses, then intermittent pressure loading using a schedule in which the periods of pressure loading and unloading are of equal duration would not result in residual ventricular hypertrophy. Alternatively, if progression of ventricular hypertrophy occurs at a faster rate than regression, net intermittent pressure loading would result in significant ventricular hypertrophy.

To examine this question, we studied chronically instrumented docile cats in which we could increase the right ventricular pressure intermittently by producing pulmo-

arv artery constriction with an inflatable balloon. Using this model, we were able to estimate the net rate at which hypertrophy progresses and regresses by using different intermittent pressure loading schedules. The results indicate that intermittent pressure loading is a potent stimulus to the development of ventricular hypertrophy and that the net rate at which ventricular hypertrophy progresses is faster than the net rate at which it regresses.

Methods

GENERAL PROTOCOL

Forty cats of both sexes were included in the study. Eight cats served as unoperated controls and eight cats

Effects of Intermittent Pressure Loading on the Development of Ventricular Hypertrophy in the Cat

MELVIN L. MARCUS, DWAIN L. ECKBERG, JAMES L. BRAXMEIER, AND FRANCOIS M. Abboud

SUMMARY Although the effects of persistent pressure loading on the development of ventricular hypertrophy have been studied extensively, the effects of intermittent pressure loading have not been examined. To study the effects of intermittent pressure loading we subjected the right ventricle of cats to intermittent pulmonary artery constriction over a 2-week period. Two intermittent pressure loading schedules were employed. The first consisted of a right ventricular systolic pressure of 60 mm Hg for 3.5 days and normal right ventricular pressure for 3.5 days; and the second consisted of a right ventricular systolic pressure of 60 mm Hg for 2.3 days and normal right ventricular pressure for 4.7 days. The intermittent pressure-loaded cats were compared with normal unoperated controls, sham-operated controls, and cats with a persistent right ventricular pressure load for either 1-week or 1- to 2-month duration. The data indicate that intermittent pressure loading caused significant right ventricular hypertrophy. Since significant residual ventricular hypertrophy was present in both intermittent pressure loading groups, regression of ventricu-

lar hypertrophy involves a slower process than the progression of hypertrophy.
Contractile and biochemical responses of cardiac and skeletal muscle to isoproterenol covalently linked to glass beads.

W R Ingerebretsen, Jr, E Becker, W F Friedman and S E Mayer

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