Biologically Active Catecholamines Covalently Bound to Glass Beads
(cyclic AMP/tissue culture/immobilized catecholamines/canine hearts)

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ABSTRACT Catecholamines bound covalently to glass beads have been found to have biological activity in several systems. Experimental evidence has been found that immobilized epinephrine and isoproterenol accelerate the heart rate in dogs, chick embryo, and chick heart cells grown in culture, whereas immobilized propanolol results in a decrease in heart rate. Isoprotorenol bound to glass beads has also been shown to markedly increase the level of adenosine 3':5'-cyclic monophosphoric acid in glial cells. The effects of the immobilized catecholamines are of longer duration than when the compounds are administered in solution. The present data indicate that the compounds are exerting their action when bound to the beads.

The effects of catecholamines on the different tissues and organs can be explained through their action via α- and β-receptors. This action is directly on sympathetic effector cells and follows interaction with specific receptor sites that are thought to be in the cell membrane (1). The most recent information indicates that the adrenergic β-receptor is probably an integral component of the adenylcyclase system in those tissues where β-receptors occur, and that β-adrenergic effects in general result from an increase in the intracellular level of adenosine 3':5'-cyclic monophosphoric acid (cAMP) (2).

Epinephrine and isoproterenol are known to increase heart rate and contractile force, and they can induce automaticity in quiescent cells by stimulating the β receptors of the myocardium, the cells of the pacemaker, and conducting tissues. Conversely, propanolol hydrochloride is a β adrenergic blocking agent and reduces the heart rate and contractile force (1).

Recently, it has been shown that some enzymes and small molecules retain activity when immobilized covalently on the surface of glass beads (3). We have obtained evidence that epinephrine, norepinephrine, isoproterenol, and propanolol remain biologically active while covalently coupled through a diazo coupling to porous glass particles. Biological activity has been shown by four methods: (i) beating heart cells grown in tissue culture; (ii) whole chicken embryonic hearts; (iii) hearts in open-chest anesthetized dogs; and (iv) stimulation of cAMP formation in glial tumor cells grown in vitro.

MATERIALS AND METHODS

The hydrochloride salts of L-epinephrine (Sigma), DL-isoproterenol (Sigma), propanolol (Ayerst Laboratories) and L-norepinephrine (Sigma) were covalently attached to alyl amine glass (Corning) by a procedure similar to that of Weetall (4). The procedure used for binding epinephrine was as follows: Aryn amine glass, 0.50 g, was placed in 10 ml of 2 N HCl with 0.125 g of NaNO₂ (Baker) and allowed to stand at 0° under vacuum for 20 min. The activated glass was then filtered and washed with 200–300 ml of ice water and placed in 0.05 M Tris buffer (pH 7.5) containing l-epinephrine (usually 10 mg) at 0°. The reaction was allowed to stand for 1 hr without exposure to visible light. The red colored glass was filtered and washed with 1 liter of distilled water. The glass was stored as a moist cake at 0°. The glass particles were washed before use, in order to insure the absence of free epinephrine. A similar procedure was used for propanolol, norepinephrine and dl-isoproterenol. A possible structure for the bound epinephrine is given in Fig. 1.

D-[7-3H]epinephrine, D-bitartrate (New England Nuclear; 13 Ci/mmol) was diluted 400 to 1 with unlabeled epinephrine. Exactly 0.139 g of alyl amine glass, with 0.035 g of NaNO₂, was reacted with 5.64 mg of epinephrine. 5 ml Of the Dl-epinephrine (1.36 × 10⁻¹⁴ mol) exhibited 3 × 10⁴ cpm.

Single beating heart cells from chicken embryos of from 5–18 days were obtained in culture at a density of 4000 cells/mm². Cells were cultured in 30-ml Falcon flasks in accordance with the dissection and disaggregation procedures described by DeHaan (5). Cells were cultured in a chemically defined nutrient medium SM20 (6). Cultures were incubated at 37°; the medium was changed every 24 hr. Cells used for this purpose were incubated at least 5 days after culture to let the beating normalize. Details of this heart cell preparation will be presented in a later publication.

Whole embryonic hearts from embryos of various ages were also used. Hearts were removed as above and placed in medium that was held at constant temperatures on a heated microscope stage. Rates of contraction of embryonic hearts and cells were recorded on a storage oscilloscope with

![Diagram](http://example.com/diagram.png)

**Fig. 1.** Proposed structure of L-epinephrine linked to glass beads, “epinephrine-on-glass.” It is possible that the linkage may be through either the 5 or 6 position.
a Grass DPAB Preamplifier and a Grass Regulated Power Supply.

Dogs were anesthetized with sodium thiamylal (25 mg/kg) and then subjected to artificial respiration. A right thoracotomy was performed through the fourth intercostal space, and the heart was suspended in a pericardial cradle. The epicardium over the right atrium was carefully removed. Electrocardiograms and blood pressure were continuously recorded on an oscillographic recorder (Clevite 200 Brush Instruments). The glass beads, with the various drugs attached, were successively applied to the area of the sinus node.

Gial cells, clone C6, were those derived by Benda et al. (7) and supplied by Professor Gordon Sato. The cells were in monolayer and were adapted to suspension culture. The cells were grown in Flow Laboratories' Spinner Modified Minimal Essential Medium, supplemented with 8% fetal calf serum, 2% fetal calf serum. A double buffering system of 6 g/liter HEPES (N-2-hydroxy-ethylpipеразин-N'-2-ethanesulfonic acid) (CalBioChem) and 2 g/liter sodium bicarbonate was used in lieu of CO₂. The medium also contained 100 U/ml penicillin G and 50 μg/ml streptomycin sulfate. The C6 cells have an approximate doubling time of 16 hr.

For purposes of the experimental study, 2 × 10⁷ gial cells were suspended in 50 ml of growth medium containing 1 nM theophylline and incubated for 60 min at 37°C. Compounds to be tested were then added, and incubation was continued for another 15 min. Immobilized compounds on glass were tested as follows: Cells were centrifuged at low speed for 5 min; the medium was then aspirated, leaving the pelleted cells to

which the glass beads were added. The cells and beads were gently mixed and incubated for 15 min. 1 ml of 5% Cl₃COOH was added to the pelleted cells; this mixture was then sonicated for 1 min, centrifuged at high speed for 5 min, and the supernatant was removed and extracted with ether. Cyclic AMP assays were done as described (8). After sonication, the precipitate was assayed for protein by the method of Lowry et al. (9).

RESULTS

Effects of "catecholamines-on-glass" on embryonic chick hearts

Addition of the "isoproterenol-on-glass" to a 15-day old chick embryo heart resulted in an increase in heart rate as shown in Fig. 2. The increased rate was sustained for 20 min and decreased to 10 beats/min when the "isoproterenol-on-glass" was removed. In a similar manner, addition of 20 "isoproterenol-on-glass" beads to a culture of beating chick embryo heart cells resulted in an increase in heart rate from 60 to 89 beats/min. Other cells surrounding the experimental group, but not in contact with the beads, showed no increase in the rate of contraction. Glass beads not reacted with catecholamines had no effect upon the chick embryo heart rate (Fig. 3). Antithetical to the increases in heart rate noted with epinephrine, norepinephrine, and isoproterenol, we were able to demonstrate that "propranolol-on-glass" induces a slower heart rate in the chick embryo. Fig. 4 shows the results of a typical experiment with immobilized propranolol. The embryo heart rate was constant for 30 min before the addition of "propranolol-on-glass." The heart rate decreased from 59 to 36 beats/min when the beads were applied, and remained at the decreased level until the beads were removed at which time the rate increased to 50 beats/min, which was close to the untreated rate.

In order to demonstrate that contact between chick embryo hearts and the "catecholamines-on-glass" was essential for the changes in heart rate, two 12-day chick embryo hearts were placed in medium SM20 1 inch apart. Hearts A and B had base rates of 63 and 59 beats/min, respectively. Addition of "epinephrine-on-glass" beads to heart B increased the rate to 78 beats/min. No change in rate was noted for heart A. "Epinephrine-on-glass" (120 mg) was added to the SM20 medium, but it was not in contact with either heart. No variations in the heart rates were apparent; however, epinephrine solution added to the medium (1 × 10⁻⁴ M) resulted in an increase in both chick embryo heart beat rates. Necessity of contact between the "catecholamines-on-glass" and the embryonic hearts was also demon-

**TABLE 1. Effect of catecholamines and immobilized isoproterenol on cAMP concentrations of rat glioma, clone C6**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pmol/10⁶ Cells</th>
<th>pmol/mg of Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O Control</td>
<td>0.9</td>
<td>6</td>
</tr>
<tr>
<td>0.1 mM Epinephrine</td>
<td>64</td>
<td>400</td>
</tr>
<tr>
<td>0.1 mM Isoproterenol</td>
<td>100</td>
<td>625</td>
</tr>
<tr>
<td>&quot;Untreated glass beads&quot;</td>
<td>0.9</td>
<td>6</td>
</tr>
<tr>
<td>&quot;Isoproterenol-on-glass&quot;</td>
<td>212</td>
<td>1325</td>
</tr>
</tbody>
</table>

2 × 10⁷ C6 suspension cells were treated with the above compounds as outlined in Methods. 63 mg Of "isoproterenol-on-glass" beads represents about 0.3 mg of isoproterenol. 0.1 mM isoproterenol in 50 ml represents 1 mg of isoproterenol.
strated by attaching six "epinephrine-on-glass" beads to a small glass rod on the end of a micromanipulator. It was possible to touch the beads to the beating hearts and then remove them; this resulted in a dramatic on-off switch. When the beads were in contact with the heart, that portion of the heart increased in rate and velocity of contraction. If the area of the sino-atrial node was touched, the rate of beating of the whole heart increased. As soon as the beads were removed the hearts resumed their normal rate of contraction. As long as the beads were in contact with the hearts, the rates of contraction remained elevated. Control glass beads that had not been reacted with catecholamines had no effect on the hearts.

To rule out the possibility that the catecholamines were being absorbed by the glass and then released from the beads when brought in contact with the hearts, we allowed glass beads unreacted with catecholamines to stand overnight in a solution of epinephrine (0.67 mg/ml). They were washed in the usual manner. When they were applied to chick embryonic hearts there was no alteration in heart rate.

Effects of "catecholamines-on-glass" on canine hearts

Fig. 5 shows the results of repeated additions of "isoproterenol-on-glass" to the sino-atrial node area of a canine heart in vivo. The first response to the "isoproterenol-on-glass" occurred within 15 sec, whereas repeated applications resulted in slower responses. The heart rate remained elevated as long as the beads were in contact with the nodal area, usually for 10 min. When the beads were removed the heart rate dropped to a new base rate. Fig. 6 shows the results of a similar experiment. After two successive applications of "isoproterenol-on-glass," "propranolol-on-glass" was added to the sinus node of the canine heart. This resulted in a decrease in heart rate from 143 to 114 beats/min. After 10 min, the beads were removed and the heart rate returned to 140 beats/min. The heart then responded immediately to "isoproterenol-on-glass" with an increased rate of heart beat.

Effect of "isoproterenol-on-glass" and other compounds on the cAMP concentration of glial cells clone C6

The effects of "isoproterenol-on-glass" and other compounds on the intracellular cAMP concentrations of rat glial tumor cells clone C6, are shown in Table 1. The basal level of cAMP in clone C6 is similar to that reported (10) for this clone, and similar to the basal levels in other tissues (2). Concentrations of cAMP increased more than 60-fold in the presence of epinephrine or isoproterenol and greater than 200-fold in the presence of "isoproterenol-on-glass" when 2 × 10⁷ suspension cells were incubated 15 min with these compounds. Untreated glass beads had no effect on the cAMP levels.

Rates of displacement of epinephrine from glass beads

To demonstrate that epinephrine was not coming off the glass beads, we immobilized [³H]epinephrine on the porous surface as described in Methods. [³H]Epinephrine beads with 111,973 cpm [toluene-2,5-diphenyloxazole (PPO)-Triton X-100 medium] were placed in 10 ml of distilled water and, at various time intervals, 20-μl aliquots were withdrawn and counted. Over a 30-min period, no detectable differences between background radiation and that of the aqueous

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**Fig. 4.** (A) 15-day embryonic chicken heart rate (54 beats/min) before administration of "propranolol-on-glass." (B) Heart rate (36 beats/min) after application of "propranolol-on-glass."

**Fig. 5.** An example of the influence of "isoproterenol-on-glass" on heart rate in the dog. The base heart rates are indicated by the lower levels on the graph. (A) denotes times when "isoproterenol-on-glass" was applied to the sino-atrial node of the dog heart. (B) denotes the times when the glass beads were removed from the hearts.

**Fig. 6.** An example of the influence of "isoproterenol- and propranolol-on-glass" on heart rates in the dog. (A) denotes the point at which "isoproterenol-on-glass" was applied to the sino-atrial node of the dog heart; (B) represents points at which the glass beads were removed from the heart; (C) denotes the point at which "propranolol-on-glass" was applied to the sino-atrial node of the heart.
medium was detectable (Fig. 7). After 76.8 hr, 167 cpm appeared in the aqueous solution, which represents \(1.47 \times 10^{-10}\) mol/ml of epinephrine. "Epinephrine-on-glass" was allowed to stand 18 hr in 20 ml of culture medium SM20; addition of this treated medium to a culture of beating heart cells had no effect on the rate of contraction. Epinephrine hydrochloride in SM20 medium does not decompose under the same conditions at concentrations of \(1 \times 10^{-4}\) M; addition of this epinephrine-containing medium to chick heart cells resulted in an increased rate of contraction.

**DISCUSSION**

The lower levels at which epinephrine produces responses in the chick heart are reported to be \(2 \times 10^{-10} M\) (11) to \(2 \times 10^{-4} M\) (12). In the present study, with chick embryo hearts 5-18 days old, a minimum recordable response was noted with \(1 \times 10^{-9}\) M epinephrine. Studies with labeled \(^3\)H]epinephrine covalently bound to the glass showed no liberation of epinephrine in a 30-min period, and only \(1.47 \times 10^{-10}\) mol/ml appeared in the aqueous solution after 76.8 hr. Although it is possible that epinephrine may be very slowly released from the glass, we believe that the levels of release are well below the minimum response dose.

Our evidence seems to point to the fact that epinephrine bound to the glass beads is responsible for the changes in heart rate. It should be pointed out, however, that enzymes present on the cell surface may result in the liberation of the covalently attached epinephrine. We have examined the SM20 medium after placing labeled \(^3\)H]epinephrine-on-glass" on chick embryo hearts and could find no radioactivity associated with the SM20 medium. These findings do not rule out the fact that epinephrine may remain associated with the cell membranes and does not appear in the medium, nor does it rule out the possibility of local concentration effects as a mode of action of the catecholamines on the membranes.

The immobilized catecholamines, epinephrine and isoproterenol, produce an increase in the heart rates of dogs and chick embryo hearts, as well as in individual beating heart cells. In all cases, except as noted in Fig. 5, the increase in heart rate is instantaneous and remains unchanged until the glass beads are removed. This is illustrated in Figs. 5 and 6, where the dog heart rates are maintained elevated for periods of at least 10 min. This sustained response would not be anticipated if it were the result of free isoproterenol (not bound to glass). The intravenous administration of isoproterenol in a dose of 0.1 μg/kg increases the heart rate to a peak reached in 15-30 sec. 50% of the effect is gone in 60 sec and 90% in 3 min. In addition to the heart rate being sustained for long periods of time, no change in arterial pressure was noted, which would not be expected if isoproterenol was absorbed and acting on the myocardium and peripheral arteries. In contrast to the increases in heart rate, "propranolol-on-glass," a β blocking agent, causes a decrease in both chick embryo and dog heart rates (Figs. 4 and 6). The decrease in heart rate was also maintained only for periods in which the beads were in contact with the hearts, which would not be expected with free propranolol, considering the long half-life of this substance in vivo. The fact that decreases as well as increases in heart rate can be brought about with catecholamines covalently bound to glass beads would indicate quite strongly that these changes are a result of an interaction with the cell's outer membrane. The striking increases in the intracellular concentrations of cAMP upon addition of "isoproterenol-on-glass" to rat glial tumor cells in culture is another example of the biological activity of the immobilized compounds. The increases in the intracellular concentration of cAMP are in the same range found by Gilman and Nirenberg (10) for similar concentrations of catecholamines with the C6 clone. It is of interest to note that the increased levels of the cAMP with the "isoproterenol-on-glass" are double those levels obtained with 0.1-mM doses of the isoproterenol in solution. All our experiments point to the conclusion that these catecholamines are biologically active while remaining covalently bound to the glass.

It is not, therefore, unreasonable to conjecture that this technique presents a possible new method for administration of various drugs to specific cells. We are exploring the potential clinical uses of immobilized catecholamines on hearts, on smooth muscles such as in the intestine, and on sphincter-type control centers. For application we have constructed a glass "wand" with immobilized catecholamines that can raise or lower the heart rates in dogs, depending upon the nature of the drug. We are working on the immobilization of other hormones, steroid and protein, and various other drugs that may act through membranes to see if they maintain biological activity. The immobilized catecholamines might present a totally new and precise method for mapping responses in specific areas of the brain. Numerous immobilized enzymes have been shown to maintain activity while bound to glass beads. In this connection, we have implanted "glucose oxidase-on-glass" in a mouse peritoneal cavity and after 2 days removed the beads. No significant loss of activity was found with the recovered beads. It is of interest to note that ACTH (13) and, more recently, insulin (14) covalently bound to Sepharose have been shown to produce effects on cells in culture. Immobilized compounds appear not only to have promise of potential clinical use but may prove to be useful tools in elucidation of the mechanism by which compounds exert their specific action on membranes.

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