Lack of Detectable Change in Cyclic AMP During the Cardiac Inotropic Response to Isoproterenol Immobilized on Glass Beads

(cat papillary muscles/paired electrical stimulation/force propagation/heart cell cultures/drug-receptor interactions)

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ABSTRACT Changes in contractility and the levels of adenosine 3':5'-cyclic monophosphoric acid (cAMP) were assessed in isolated cat cardiac muscle in response to soluble isoproterenol and isoproterenol immobilized on glass beads. Drug-induced positive inotropic responses were compared to the maximum isometric force achieved with paired electrical stimulation, a potent physiological inotropic stimulus. Isoproterenol (1 μM) in solution increased the force of contraction 0.332 ± 0.165 g at 60 sec in eight muscles tested, which at 60 and 120 sec averaged 65.5 ± 6.5% and 82.9 ± 8.8%, respectively, of the force with paired electrical stimulation. Isoproterenol immobilized on glass beads gave positive inotropic responses similar to those for the soluble form of the drug. Placement of only three isoproterenol-glass beads on the muscles increased the force of contraction 0.742 ± 0.166 g at 60 sec (n = 11), which at 60 and 120 sec averaged 45.1 ± 7.0% and 58.6 ± 6.4%, respectively, of the force with paired electrical stimulation. The magnitude of this response indicates that the increased force was developed by at least 60% of the cells in each muscle. Control levels of cAMP were 0.527 ± 0.049 pmol/mg of tissue wet weight, n = 11. cAMP levels 60 sec after 1 μM soluble isoproterenol was added were 1.212 ± 0.085 pmol/mg; in contrast, the levels of cAMP in response to isoproterenol immobilized on glass beads at 60 sec were 0.490 ± 0.060 pmol/mg, not significantly different from control levels. These data indicate that cAMP may not be involved in the propagation of the inotropic response that must have occurred in these cardiac muscles and raise questions as to the physiological significance of the large cAMP increases that occur in response to soluble drugs and hormones.

Catecholamines immobilized on glass beads have been shown to elicit positive inotropic and chronotropic responses in several cardiac preparations in vitro and in vivo (1, 2). The catecholamine-glass bead complexes have been demonstrated to be extremely stable, and their exact structure has been elucidated (3, 4). Soluble azo-substituted catecholamine derivatives have also been synthesized and shown to be biologically active, demonstrating that the catecholamine linkage to the glass beads is in an active conformation (4). Throughout these initial studies it was repeatedly observed that single 300 μm diameter isoproterenol-glass beads, containing only picomole amounts of covalently bound isoproterenol, were capable of inducing substantial positive inotropic responses on isolated cat cardiac muscle (2). These findings, which have been shown not to be due to the attainment of pharmacological concentrations of catecholamine in solution through catecholamine release (3, 4), have been difficult to explain in terms of generally assumed pharmacological principles. Although cardiac muscle is thought to function as a syncytium with the ability to transmit waves of depolarization and force to adjacent cells via the tight junctions and intercalated discs (5), it has been a general premise that drug effects result from the interaction of the drug with multiple receptor sites on each cell and involve most, if not all, cells present in the tissue in order to elicit a pharmacological response. However, the catecholamines immobilized on glass beads, irrespective of whether or not minute catecholamine leakage occurs, must be exerting their measurable effects on the cat papillary muscles by direct interaction with a very finite number of cells. It can be calculated that of the over one million cells contained in an average experimental cat papillary muscle, the maximum number of cells that one isoproterenol-glass bead could interact with directly is less than 100*. This implies that substantial pharmacological responses can be elicited by isoproterenol directly contacting less than 0.01% of the cell population.

Adenosine 3'-5'-cyclic monophosphoric acid (cAMP) has been implicated by a number of laboratories as the mediator of catecholamine-induced positive inotropic responses in cardiac tissue (6-8), and recently as the beat-to-beat regulator of contraction in certain frog heart preparations (9, 10). While the exact role of cAMP in cardiac contractility has been questioned (see ref. 11 for review), it has proven extremely difficult to clearly dissociate the inotropic effects of catecholamines from cAMP increases. It might be assumed that if cAMP were the mediator of catecholamine-induced positive inotropic effects in the heart, alterations in the intracellular cAMP levels would be detectable in response to catecholamines immobilized on glass beads, especially if catecholamine leakage from the glass beads were partially responsible for the reported biological activity of these agents.

This preliminary report will present evidence indicating that, in contrast to soluble isoproterenol, there is no detectable increase in the level of cAMP in cat papillary muscles in response to isoproterenol-glass beads, even though there is a substantial increase in the contractile force in response to both the immobilized and the soluble agent. These increases in force approached the maximum obtainable physiologically with paired electrical stimulation (PES) (12), and suggest that most of the cells in the muscle are involved in the contractile

Abbreviation: PES, paired electrical stimulation.

* Based on a cell 10 × 30 μm and a papillary muscle 1.0 × 10 mm.
responses. These results will be presented in more detail elsewhere.

**MATERIALS AND METHODS**

Papillary muscles of 1.2 mm diameter or smaller were rapidly dissected from the right ventricles of 1- to 3-kg domestic cats anesthetized with intraperitoneally administered sodium pentobarbital (40 mg/kg). The papillary muscles were positioned horizontally in a Lucite bath and arranged to contract isometrically. One end of the muscle was held by a Lucite clip connected to a force transducer (Statham), and the tendinous end was tied by 5-0 silk thread to a micrometer, thus allowing muscle length to be altered. The muscles were stimulated to contract at 12 times per minute by means of a Grass stimulator and two platinum electrodes placed along the parallel aspect of the muscle, providing transverse field stimulation. Stimulus voltage was maintained at 2 mV above threshold with a 5 msec duration. Peak isometric force was recorded along with a time marker on a forced ink oscillographic recorder (Clevite 200, Brush Instruments). The muscle bath contained Krebs solution (20 ml) at 30°C maintained at pH 7.4 when bubbled continuously with a mixture of 95% O₂, 5% CO₂. Each muscle was lengthened to attain peak isometric tension (L-max) by performing a standard length-tension curve.

After muscle stabilization, the maximum positive inotropic response was determined by means of paired electrical stimulation (13). The paired stimulus maintained the stimulus voltage and stimulus rate as above, but with the addition of a second stimulus at a delay of 200–800 msec after the initial stimulus. The exact delay that resulted in maximal isometric tension development was determined by increasing the delay in 50 msec steps between 200 and 800 msec. Paired electrical stimulation was followed by the addition of either 1 μM isoproterenol (Sigma) or three l-isoproterenol-glass beads prepared as described (4, 14) to obtain a time course of the drug action. After removal of the drug and muscle restabilization, paired electrical stimulation was repeated, followed by the addition to the muscle of either free 1 μM isoproterenol or three isoproterenol-glass beads. The muscles were rapidly frozen at the times indicated in the text, and cAMP was determined by the radioimmune assay of Steiner et al. (15), as supplied by Schwarz/Mann.

Chick embryo heart cells were cultured as described (1). Protein was measured by the method of Lowry et al. (16).

**RESULTS**

**Effects of Soluble Isoproterenol and Paired Electrical Stimulation on the Force of Contraction of Cat Papillary Muscles.** The addition of 1 μM isoproterenol to muscle baths containing isometrically contracting cat papillary muscles resulted in increases in the contractile force. The time course and magnitude of this positive inotropic response is depicted in Fig. 1. The average control isometric force at L-Max for these muscles was 3.0 ± 1.0 g (mean ± SEM, n = 8). In response to soluble isoproterenol, the force of contraction was augmented within approximately 10 sec after the drug addition (i.e., on the second contraction). The force continued to increase, reaching a maximum within 120 sec. When force mea-

† L-Max is the optimum resting muscle length (L) that gives the maximum active isometric force when a series of twitches are performed over a range of resting muscle lengths.
maximum at least 90 sec prior to the peak inotropic response, and cAMP remained elevated beyond 3 min. The cAMP response was clearly maximal at 60 sec with a value of $1.212 \pm 0.086$ pmol/mg of wet weight, $n = 7$; therefore this time period of 60 sec will be used later for comparative purposes.

Effects of Isoproterenol-Glass Beads and Paired Electrical Stimulation on the Force of Contraction of Cat Papillary Muscles. As was seen with soluble isoproterenol, the addition of three isoproterenol-glass beads (about 300 μm diameter each) to the surface of isometrically contracting cat papillary muscles resulted in a significant positive inotropic response. The time course of the inotropic response to isoproterenol bound to glass beads is compared in Fig. 3 to the PES response in the same muscles. The isometric force at $L_{\text{Max}}$ was $2.21 \pm 0.29$ g, $n = 11$. As with soluble isoproterenol, the force of contraction was increased within approximately 10 sec (on the second beat) after glass bead addition to the muscle surface and continued to increase, reaching a maximum within 120 sec. The change in isometric force induced by isoproterenol-glass beads at 60 and 120 sec was $0.742 \pm 0.166$ g and $1.00 \pm 0.23$ g, respectively.

The effects of PES on these same muscles (Fig. 3) again allow for comparison of the force achieved with the isoproterenol-glass bead to the maximal possible force development of the muscle. PES resulted in a change in force averaging $1.618 \pm 0.47$ g, $n = 11$. The percentage of PES, and therefore of maximum force achieved by three isoproterenol-

### Table 1. cAMP and positive inotropic changes in cat papillary muscles in response to paired electrical stimulation, soluble isoproterenol, and isoproterenol immobilized on glass beads

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Change in isometric force at 60 sec [peak force/L-Max force (g)]</th>
<th>% Increase in peak force at 60 sec</th>
<th>% of PES at 60 sec</th>
<th>% of PES at 120 sec</th>
<th>cAMP 60 sec after drug addition (pmol/mg of tissue wet weight)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>(L-Max/Max force (g))</td>
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<tr>
<td>Control muscles</td>
<td>1.86 ± 0.48</td>
<td>0.527 ± 0.049 (0.325–0.79)</td>
<td>0.0 ± 0.0</td>
<td>0.166</td>
<td>0.742 ± 0.085</td>
<td></td>
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<tr>
<td></td>
<td>(0.5–4.0)</td>
<td>n = 11</td>
<td></td>
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</tr>
<tr>
<td>A. PES</td>
<td>3.0 ± 1.0</td>
<td>1.46 ± 0.45 (0.22–3.4)</td>
<td>0.0 ± 0.0</td>
<td>156.9 ± 6.0</td>
<td>82.9 ± 8.8</td>
<td>1.212 ± 0.085</td>
</tr>
<tr>
<td></td>
<td>(0.4–8.18)</td>
<td>n = 8</td>
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<tr>
<td>1 μM isoproterenol</td>
<td>3.0 ± 1.0</td>
<td>157.9 ± 6.3 (111.1–155.0)</td>
<td>157.9 ± 6.3</td>
<td>65.5 ± 6.5</td>
<td>82.9 ± 8.8</td>
<td>1.212 ± 0.085</td>
</tr>
<tr>
<td>(~30 nmol)</td>
<td>(0.4–8.18)</td>
<td>n = 8</td>
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<tr>
<td>B. PES</td>
<td>2.21 ± 0.29</td>
<td>1.618 ± 0.347 (1.16–4.14)</td>
<td>177.4 ± 16.3</td>
<td>45.1 ± 7.0</td>
<td>58.6 ± 6.4</td>
<td>0.490 ± 0.060</td>
</tr>
<tr>
<td></td>
<td>(1.1–4.4)</td>
<td>n = 11</td>
<td></td>
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<tr>
<td>Three isoproterenol-glass beads (~18 pmol of isoproterenol)</td>
<td>2.21 ± 0.29</td>
<td>0.742 ± 0.166 (1.082–1.78)</td>
<td>133.5 ± 6.4</td>
<td>45.1 ± 7.0</td>
<td>58.6 ± 6.4</td>
<td>0.490 ± 0.060</td>
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<tr>
<td></td>
<td>(1.1–4.4)</td>
<td>n = 11</td>
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Values represent the mean ± SEM; range is in parentheses; $n =$ number of papillary muscles.
glass beads, was 45.1 ± 7.0% and 58.6 ± 6.4% for 60 and 120 sec, respectively. These data are summarized in Table 1.

**Effects of Isoproterenol-Glass Beads on the Intracellular cAMP Levels in Cat Papillary Muscles.** In sharp contrast to the effects of soluble isoproterenol on the intracellular cAMP in the cat cardiac muscles, the addition of three isoproterenol-glass beads to the papillary muscles resulted in no detectable change of the cyclic nucleotide levels. The time course of the cAMP response to isoproterenol-glass beads is compared in Fig. 4 to the time course of the contractile inotropic response. Control cAMP levels averaged 0.327 ± 0.049 pmol/mg of wet weight, n = 11. The levels of cAMP in 10 muscles frozen at 60 sec after glass bead addition averaged 0.490 ± 0.060 pmol/mg of wet weight. The cAMP time course (Fig. 4) further illustrates that no detectable increase in cAMP occurred during the entire inotropic response to the glass bead-isoproterenol. These data are summarized in Table 1.

Due to the standard error inherent in these measurements, a number of carefully paired muscles were tested for cAMP increases in response to glass bead-isoproterenol. Two pairs of muscles, each pair of the same size and from the same heart, were tested with glass bead-isoproterenol. One muscle from each pair served as a control; the other muscle was covered for 60 sec with as many isoproterenol-glass beads as could be placed on the muscle's surface (approximately 500 beads). In experiment I the cAMP levels were 0.610 and 0.592 pmol/mg of wet weight, respectively, for the control muscle and that stimulated by glass bead-isoproterenol. In experiment II the levels were 0.395 and 0.403 pmol/mg, respectively.

**cAMP Concentrations in Synchronously Beating Chick Embryo Heart Cell Cultures in Response to Soluble Isoproterenol and Isoproterenol Immobilized on Glass Beads.** When either 10 μM isoproterenol in solution or 10 isoproterenol-glass beads were added to a confluent plate of synchronously beating chick embryo heart cells, similar positive chronotropic responses were detectable throughout the cell population, as reported (1). Control cAMP levels in these cells were 2.55 ± 0.21 pmol/mg of protein. In response to 10 μM isoproterenol the cAMP levels increased in 60 sec to 6.90 ± 0.58 pmol/mg of protein. In contrast, with 10 isoproterenol-glass beads the cAMP levels at 60 sec were only 2.45 ± 0.23 pmol/mg of protein. When a sufficient number of isoproterenol-glass beads were added to the cultures so that every visible area was in contact with a glass bead (about 10,000 beads), the cAMP levels averaged 8.06 ± 0.67 pmol/mg of protein.

**DISCUSSION**

The magnitude of the inotropic response to the isoproterenol immobilized on glass beads related to the peak inotropic response of PES clearly suggests that the force changes are the result of changes in force propagated throughout the cardiac muscle. The about 60% of PES force obtained with the isoproterenol immobilized on glass beads indicates that most of the papillary muscle cell population, i.e., about 600,000 cardiac cells of an idealized papillary muscle of 1,000,000 cells, must have been activated to achieve the observed responses. We suspect, however, that there was a gradient of force response throughout the heart muscle, with cells directly activated by the immobilized drug providing a maximal force response, and those more distant exhibiting progressively less response.

**Fig. 4.** The change in isometric force (O) with respect to time in response to three isoproterenol-glass beads is compared to the cAMP levels (●) in response to the same agent. Control cAMP levels are indicated at time 0. Error bars denote SEM.

For the present argument it is not essential that the isoproterenol be considered to exert its action while coupled to the glass beads. Of the about 1.0 attomole of isoproterenol released per min per glass bead, less than 0.01 attomole would be available for interaction with the muscle surface (4), and if active its effect would be limited by diffusion and tissue binding to the same cell population as the glass-bead-immobilized form of the drug. That this view is correct is supported by the present data (Fig. 4), in which there is no increase in the cAMP levels being found with the immobilized drug, whereas cAMP increases could clearly be expected if the glass beads were leaching catecholamines significantly.

Our evidence so far indicates that the isoproterenol-glass beads probably act via the β-adrenergic receptor system in producing the cardiac inotropic responses, since these responses are blocked in the papillary muscles by the β-adrenergic antagonist, propranolol (2–5 μM); in addition, it has been shown that while immobilized, only the l-enantiomers of the catecholamines and not the d-enantiomers can exert positive inotropic effects on isolated cardiac tissue (Venter and Weiland, unpublished observations).

The question of cAMP involvement in the cardiac inotropic response to catecholamines is not settled by these experiments. These data do suggest, however, that cAMP was not elevated and therefore may not be involved in the response of many of the cells, which must have taken part in the force increase resulting from the bound drug. Thus, cAMP did not appear to mediate the propagated inotropic response that must have occurred in these isolated cardiac muscles. This is not to say that cAMP could not have been involved in the initiation of the inotropic response in the cells directly in contact with the isoproterenol-glass beads. The data obtained with isolated heart cells in culture indicate that cAMP can be increased in cardiac tissue when many cells are in direct contact with the isoproterenol-glass beads. If this is extended to the cat papillary muscles, it could indicate cAMP is increased in those few cells in actual contact with the isoproterenol-glass bead. Although large increases in cAMP occurred in response to soluble isoproterenol (Fig. 2), the corresponding inotropic response was not substantially greater than the inotropic response obtained with three isoproterenol-glass beads (Fig. 3); thus, it is difficult to assign definite...
relations between cAMP increases in the muscles treated with soluble isoproterenol and the magnitude of the inotropic responses.

It is not unreasonable to assume that cAMP changes, if they are occurring with the glass bead-isoproterenol, would not be detected. The actual contribution of each single cardiac cell to the total cAMP response with free isoproterenol can be calculated, and from this number the minimum number of cells that must have cAMP increases in order to detect a change can be calculated to be on the order of 100,000 cells or 10% of the total. Clearly, if the glass bead-isoproterenol is interacting with <100 cells per bead, cAMP changes in those few cells would go completely undetected. This is further emphasized in the experiments where the entire exposed surface of the papillary muscles was covered with glass beads, yet no changes in cAMP were detectable. In contrast, when the entire surface of the heart cell cultures was covered with the isoproterenol-glass beads, large cAMP increases were detectable. It can be calculated that about 35,000 cells (<3.5% of the total cells) of our idealized papillary muscle would be in contact with glass bead-isoproterenol if the entire upper (dorsal) surface was covered with glass. Moreover, if the glass beads were, in fact, releasing catecholamines as suggested by Yong (18), there would have been more than an adequate amount of free isoproterenol released directly into the muscle tissue to result in maximal cAMP increases. This did not occur, further supporting the concept of a limited area of isoproterenol action either while covalently attached to the beads, or within the volume between the bead and the muscle surface (1-4).

These present results suggest that significant positive inotropic responses in most of the cell population of isolated cat papillary muscles can be elicited by immobilized isoproterenol interacting with less than 0.01% of the cell population. Since cAMP did not increase, these data further suggest that cAMP may not be involved in the propagation of the drug effect in this tissue. Our results also raise questions concerning the physiological significance of the large increases in cAMP that occur in response to soluble drugs and hormones.

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