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Cardiac Sites of Catecholamine Action: Diffusion Models for Soluble and Immobilized Catecholamine Action on Isolated Cat Papillary Muscles

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SUMMARY

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Models for isoproterenol and polymer-immobilized isoproterenol diffusion into cat cardiac muscle have been developed by applying the diffusion rates in water and the tortuosity factor for cat cardiac muscle extracellular diffusion. The theoretical extent of drug diffusion into cardiac muscle with time has been compared with the kinetics of cyclic 3',5'-AMP formation and the development of the positive inotropic response to these agents. These studies indicate that peak cardiac inotropic responses may be obtained in cat papillary muscles when the majority of drug molecules are still at the muscle surface and less than 40% of the equilibrium concentration is present in the tissue. The maximum cyclic AMP response occurs with less than 15% of the equilibrium concentration of isoproterenol present and with the majority of the muscle cells exposed to subthreshold levels of drug. These findings suggest that under non-steady-state conditions soluble isoproterenol as well as immobilized catecholamines may cause increased contractility by propagation mechanisms. Biochemical events such as increases in the concentration of cyclic AMP may not reflect events occurring throughout the cardiac muscle. The diffusion arguments indicate that the common assumptions concerning a homogeneous distribution of drug-receptor interactions producing cardiac contractile events oversimplify the events that occur under actual experimental conditions. A model for glass bead-immobilized catecholamine action based on catecholamine leakage is also presented. It indicates that while massive drug leakage does not account for the biological responses of these agents, it may be impossible to elucidate the exact mode of drug action in the microenvironment enveloping the bead surface.

INTRODUCTION

The action of catecholamines covalently immobilized on either soluble polymers or insoluble glass beads indicates that in isolated cardiac muscle positive inotropic responses can be propagated from a site of local catecholamine stimulation (1-4). These studies with the immobilized drugs have raised several questions concerning the exact sites and mechanisms of catecholamine action in cardiac muscle. The time of onset of the maximum catecholamine responses in isolated cat cardiac muscle preparations appears to be essentially identical whether the catecholamine is applied in solution or while immobilized on

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glass beads, suggesting similar mechanisms of action for both agents. However, the exact mode of catecholamine response initiation may depend in part on the manner of introduction of the drug to the tissue. For example, although soluble isoproterenol and glass bead-immobilized isoproterenol produce essentially identical inotropic responses in isolated cardiac muscle, the response to soluble isoproterenol is preceded or accompanied by an increase in the intracellular level of cyclic 3',5'-AMP whereas no cyclic AMP changes are detectable in response to the application of glass bead-immobilized catecholamines to the muscle surface (2).

In studies with isoproterenol covalently immobilized on soluble amino acid copolymers, it has been demonstrated that a 12,800 mol wt isoproterenol azoamino acid polymer can produce a maximal drug-induced positive inotropic response within the same time period as that required for a response to isoproterenol (211 mol wt) in solution (4). As with the glass bead-immobilized isoproterenol, this inotropic response is not accompanied or preceded by an increase in cyclic AMP (4). These data (4) support the hypothesis (2) of a propagated inotropic response in cardiac muscle as a result of catecholamine action on the superficial muscle layers (4). The propagated inotropic response hypothesis and the findings cited above necessitate an investigation into whether there are different sites and mechanisms of catecholamine action, based upon the rate of access of the drug to the tissue.

In this report the sites and mechanisms of soluble and immobilized catecholamine action on isolated cardiac muscle are considered with the development of models, based in part on studies (experimental and theoretical) of the limits of drug diffusion.

MATERIALS AND METHODS

Inotropic studies. Right ventricular papillary muscles were dissected from hearts of domestic cats (1–3 kg) anesthetized with intraperitoneal sodium pentobarbital (40 mg/kg). The papillary muscles were placed horizontally in a muscle bath and arranged to contract isometrically. One end of the muscle was held by a Lucite clip attached to a force transducer (Statham), and the tendinous end was tied by a 4-0 silk thread to a micrometer, thus allowing muscle length to be altered. The muscles were stimulated to contract 12 times/min by means of a Grass stimulator, with two platinum electrodes placed parallel to the muscle so as to provide transverse field stimulation. Peak isometric force was recorded on a forced-ink oscillographic recorder (Brush Instruments). The muscle baths contained Krebs' solution (20 ml), which was maintained at 30° and pH 7.4 when bubbled continuously with a mixture of 95% O_2–5% CO_2. Each muscle was lengthened to attain peak isometric tension (L_{max})[1] by constructing a standard length-tension curve.

Studies on soluble and polymer-immobilized isoproterenol. For drug studies, either 100 µl of *dl*-isoproterenol HCl (Sigma) or 100 µl of *l*-isoproterenol diazotized to a 12,800 mol wt random copolymer of hydroxypropylglutamine with *p*-aminophenylalanine, prepared as previously described (4), was added to the muscle baths to achieve the desired concentrations, and the inotropic response was monitored with time. For response decay studies, the drugs were incubated with the papillary muscles for exactly 10 min, after which the muscle baths were drained, refilled with fresh oxygenated Krebs' solution (previously warmed to 30°), drained, and refilled with Krebs' solution. This procedure took less than 10 sec. Following the washing procedure, the force of contraction was monitored until the control inotropic state was again obtained.

Isoproterenol uptake studies. *dl*-[7-^{3}H]Isoproterenol (2 Ci/mmole; New England Nuclear) was added to muscle baths (volume, 5 ml) to obtain a final concentration of 1 µM [^{3}H]isoproterenol. Right ventricular papillary muscles were dissected from hearts of domestic cats (1–3 kg) anesthetized with intraperitoneal sodium pentobarbital (40 mg/kg). Muscles were se-

[1] L_{max} is the optimum resting muscle length (L) that gives the maximum active isometric force when a series of twitches is elicited over a range of resting muscle lengths.

lected within a narrow range of 0.9–1.0 mm in diameter, and their weight was determined. Muscles were suspended in a well-stirred [^{3}H]isoproterenol solution for periods ranging from 5 sec to 20 min. Three muscles were used for each time point. Immediately on removal from the radioactive solution, the muscles were rapidly rinsed in Krebs' solution (4°), placed in scintillation vials with 1 ml of Protosol (New England Nuclear), and incubated overnight at 37°. The muscle-Protosol solution was neutralized with glacial acetic acid. Twelve milliliters of Aquasol (New England Nuclear) were then added, and the radioactivity was determined on a liquid scintillation counter (efficiency, 33%). Total radioactivity per muscle was corrected on the basis of muscle wet weight.

RESULTS

Diffusion theory. Isolated cat papillary muscles are cylindrical, have an average diameter of 1 mm, and are generally up to 1 cm in length. When a muscle is suspended in a large muscle bath in Krebs' solution containing a drug such as a catecholamine, the bathing solution can be considered, for the purposes of diffusion, to be an infinite reservoir of drug at a constant concentration. The diffusion of the catecholamine into the muscle tissue can be assumed to be everywhere radial. The drug concentration in the muscle becomes a function of the muscle radius r and time t, and can be described by the equation for diffusion into a cylinder, given by Crank (5) as

$$\frac{\partial C}{\partial t} = \frac{1}{r}\frac{\partial}{\partial r} rD\frac{\partial C}{\partial r} \qquad (1)$$

where D is the diffusion coefficient of the drug. In 1928 Hill described the diffusion of oxygen[2] and lactic acid into and out of muscle tissue and found the diffusion rates of experimental substances to be substantially slower in muscle than in solution (6). The diffusion rate of neurotransmitters in tissue is also generally slower than in water. Acetylcholine diffusion into rat diaphragm muscle and norepinephrine diffusion through the medial layer of the aorta have been estimated to be approximately ⅐ and ⅒, respectively, of their diffusion rates in water (8, 9). However, while it is likely that the rate of isoproterenol diffusion through cardiac muscle would be equally reduced, I have chosen for purposes of testing my hypothesis to assume the faster diffusion rates in water and only to apply a tortuosity factor to the diffusion coefficient. Page and Bernstein (10, 11) have published extensive studies on the properties of cat cardiac muscle *in vitro* and have estimated the extracellular space, the lower limits of channel diameter of the extracellular space, and a tortuosity factor for extracellular diffusion. The tortuosity factor is an indication of the increase in the mean diffusion path resulting from the fact that extracellular molecules must diffuse around rather than through muscle cells, hence taking a tortuous route. When Eq. 1 is applied to a nonhomogeneous material such as the cat papillary muscle, D must be considered to be an apparent diffusion coefficient D', as its exact value will depend on the particular geometrical structure of each muscle. The apparent diffusion coefficient D' can be estimated from

$$D' = \frac{D}{\lambda^2} \qquad (2)$$

where λ is the tortuosity factor. For cat cardiac muscle, Page calculated $\lambda = 1.44$ (10).

The solutions for Eq. 1 can be written in terms of the dimensionless parameters $D't/a^2$ and r/a, where a equals the radius of the cylinder in centimeters; if we are r cm from the center, we can describe this position as r/a (5). The unit of time $D't/a^2$ is the apparent diffusion coefficient D' times the real time in seconds over the square of the radius. The solutions of Eq. 1 were obtained graphically (5, 12) in terms of $D't/a^2$ and r/a. Solutions for a series of diffusion situations are therefore readily available (5).

[2] This study by Hill describes oxygen diffusion as a function of muscle thickness (6). His data indicate that the oxygen tension, muscle diameter, bath temperature, and stimulus rate utilized in the present study are adequate to prevent hypoxia (6). In addition, cat papillary muscles studied under the present conditions are metabolically intact in terms of high-energy phosphate stores (7).

Isoproterenol diffusion into isolated cat papillary muscles. The diffusion coefficient for isoproterenol (mol wt 211) can be approximated from the diffusion coefficient for glucose (mol wt 180), where $D_{25,w} = 6.73 \times 10^{-6}$ cm^2/sec (13). The apparent diffusion coefficient D' can be calculated for cat cardiac muscle by Eq. 2; D' therefore becomes 3.25×10^{-6} cm^2/sec. By applying this D' value and the graphical solution of Eq. 1 to a cat papillary muscle 1 mm in diameter, the theoretical extent of drug diffusion with time can be estimated. Figure 1 represents such an application. Because diffusion processes are theoretically independent of concentration, the data in Fig. 1 are applicable to any concentration (C_0) of the drug in the tissue bath. From Fig. 1, one can estimate that it would take on the order of 10 min for isoproterenol to reach at least 98% of the equilibrium (bath) concentration throughout the entire papillary muscle.

The time courses for the positive inotropic response and intracellular cyclic AMP response to 1 μM isoproterenol are compared in Fig. 2 with the time course for the theoretical fractional uptake of isoproterenol by the muscle. An apparent disparity exists between the time course of the muscle responses and the theoretical fractional uptake of isoproterenol (Fig. 2). At the time when the positive inotropic response reaches a maximum level [usually within 120 sec (2)], the theoretical fractional uptake of isoproterenol is only 70% of the equilibrium value. More striking, however, is the time course of the cyclic AMP response. Intracellular cyclic AMP concentrations reach a maximum in cat papillary muscles 15 sec after isoproterenol addition (2), when less than 30% of the theoretical equilibrium level of isoproterenol would be present in the tissue. From Fig. 1, at 15 sec the isoproterenol concentration only 200 μm from the surface would be less than 10% of the muscle bath concentration.

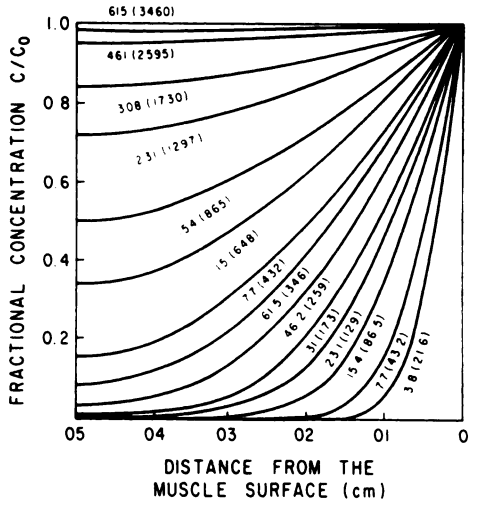

FIG. 1. *Calculated distribution of isoproterenol and polymeric isoproterenol (12,800 mol wt) in a 1-mm-diameter isolated cat papillary muscle at 25°*

Concentration distributions at various times with initial surface drug concentration C_0, and C, the concentration at different calculated values of r/a in centimeters from the muscle surface. The numbers on the curves indicate the point in time in seconds; numbers without parentheses show the time for soluble isoproterenol diffusion, and those in parentheses show the time for 12,800 mol wt polymeric isoproterenol diffusion.

In order to estimate whether the assumptions used in applying Eq. 1 are reasonable for the actual experimental conditions, the fractional uptake of ^{3}H-labeled isoproterenol was determined in cat papillary muscles. The time course of the actual fractional uptake of isoproterenol (Fig. 2) was found to be substantially slower than that calculated from Eq. 1. At 15 sec following drug addition, when the cyclic AMP levels are maximal, the determined isoproterenol uptake is only approximately 15% of its equilibrium value and only 44% of the theoretical uptake. By 120 sec, when the inotropic response is maximal, the levels of isoproterenol have reached only 40% of the equilibrium value and only 57% of the theoretical levels (Fig. 2).

The theoretical fractional isoproterenol uptake is based on a geometrically perfect papillary muscle and assumed diffusion properties that it may be unreasonable to expect of a random series of experimental muscles of variable dimensions. The results with labeled isoproterenol indicate that the theoretical model substantially overestimates the rate of isoproterenol diffusion in isolated cardiac muscles, hence increasing the disparity between inotropic responses and cyclic AMP responses and isoproterenol uptake.

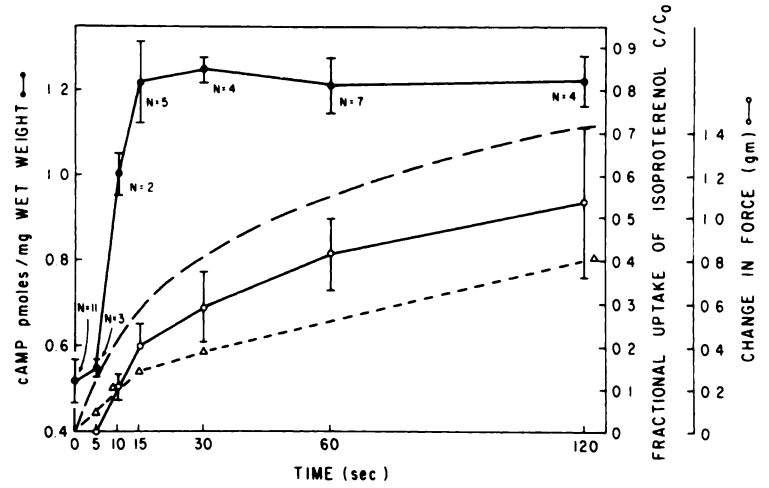

FIG. 2. *Time course of positive inotropic response and intracellular cyclic AMP response to 1 μM isoproterenol compared with theoretical and experimental fractional uptake of isoproterenol into a cat papillary muscle*

The change in isometric force (○) in eight isolated cat papillary muscles in response to 1 μM isoproterenol is compared with the change in intracellular cyclic AMP (cAMP) levels (●). Average contractility for the eight muscles did not increase after 120 sec. Control cyclic AMP levels are shown at zero time. Error bars denote standard errors of the mean. The theoretical fractional uptake of isoproterenol (- - -) and the experimental fractional uptake (Δ-----Δ) are presented with respect to time. The data points for fractional uptake of isoproterenol represent the average of six muscles. The data on cyclic AMP levels and the positive inotropic response are from Venter *et al.* (2).

These results are not altogether surprising, because of the existing evidence in the literature concerning reduced diffusion rates of drugs into tissue (6, 8, 9); however, the differences themselves are important and are supportive of the "propagation model."

Contractile responses to isoproterenol. Isoproterenol produces a positive inotropic response in a dose-related manner between 1 nM and 1 μM isoproterenol (Fig. 3). The dose-response relationship for isoproterenol in producing positive inotropic responses in isolated cardiac muscle (Fig. 3) can be utilized in conjunction with Figs. 1 and 8 in testing the theoretical diffusion model at a number of isoproterenol concentrations (see Table 1 and DISCUSSION).

Time course of isoproterenol action. The relationship between isoproterenol concentration and the time course of the inotropic response is not a simple one, and not what one might predict a priori for a diffusion-controlled bimolecular reaction. A study of the association rates of adrenergic ligands with cardiac *beta* adrenergic receptors by

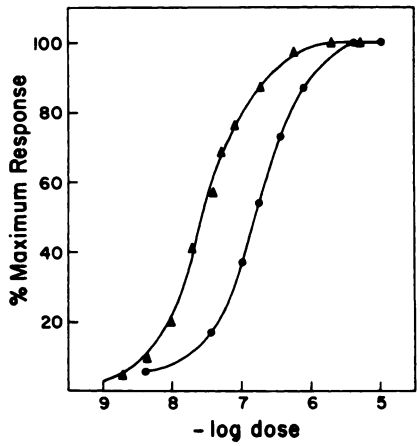

FIG. 3. *Dose-response relationship for dl-isoproterenol (▲) and polymeric isoproterenol (●) in producing positive inotropic responses in isolated cat papillary muscles*

Each data point represents the mean of four experiments. The 100% response was identical for both agents on each muscle tested.

Ghai and Venter[3] illustrates the expected

[3] G. Ghai & J. C. Venter (1978). Submitted for publication.

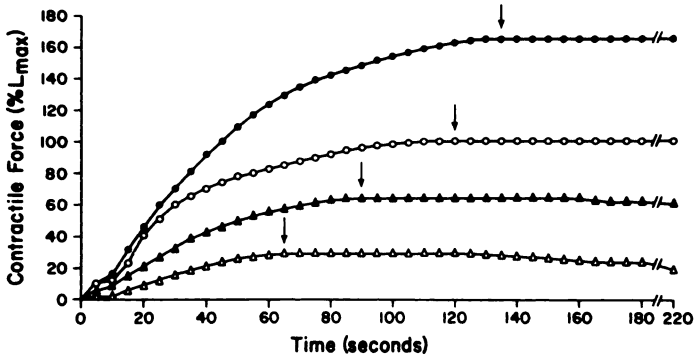

FIG. 4. *Time course of positive inotropic response to increasing concentrations of soluble isoproterenol*
The concentrations of isoproterenol were 10 nM (△), 20 nM (▲), 50 nM (○), and 100 nM (●). The arrows indicate the points in time when steady-state contraction was achieved with each dose. The data shown are from a single representative papillary muscle in six experiments.

outcome for a simple bimolecular reaction; i.e., as the drug concentration is increased with a constant number of *beta* adrenergic receptors, the time to reach equilibrium is substantially shortened.[3] However, when the concentration of isoproterenol is increased from 10 to 100 nM, the time required to achieve a steady-state contractile response is progressively increased from 60 to 130 sec (Fig. 4).

These results indicate that the inotropic response cannot be considered only to be a function of the rate of drug access to the entire muscle or only of a simple bimolecular reaction such as isoproterenol binding to *beta* adrenergic receptors.

Polymer-immobilized isoproterenol diffusion into isolated cat papillary muscles. Isoproterenol can be coupled via an azo linkage to soluble amino acid copolymers of varying molecular weights, with retention of biological activity (1, 3, 4). When these agents are properly purified, their biological activity is due to the covalently coupled form of isoproterenol (1, 4); the high molecular weight polymeric isoproterenol derivatives can therefore be utilized to study sites of catecholamine action by exploiting the diffusion limitations for large molecules. The diffusion coefficient for a 13,000 mol wt isoproterenol polymer (4) can be approximated from the diffusion coefficient for beef heart cytochrome *c* (mol wt 13,370): $D_{20,w} = 1.2 \times 10^{-6}$ cm^2/sec (14). By Eq. 2, applying the tortuosity factor[4] for cat cardiac muscle, the apparent diffusion coefficient can be estimated to be $D' = 5.78 \times 10^{-7}$ cm^2/sec for the high molecular weight polymeric isoproterenol. The theoretical time course for diffusion of the polymeric isoproterenol into the papillary muscles can be described in a manner similar to that for the parent isoproterenol. The calculated extent of polymeric drug diffusion at various times into the 1-mm-diameter papillary muscle is indicated in Fig. 1. According to this theoretical model, it would take on the order of 1 hr for the polymeric isoproterenol to approach an equilibrium concentration by diffusion into the isolated cardiac muscles.

The theoretical fractional uptake of the polymeric isoproterenol derivative is compared in Fig. 5 with the time course of the positive inotropic response to this agent. The addition of 2 μM isoproterenol immobilized on the 12,800 mol wt amino acid copolymer to a muscle bath containing an isometrically contracting cat papillary muscle resulted in increases in contractile force. The time course and magnitude of the positive inotropic responses are depicted in Fig. 5. In response to polymeric isoproterenol, the force of contraction was augmented within approximately 30 sec following drug addition (i.e., on the fourth to sixth contraction). The force continued to increase, reaching a maximum within 180 sec.

[4] Page has calculated that diffusion channels in cat cardiac muscles are identical for compounds from sodium ions to a 60,000 mol wt dextran polymer (10, 11).

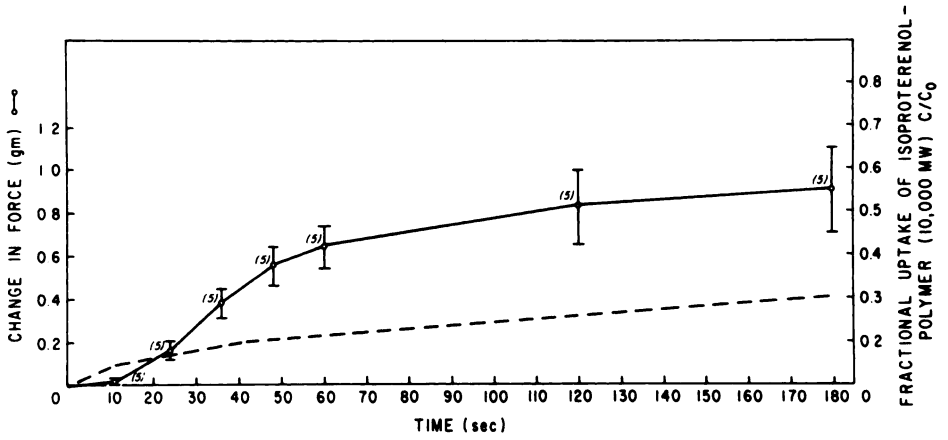

FIG. 5. *Time course of positive inotropic response to 12,800 mol wt polymeric isoproterenol in five isolated cat papillary muscles compared with theoretical fractional uptake of 12,800 mol wt polymer derivative*

When force measurements were continued beyond 180 sec, no further increases were noted. The average changes in isometric force (peak force minus L_{max} force) at 60, 120, and 180 sec were 0.64 ± 0.10, 0.83 ± 0.17, and 0.90 ± 0.2 g.

Figure 5 indicates that when a maximal inotropic response is achieved with the polymeric isoproterenol derivative, the theoretical fractional uptake of drug would be only 30%. As can be extrapolated from Fig. 1, the fractional distribution is also very low.

In order to demonstrate in cardiac muscle the different rates of diffusion of isoproterenol compared with the high molecular weight polymer derivative, drug washout experiments were performed. Isoproterenol or polymeric isoproterenol was added to the baths of contracting isolated papillary muscles, and the positive inotropic response was assessed. Following a 10-min incubation with either drug the muscle baths were drained, refilled, immediately drained, and again refilled with fresh, warmed, oxygenated Krebs' solution, and the decay time of the inotropic response was monitored. When experiments were performed with polymeric isoproterenol, the inotropic responses immediately returned to control levels (within 2 min). In contrast, the response decay time after soluble isoproterenol treatment was at least one order of magnitude longer. The time to reach the control level of contraction averaged more than 20 min (1). A sample experiment illustrating the response decay times is shown in Fig. 6. While the response decay times may represent events in addition to back-diffusion of drug from the muscles, these results can be considered additional evidence that the polymeric isoproterenol acts in the immobilized form and on or near the muscle surface. If the responses to polymeric isoproterenol were due to a dissociated species of isoproterenol, the response decay curves would be similar to those for free isoproterenol (1). These findings also demonstrate that the theoretical diffusion rates for the polymeric isoproterenol derivatives are considerable overestimates. The immediate diminution in response to polymeric isoproterenol suggests that the polymeric drug had not significantly penetrated the muscle during the 10-min incubation period.

The dose-response relationship for the polymeric isoproterenol in producing a positive inotropic response is illustrated in Fig. 3. The shape of the dose-response curve suggests that isoproterenol and polymeric isoproterenol produce the contractile responses by similar mechanisms.

Model for glass bead-immobilized catecholamine action by catecholamine release. Glass beads containing covalently or noncovalently "immobilized" catecholamines are known to liberate catecholamine molecules at constant rates (15). The actual rate of drug release is dependent on many factors, primarily on the amount of material bound to the glass and the exact

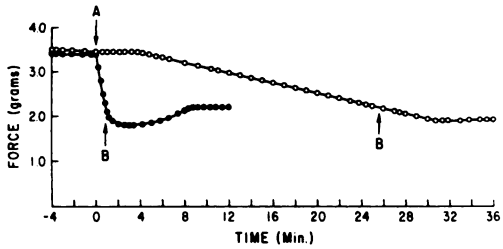

FIG. 6. *Response decay time following drug washout in isolated cat papillary muscle exposed to free isoproterenol or 12,800 mol wt polymeric isoproterenol*

Isolated right ventricular cat papillary muscles were subjected to the indicated compound for 10 min. The papillary muscle bath was drained, refilled with fresh oxygenated Krebs' solution, drained, and again refilled with fresh Krebs' solution warmed to 30°. The force of contraction was monitored until the control inotropic state was obtained. The dose of soluble isoproterenol (0.3 μM) was chosen to match the inotropic response obtained with the polymer-immobilized isoproterenol and was the minimum dose that equaled the response. The following response decay times are shown for *l*-isoproterenol (○) or 12,800 mol wt polymeric *l*-isoproterenol (●): *A*, time of drug washout from the muscle baths; *B*, time at which the contractile force returned to control levels. The inset is a sample polygraph tracing from a polymeric isoproterenol experiment, illustrating the point at which the bath was washed (*A*) and the time when the contractile force returned to control levels (*B*). The papillary muscles were maintained at 30° and stimulated to contract at 12 contractions/min in a well-oxygenated environment.

washing procedures used in glass bead–catecholamine preparation (3). Under standard conditions of binding and acid washing, the glass beads bind approximately 6 pmoles of catecholamine per 300-μm-diameter bead and liberate the catecholamine at a rate of 0.008%/hr at 25–37° (3, 15). Although the exact mechanism of biological action of the catecholamine–glass beads is in question, massive drug leakage has been ruled out experimentally (2, 3, 15). However, it has been unclear whether the minute amounts of catecholamines constantly liberated from the glass beads could attain a sufficient molar concentration in the vicinity of the cardiac muscles to account for the biological activity of these agents. Because of the extremely small number of catecholamine molecules liberated from the glass, experimental conditions that would permit accurate measurements of the disposition of these molecules are not readily attainable. However, estimates can be made of the existing conditions from the diffusion equation. The solution of the equation for a diffusing substance liberated at a continuous rate from a point source is given by Crank (5) as the integral of the diffusion equation for an instantaneous point source with respect to time *t*. Therefore, if the diffusing substance is liberated continuously from a point in an infinite volume at a ϕ per second, the concentration at a distance r from the source at time t is obtained by

$$C = \frac{1}{8(\pi D)^{3/2}} \cdot \int_0^t \phi(t') e^{-r^2/4D(t-t')} \frac{dt'}{(t-t')^{3/2}} \tag{3}$$

If ϕ is constant and equal to q, then

$$C = \frac{q}{4\pi Dr} \operatorname{erf} c \frac{r}{2(Dt)^{1/2}} \tag{4}$$

The concentration under any conditions may be found from tables of the error function (16), where

$$\operatorname{erf} x = \frac{2}{\pi^{1/2}} \int_0^x e^{-u^2} du \tag{5}$$

and erf $cx = 1 - erf\ x$. It should be noted that erf $\infty = 1$.

We know that for a single acid-washed catecholamine–glass bead the catecholamine release rate averages 0.48 fmole/hr (15), or 0.133 amole/sec.[5] Because of the porous nature of the glass beads, more than 90% of the catecholamine is contained in the inner pores of the glass (3). If we assume that free drug diffusion from the glass beads is radial and that only a fraction of the bead surface will be in contact with the tissue (3), it seems reasonable that only a fraction of the released amine will have direct access to the tissue. Solutions for Eq. 4 were therefore obtained for q = 0.0013 and 0.013 amole/sec, where the fraction of released catecholamine with access to the tissue equals 1/100 and 1/10, respectively, of the total

[5] One attomole is equal to 10^{-18} mole.

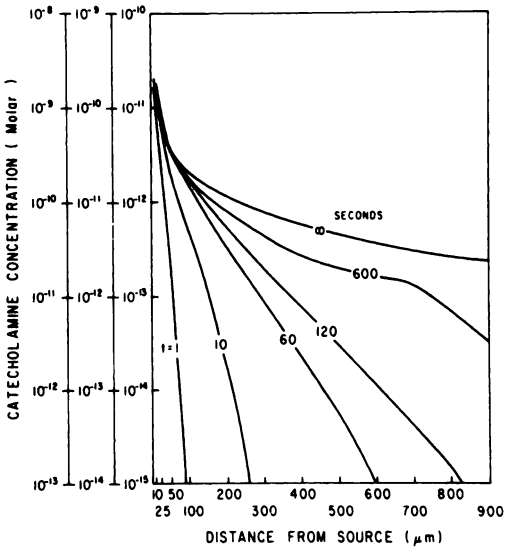

FIG. 7. *Calculated diffusion of catecholamine into cardiac muscle from single catecholamine-glass beads*

The curves represent calculated solutions for the diffusion equation

$$C = \frac{q}{4\pi Dr} \operatorname{erf} c \frac{r}{2(Dt)^{1/2}}$$

for a substance liberated at a continuous rate from a point source into an infinite volume (5). Values given on the ordinate represent (from left to right) concentrations from the solution of Eq. 4 for q = 0.13, 0.013, and 0.0013 amole/sec. The curves indicate the molar concentration of drug obtained for different times from 1 sec to infinity. Values on the abscissa represent the distance into the muscle tissue from the single glass bead source of diffusing material. The values of q given above represent the maximum to the minimum experimentally determined catecholamine release rates from a single catecholamine-glass bead.

amount released from the bead per second. In addition, a solution for Eq. 4 was obtained for q = 0.13 amole/sec. These solutions are represented graphically in Fig. 7 as the molar concentration of catecholamine obtained at different times, from 1 sec to infinity, for various distances into the effector tissue. Therefore the solutions given in Fig. 7 are representative of the experimentally encountered release rates from catecholamine-glass beads.[6] From Fig. 7 it can be seen that the catecholamine concentration falls rapidly within even very small distances from the bead. However, for distances of less than 10 μm the concentration asymptotically approaches the concentration of the source. Because the glass bead presents a surface area, even at every small distances from its surface it can no longer be considered a point source of drug. However, within these limits the molar concentration of released drug is adequate to explain the biological action of the beads only in a tissue with the ability to propagate the response from the site of local stimulation throughout the remainder of the tissue; the extent of drug diffusion into the muscle tissue (Fig. 7) is not adequate to explain the magnitude of a nonpropagated inotropic response.

[6] The maximal release rate obtained for nonwashed catecholamine-glass bead preparations is on the order of 0.3%/hr, which for one glass bead represents q = 0.5 amole/sec.

DISCUSSION

Models for sites of catecholamine action. The hypothesis that the response to glass bead-immobilized and polymer-immobilized catecholamines is propagated is supported by the diffusion models for these agents. The similarity between time courses for maximal positive inotropic responses in the cat papillary muscles suggests similar mechanisms of tissue activation for both the soluble and immobilized forms of isoproterenol (Figs. 2 and 5) (2).

In order to develop models for isoproterenol action, it is important to consider the geometry of the cylindrical papillary muscle. In a cylinder the majority of the volume is nearest the cylinder surface. A one-half cross section of a 1-mm-diameter cat papillary muscle divided into five 0.1-mm radial zones is illustrated in Fig. 8. Zone A is the outermost section of the muscle, and zone E is the inner core. The various zones can be described in terms of the percentage of the total muscle volume and therefore presumably the percentage of the total cell complement. Zone A represents 36.3%; zone B, 27.8%; zone C, 20%; zone D, 11.9%; and the inner core, zone E, only 4% of the total muscle volume (Fig. 8).

The inotropic and cyclic AMP responses can be correlated with the amount of tissue activated by various concentrations of isoproterenol. For soluble isoproterenol, Fig. 2 shows that the cyclic AMP response

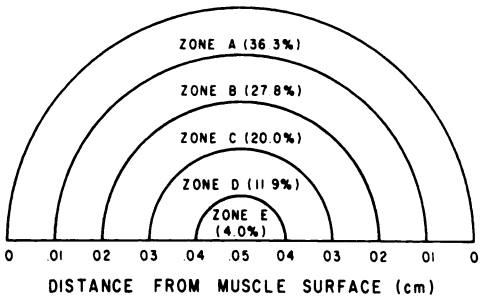

FIG. 8. *One-half cross section of a 1-mm-diameter cat papillary muscle*

The numbers in parentheses represent the percentage of the total cardiac muscle volume contained in the indicated radial zones.

reaches a maximum within 15 sec of isoproterenol addition to the muscle bath. It can be estimated from Fig. 1 that at 15 sec a concentration gradient would exist across zone A from 1 μM in the bath and on the outer muscle surface to 0.36 μM at the interface of zones A and B. The concentration across zone B falls even faster, decreasing to only 60 nM at the borders of zones B and B. In zone C the concentration would be less than 1 nM. Therefore from Figs. 1 and 2 it can be estimated that a maximum cyclic AMP tissue response can be effectively achieved by a gradient of isoproterenol from 1 μM to 60 nM over approximately 64% of the cells. These considerations, along with the observation that cyclic AMP does not continue to increase beyond 15 sec (Fig. 2) (2), suggest that the cyclic AMP increases measured in cardiac muscle may not reflect the conditions throughout the muscle tissue. It is possible that cyclic AMP increases in cardiac muscle, under these experimental conditions, could best be described as a wave of cyclic AMP moving through the radius of the muscle, with the rate of cyclic AMP metabolism equal to the rate of continued synthesis, although this awaits experimental documentation. In isolated cells in tissue culture cyclic AMP increases can be relatively transient phenomena, whereas in isolated cardiac tissue cyclic AMP levels, once achieved, remain relatively constant for at least 3 min (2). If cyclic AMP increases are causally related to cardiac positive inotropic responses, the above mechanism would suggest that cyclic AMP could act only as a trigger for contractile events; continued cyclic AMP elevations in a given cell would not have any additional effects on contractile function.

A second explanation for the cyclic AMP increase would be that the cyclic AMP response is propagated in a manner similar to that proposed for the inotropic responses. However, the results obtained with the glass bead-immobilized isoproterenol and polymeric isoproterenol have demonstrated that nearly maximal inotropic responses can be obtained under conditions in which no detectable changes occur in the concentration of cyclic AMP. This strongly suggests that cyclic AMP increases are not propagated. The cyclic AMP time course makes it difficult to reconcile much of the data used in support of a causal relationship between cyclic AMP and cardiac contractility.

For soluble isoproterenol interreacting with papillary muscles under non-steady-state conditions, not all the contractile events can be completely explained by isoproterenol interreacting with each cell involved in the contractile response and without some response propagation occurring. By obtaining the steady-state response to a given isoproterenol concentration from the dose-response curve for isoproterenol (Fig. 3) and the fractional concentration for each tissue zone with time from Fig. 1, it is possible to estimate the theoretical fractional contribution of each cardiac muscle zone to the contractile steady-state response. The predicted response level can then be compared with the experimentally observed response as a means of further testing the diffusion vs. the propagation hypothesis. This discussion will continue to use the theoretical model (Fig. 1) even though, as shown in Fig. 2, the actual isoproterenol uptake is substantially slower than predicted from the diffusion equation.

Table 1 compares contractile levels predicted from the diffusion equation with experimental observations. The times chosen in Table 1 at which to compare results are taken from a series of experiments on the time course of the inotropic response to various isoproterenol concentrations (Fig. 4).

From Table 1, with isoproterenol concentrations of 1 μM (or higher), the theoretical diffusion model appears to predict the contractile level obtained experimentally; how-

TABLE 1

Comparison of contractile responses predicted by diffusion equation for isoproterenol with experimental observations

Muscle zone[a]	Theoretical contribution to total contractile response[b] at various bath concentrations				
	1 μM	100 nM	50 nM	20 nM	10 nM
cm (%)	%	%	%	%	%
A: 0–0.01 (36.3)	35.6	35.4	35.1	31.8	31.6
B: 0.01–0.02 (27.8)	27.2	24.7	23.8	20.8	14.5
C: 0.02–0.03 (20.0)	19.2	15.7	13.6	10.5	4.3
D: 0.03–0.04 (11.9)	11.3	8.8	7.5	5.0	1.0
E: 0.04–0.05 (4.0)	3.7	2.8	2.1	1.2	0
Predicted contraction (%)[c]	97.0	87.4	82.1	69.3	51.4
Experimentally observed (%)[d]	100	100	100	100	100
Time to obtain 100% response (sec)[e]	120	120	120	100	60

[a] Tissue band in centimeters; numbers in parentheses are percentages of cylinder volume (from Fig. 8).

[b] Calculated from the diffusion model (Fig. 1) with equilibrium contraction levels obtained from the isoproterenol dose-response curve. The maximum response (Fig. 3) was normalized to 100% for each concentration shown above.

[c] Sum of the calculated percentage contributions for zones A–E.

[d] Experimentally observed values represent the maximal contractile level achieved with the indicated isoproterenol bath concentration. The interrelationships among different isoproterenol concentrations can be obtained from the dose-response curve (Fig. 3).

[e] Average times subsequent to isoproterenol addition to the muscle bath required to achieve 100% response for the indicated isoproterenol concentration (e.g., refer to Fig. 4).

ever, for all drug concentrations below 1 μM, the disparity between predicted and observed increases markedly. The effect of drug concentration on the apparent predictability is not surprising, given the sigmoidal nature of the dose-response relationship (Fig. 3). With 1 μM or higher concentrations of isoproterenol, which are in the nonlinear portion of the dose-response curve, the diffusion model cannot be adequately tested. Changes in isoproterenol concentrations of greater than one order of magnitude are required to see a substantial drop in the net contractile response (Fig. 3). With isoproterenol concentrations from the linear portions of the dose-response curve, the diffusion model falls substantially short of apparent predictive value (Table 1). With 10 and 20 nM isoproterenol the diffusion model predicts only 51% and 69% of the actual contractile levels achieved, respectively. Even if the drug diffusion completely paralleled the contractile response, the exact relationship between diffusion and contraction would depend upon whether the rate-limiting step in the muscle activation process is due to drug-receptor interactions or to postreceptor events, including cell-to-cell transfer of information. The near identity of the time courses of contractility development in response to maximum effective doses of soluble isoproterenol (Fig. 2), polymeric isoproterenol (Fig. 5), and glass bead-immobilized isoproterenol (2), as well as the disparity in the contractile time courses to high vs. low doses of isoproterenol, suggests that the rate-limiting step in increased contractility cannot be drug diffusion or drug-receptor interactions, but is a postreceptor event. Possible postreceptor events might include slow rates of calcium accumulation (a Bowditch staircase-type phenomenon). The processes of muscle activation therefore must occur very early in the diffusion process. This implies that following the activation of a certain portion of cardiac tissue, an inotropic response level will be achieved whether or not further drug diffusion occurs. For example, if isoproterenol diffusion could be effectively stopped after a given period without affecting other muscle events, the time of onset of the inotropic response and the contractile level achieved from propagation might be indistinguishable from those observed with continued drug diffusion. These conditions are essentially those obtained with the polymeric isoproterenol (Fig. 5) (4).

An analysis similar to that in Table 1 can be performed with the diffusion model (Fig. 1) for the polymeric isoproterenol and the dose-response data (Fig. 3). Table 2 is such an application. One can readily see from

this table the marked disparity between the theoretical and observed values. The diffusion model substantially underestimates the contractile responses. These data, together with the drug washout experiments (Fig. 6), indicate that the diffusion model overestimates the rate of polymer uptake into the cardiac muscle and that the contractile responses obtained cannot be explained on the basis of drug diffusion.

These data and discussions illustrate that for soluble, polymeric, or glass bead-immobilized catecholamine action on isolated cardiac muscle the events occurring in the superficial muscle layers can control the contractile events of the entire cardiac tissue without the prerequisite of continued drug diffusion.

The exact mechanism(s) involved in the propagation response is still unknown.

TABLE 2

Comparison of contractile responses predicted by diffusion equation for polymer-immobilized isoproterenol with experimental observations

Muscle zone[a]	Theoretical contribution to total contractile response[b] at various bath concentrations			
	4 μM	1 μM	400 nM	100 nM
cm(%)	%	%	%	%
A: 0–0.01 (36.3)	35.6	34.6	28.4	27.7
B: 0.01–0.02 (27.8)	24.5	19.2	14.1	9.8
C: 0.02–0.03 (20.0)	12.4	6.6	3.6	3.7
D: 0.03–0.04 (11.9)	3.0	1.3	1.1	0.96
E: 0.04–0.05 (4.0)	0	0	0	0
Predicted contraction (%)[c]	75.5	61.7	49.6	42.2
Experimentally observed (%)[d]	100	100	100	100

[a] Tissue band in centimeters; numbers in parentheses are percentages of cylinder volume (from Fig. 8).

[b] Calculated from diffusion model (Fig. 1) for 129 sec with equilibrium contraction levels obtained from the polymeric isoproterenol dose-response curve (Fig. 3). The maximum response (Fig. 3) was normalized to 100% for each concentration.

[c] Sum of the calculated percentage contributions for Zones A–E.

[d] Experimentally observed values represent the peak contractile level achieved with the indicated polymeric isoproterenol bath concentration. The interrelationships among different polymeric-isoproterenol concentrations can be obtained from the dose-response curve (Fig. 3).

Speculation as to possibilities may include cell-to-cell transfer of chemical information, perhaps in the form of calcium diffusing across tight junctions; a depolarization spreading throughout the tissue, acting to "open" more calcium channels; or a mechanical perturbation due to increased cardiac cell contraction in areas of catecholamine stimulation. As suggested (3), a threshold amount of tissue may need to be activated beyond a threshold level for propagation to occur, with the activation center for a volume of muscle physically being any portion of the muscle mass in contact with drug. In intact hearts, the muscle activation would not necessarily need to occur in the superficial layers. For example, the area surrounding an adrenergic nerve varicosity could become an activator, initiating a response propagated throughout a certain volume.

Bevan and Török (9) found that norepinephrine diffusion in blood vessels is 1/10 of that in water. These results, together with a rapid phase of blood vessel contraction, led Bevan and Waterson (17) to propose rapid myogenic propagation through the vessel wall unaccounted for by drug diffusion. The results presented here indicate that catecholamine diffusion in cardiac muscle is substantially slower than in water and that similar myogenic propagation occurs.

With drugs immobilized on solid supports such as glass beads, certain additional considerations are necessary in order to understand the sites and modes of action of these agents. If a solid is placed in a well-stirred liquid, a stationary layer of fluid will envelop the solid; therefore interactions with the solid must occur by diffusion through this layer. Both the glass beads and the cardiac muscle will be surrounded by stationary layers of fluid. The placement of a glass bead on a muscle surface will result in overlapping of these stationary layers. The stationary layer for isolated muscle preparations has been experimentally determined to be on the order of 70–100 μm thick (18), and for simpler systems, on the order of 18–52 μm thick (19). The amount of stirring in the solution cannot directly affect the stationary layer at the junction of

the bead and the muscle surface, and therefore within this junction the principal control of drug molecule movement will be diffusion. Within the zone 100 Å from the glass bead surface, catecholamine molecules are covalently attached to side arms 16–32 Å long. This same unstirred zone will contain free catecholamine molecules, the concentration possibly approaching or exceeding 10 μM (Fig. 7). When the glass beads are brought into contact with a tissue it can be essentially impossible to distinguish between covalently bound and soluble drug molecules as the active species, owing to the high molar concentration in this microzone. We can assume by extrapolation from structure-activity relationships of soluble azo-substituted catecholamines (3, 15) and from the activity of polymeric azocatecholamines (1, 4) that azo-substituted catecholamines on glass beads are in a biologically active conformation. The biological effects ensuing from the glass bead–catecholamines could theoretically be attributed to either the soluble or the immobilized drug or a combination of the two. However, for these same reasons, as long as essentially any active drug molecules are released from the surface of the beads, the solid-state immobilized drugs cannot conclusively be proven to act while covalently coupled. The same arguments further support the concept of a very localized catecholamine stimulus with the glass bead-immobilized catecholamines, either while immobilized or within the microlayer that surrounds solid objects in solution, and are consistent with the inotropic propagation hypothesis.

The localized action of glass bead–catecholamines suggested a propagated response mechanism. The diffusion considerations presented here argue that under non-steady-state conditions and heterogeneous drug distribution, soluble catecholamines also exert their effects on cardiac muscle by response propagation. The suggested mechanisms for soluble, polymeric, and glass bead-immobilized catecholamines have particular significance in attempts to correlate cellular biochemical events with tissue contractile responses. The diffusion arguments suggest that the present assumptions concerning drug-receptor interactions and contractile events may be oversimplifications of the events that occur under experimental conditions both *in vivo* and *in vitro*.

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