EFFECT OF CATECHOLAMINES IN RESTORING
THE BEATING OF CULTURED RAT HEART
CELLS TREATED WITH RESERPINE

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Abstract—The effects of catecholamines on the beating of rat heart cells in monolayer culture were examined. On adding $2 \times 10^{-5}$ M reserpine to the culture medium, the beating of heart cells stopped and as long as the cells were cultured in medium containing reserpine, beating did not resume to that observed before treatment with reserpine. When placed in a fresh medium without reserpine, some cells resumed beating after a lag period of 1 to 2 hr. Addition of $10^{-5}$ M epinephrine or dopamine to the fresh medium reduced the lag period and the beating started within 5 to 20 min. Dopa had no effect on the beating. Propranolol, a β-adrenergic blocking agent, delayed the effect of norepinephrine. Acetylcholine ($10^{-7}$–$10^{-5}$ M) or carbachol ($10^{-5}$ M) had no effect. From these results, it is suggested that catecholamines evoke the contraction of cultured heart cells, and that the rhythmical beat may be due to repeated cycles of depletion and replenishment of catecholamines.

Cultured rat heart cells beat spontaneously and rhythmically. When cells come in contact or are grown as monolayer sheets they beat synchronously (1, 2), however, the substance which evokes this autonomic contraction is unknown.

Catecholamines were shown to accelerate the rate of beating of perfused heart (3). A chronotropic effect of epinephrine was observed by Wollenberger et al. (4) in cell cultures of embryonic chick heart, and by Boder et al. (5) in those of newborn mouse heart. Sperelakis and Lehmkühl (6) did not succeed in showing any effect of epinephrine on the membrane potential of cultured chick embryo heart cells. On the other hand, the cholinergic compound, acetylcholine, was shown to depress the beating of cultured rat heart cells (2).

This paper reports studies of the effects of various compounds on the beating of cultured monolayer sheets of rat myocardial cells. It was found that reserpine stopped the beating while catecholamines and dopamine, one of precursors, reversed this effect.

MATERIALS AND METHODS

Animals and heart cell culture

One to four day old Sprague-Dawley strain rats were used. About 10 hearts were excised aseptically, placed in ice cold saline-phosphate buffer (Ca$^{++}$- and Mg$^{++}$-free) and

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dissociated into single cells by a modification of the method of Harary and Farley (7) as follows: The hearts were cut into small pieces using scissors, transferred to a 50 ml Erlenmyer flask containing 10 ml of 0.125% trypsin solution and incubated at 37°C for 20 min with shaking. The medium was decanted, fresh trypsin solution was added to the tissue, and the mixture was incubated for another 20 min. The resulting suspension of dispersed cells was decanted and centrifuged at 150 g for 5 min. The precipitated cells were washed with Hank's salt solution and suspended in a culture medium, consisting of 30% Hank's solution, 50% Eagle's MEM and 20% calf serum. Undispersed heart tissue was again subjected to the same treatment. Over 80% of cells were viable when counted under microscope using nigrosin. About 3 x 10^6 cells were placed on a gelatin-coated petri dish (3.5 cm diameter) prepared by the method of Goshima (8) or on a plastic dish (Falcon plastic, 3.5 cm diameter). The dishes were incubated under an atmosphere of 5% CO₂ and 95% O₂ at 37°C for 3 to 5 days to obtain a monolayer sheet of heart cells, beating synchronously (Fig. 1). The culture medium was changed every three days.

Estimation of the beating rate

The number of beats was counted at room temp. over a period of several min in one field in each dish, under a phase-contrast microscope at 100-fold magnification. The rate was expressed as beats per min.

Addition of reagents

l-Norepinephrine, l-epinephrine and l-dopa were dissolved in diluted hydrochloric acid to obtain neutral solutions and l-dopamine HCl and propranolol were dissolved in water. To minimize dilution of the culture medium, solutions of these compounds were added in a volume of less than 2% of that of the culture medium. Acetylcholine and carbachol were added to the medium at a concentration of 10^-7-10^-5 M and 10^-5 M, respectively.

Treatment with reserpine

Reserpine at a final concentration of 2 x 10^-5 M was added to the culture medium when restoration of the beating was observed. The dishes were left standing for 1 hr at room temp. and then after confirming that the beating had stopped, reserpine was removed by washing the cells twice with Hank's salt solution. The cells were then incubated in fresh medium containing an appropriate concentration of epinephrine, nor-epinephrine, dopamine or dopa.

Chemicals

l-Norepinephrine was purchased from Sigma Chemical Co., l-epinephrine from Merck Chemical Co., reserpine solution from Daiichi Pharmaceutical Co. Inc., and propranolol from Sumitomo Kagaku Co.
RESULTS

Effect of catecholamines on the beating rate

Addition of $10^{-5}$ M epinephrine or $10^{-5}$ M norepinephrine to beating heart cells usually increased the rate of beating (Fig. 2), however, the stimulatory effect was not uniform. The beat of some cells increased immediately, while that of others increased after a lag period or continued unchanged.

The cells usually stopped beating with prolonged culture. Addition of $10^{-3}$ M catecholamines to cells which had ceased beating within less than 24 hr previously, resumed beating within 5 min (Fig. 3). Epinephrine, norepinephrine and isoproterenol had similar effects on initiation of the beating when added at a concentration of $10^{-5}$ M. After the beating had been restored in this way it continued for at least 12 hr. Epinephrine and norepinephrine reactivated resting heart cells even when added at a concentration of $10^{-8}$ M.

FIG. 2. Effect of catecholamines on the beating rate of cultured rat heart cells.

None (a), $10^{-3}$ M epinephrine (b) or $10^{-3}$ M norepinephrine (c) was added to cells at 0 time. Each symbol expresses the relative beating rate of cells in each dish examined. Experimental conditions are described in the text.

FIG. 3. Effect of catecholamines on initiation of beating. Cells had stopped beating less than 24 hr after which $10^{-5}$ M epinephrine (a) or $10^{-3}$ M norepinephrine (b) was added at 0 time. Each symbol expresses the relative beating rate of cells in each dish examined.

FIG. 4. Effect of reserpine on the beating rate of cultured rat heart cells.

None (a), $2 \times 10^{-6}$ M reserpine (b) or $2 \times 10^{-3}$ M reserpine (c) was added to cells at 0 time. Each symbol expresses the relative beating rate of cells in each dish examined.
Effect of reserpine on the heating rate

Reserpine was added to the incubation medium to deplete catecholamines of the heart cells. As shown in Fig. 4, addition of $2 \times 10^{-6}$ M reserpine reduced the rate of beating significantly, but the cells continued beating throughout the 36 hr observation period. On addition of $2 \times 10^{-5}$ M reserpine, the beating slowed down immediately, and stopped completely within 1 hr. These facts suggest that the critical concentration of reserpine required to inhibit the autonomic beating is between $2 \times 10^{-5}$ M and $2 \times 10^{-4}$ M.

When cells were incubated in a medium containing $2 \times 10^{-4}$ M reserpine, they did not resume beating within 10 hr, but after 24 hr most cells had started beating again very slowly. These facts indicate that $2 \times 10^{-5}$ M reserpine was sufficient to reduce the catecholamines in heart cells to below the threshold level necessary to initiate and maintain the beating.

Restoration of beating by catecholamines

Beating cells were exposed to $2 \times 10^{-3}$ M reserpine for 1 hr to stop the beating, and were then washed and incubated in a fresh medium without reserpine. As shown in Table 1, cells resumed beating after a lag period of 20 min (1 plate) to 1–2 hr (6 plates). The lag period may reflect the time required to restore catecholamines in the cells to the threshold level.

As shown in Table 1, on addition of $10^{-8}$ M or $10^{-7}$ M epinephrine to the fresh medium, cells in all plates started beating within 5 to 30 min. The time lag before beating started seemed to be longer when adding $10^{-7}$ M epinephrine, and when adding $10^{-6}$ M, beating did not start. Heart cells which had been treated with reserpine responded to

| TABLE 1. Effect of catecholamines in restoring the beat of reserpine-treated heart cells. |
|-----------------------------------------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|
| Addition          | Time (min)    |               |               |               |               |               |               |
|                  | 0      | 5      | 10     | 20     | 30     | 60     | 120    |
| None             | 0*     | 10**   | 0/10   | 0/10   | 1/10   | 1/10   | 5/10   |
| Epinephrine      |         |         |         |         |         |         |         |
| $10^{-8}$ M      | 0/1    | 0/1    | 0/1    | 0/1    | 0/1    | 0/1    | 0/1    |
| $10^{-7}$ M      | 0/5    | 0/5    | 1/5    | 4/5    | 4/5    | 4/5    | 4/5    |
| $10^{-6}$ M      | 0/3    | 0/3    | 1/3    | 3/3    | 3/3    | 3/3    | 3/3    |
| $10^{-5}$ M      | 0/5    | 1/5    | 2/5    | 4/5    | 5/5    | 5/5    | 5/5    |
| Norepinephrine   |         |         |         |         |         |         |         |
| $10^{-8}$ M      | 0/2    | 0/2    | 0/2    | 0/2    | 0/2    | 0/2    | 0/2    |
| $10^{-7}$ M      | 0/5    | 0/5    | 3/5    | 5/5    | 5/5    | 5/5    | 5/5    |
| $10^{-6}$ M      | 0/3    | 1/3    | 3/3    | 3/3    | 3/3    | 3/3    | 3/3    |
| $10^{-5}$ M      | 0/4    | 2/4    | 3/4    | 3/4    | 4/4    | 4/4    | 4/4    |

Cells were washed and put in fresh medium without reserpine but with epinephrine or norepinephrine at 0 time.
* Numbers of dishes in which beating was restored.
** Numbers of dishes examined.
similar concentration ranges of norepinephrine and epinephrine. Thus the threshold concentrations of these compounds required for beating appeared to be similar, that is about $10^{-5}$ M.

Among the precursors of catecholamines tested at a concentration of $10^{-5}$ M, dopamine was found to have an effect similar to that of epinephrine and norepinephrine (Table 2).

When catecholamines ($10^{-5}$ M) were added to the cells incubated in medium containing reserpine, the beating of the heart cells was restored, but at a much slower rate than normal (Fig. 5).

| Addition       | Time (min) |   |   |   |   |   |   |   |
|----------------|------------|---|---|---|---|---|---|---|
| None           | 0          | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| Dopa $10^{-5}$ M | 0.2       | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| Dopamine $10^{-5}$ M | 0.2     | 2.2 | 2.2 | 2.2 | 2.2 | 2.2 | 2.2 |

Experimental conditions equal those in Table 1 except that dopamine was added to cells.

* Numbers of dishes in which beating was restored.
** Numbers of dishes examined.

Fig. 5. Effect of catecholamines in restoring the beat of reserpine-treated heart cells.

Cells were incubated with reserpine for 1 hr and then epinephrine (a) or norepinephrine (b) was added at a concentration of $10^{-5}$ M (●) or $10^{-6}$ M (○) in the presence of reserpine. The initial beating rate is that before treatment with reserpine.

Effect of propranolol on restoration of the beating

Propranolol, a $\beta$-adrenergic blocking agent, reduced the beating rate. With $10^{-5}$ M propranolol the beating usually stopped immediately (not shown). Heart cells were incubated with $2 \times 10^{-5}$ M reserpine, for 1 hr, after which they were washed and incubated
in fresh medium without reserpine, but containing 10^{-5} M norepinephrine, 10^{-8} M propranolol, or norepinephrine plus propranolol. As shown in Table 3, in medium containing norepinephrine, cells started beating again within 5 to 10 min, while in medium containing propranolol plus norepinephrine, the cells started beating after 10 to 20 min.

**Cholinomimetics**

Carbachol (10^{-5} M) and acetylcholine (10^{-7}–10^{-5} M) with eserine (10^{-2} M) did not affect the beating rate of heart cells under the experimental conditions used herein (Fig. 6 and Fig. 7).

![Fig. 6. Effect of carbachol on the beating rate.
None (a), 10^{-7} M carbachol (b) or 10^{-5} M cabachol (c) was added to cells at 0 time. Each symbol expresses the relative beating rate of cells in each dish examined.](image)

![Fig. 7. Effect of acetylcholine on the beating rate.
The beating rate was counted after addition of 10^{-8} M eserine at 0 time. 10^{-5} M acetylcholine and 10^{-5} M acetylcholine were added after 10 min and 20 min, respectively. Each symbol expresses the relative beating rate of cells in each dish examined.](image)
DISCUSSION

Rhythmical and spontaneous contraction is a unique characteristic of heart muscle. It is well known that stimuli initiated rhythmically at the sinus node pass through the atrium to Tawara's node and terminate at myocardial cells in His' bundles and Purkinje's fibers, causing heart contraction.

Harary and his colleagues (1, 2) obtained evidence of the rhythmical beating of primary cultures of heart cells from the newborn rat. They showed that single cells beat at independent rates but that on physical contact between the cells the beats became synchronous. When the cells grow to form network of cells, a beating center is established. These results clearly show the autonomy of the beat of individual cells.

The chemical stimulus evoking the rhythmic contraction of heart cells is unknown. Catecholamines have a chronotropic effect on intact heart. Wollenberger et al. (4) examined the effects on cultured chick embryonic heart cells and found that epinephrine or digitalis has a stimulatory effect on the beating rate in addition to the fact that treatment with reserpine or dichloroisoproterenol depresses the effect of digitalis. It was thus suggested that digitalis acts on the heart by some mechanism which releases catecholamines.

The authors also examined herein the effect of catecholamines using monolayer sheets of rat heart cells and found that the effect of catecholamines on the beating rate was not uniform or significant. The variable and additive effects of catecholamines can be explained by presuming that before addition of catecholamines only some of the reactive sites for catecholamines would be occupied, and that only the cells with all the sites occupied would not respond to extra catecholamines.

To prove that catecholamines can initiate the beat of heart cells the beat must first be stopped using some treatment which depletes the cells of catecholamines. We obtained cells depleted of catecholamines in two ways: by prolonged culture, and by treatment with reserpine. Cells in culture can beat for several days but eventually they stop beating even under favorable culture conditions. When catecholamines were added to cells which had stopped beating less than 24 hr before, the cells started beating again at almost the normal rate within 5 min. This resuscitative effect of catecholamines suggested that the beating stopped, not because the cells were depleted of catecholamine receptors but because they were depleted of catecholamines. The results also suggested that resting cells poorly synthesize these amines from their precursors (tyrosine and phenylalanine) which are present in the medium.

Addition of reserpine at a concentration of more than $2 \times 10^{-6}$ M, stopped the beating. The effect of reserpine was counteracted by adding $10^{-5}$ M catecholamines. Cells which had been treated with reserpine started beating again after a long lag period but catecholamines added at concentrations of about $10^{-7}$ M reduced the lag period to a few min. This suggested that when cells are depleted of catecholamines their property of spontaneous beating is lost, and that when intracellular catecholamines immediately increase to the threshold level, due to synthesis in the cells or addition to the medium, the
cells resume beating.

Among the catecholamines and their precursors, epinephrine, norepinephrine and dopamine were all equally effective in evoking the beating, but dopa had no effect. This suggests that the synthesis of catecholamines from tyrosine in cultured heart cells is limited at the step of dopa-decarboxylase and that this enzyme may limit the level of catecholamines in isolated heart cells.

Propranolol inhibited the effect of catecholamines in evoking the beat. This suggests that a β-adrenergic mechanism is important for the autonomic contraction of cells. The fact that acetylcholine or carbachol had no effect, indicated that a cholinergic mechanism is not involved in the spontaneous beating of isolated heart cells.

This report shows that catecholamines evoke the contraction of cultured rat heart cells. The threshold level of catecholamines necessary for spontaneous beating appear to be in the order of $10^{-7}$–$10^{-6}$ M. Dopa-decarboxylase may therefore control the level of catecholamines in the cells. Based on these results, we present the hypothesis that a cycle of depletion (discharge) and replenishment (charge) of catecholamines in heart cells induces the rhythmical contraction of heart beats. To prove this hypothesis it must be demonstrated that there is a rhythmical change in the level of intracellular catecholamines and in its, metabolic activities in the cells and that these changes are synchronized with the beating rate.

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REFERENCES
1) Harary, I. and Farley, B.: Science 132, 1839 (1960)
2) Harary, I. and Farley, B.: Science 131, 1674 (1960)
3) Tsiganoff, S.V.: Zhurnal, Exptl. Biol. Med. 8, 437 (1928)
4) Wollenberger, A. and Halle, W.: Mschr. Dent. Akad. Wiss. (Berlin) 5, 38 (1963)
5) Bodor, G.B., Harley, R.J. and Johnson, I.S.: Nature 231, 531 (1971)
6) Sperelakis, N. and Lehmkuhl, D.: Am. J. Physiol. 209, 693 (1965)
7) Harary, I. and Farley, B.: Exptl. Cell Res. 29, 451 (1963)
8) Goshima, K. and Tonomura, Y.: Exptl. Cell Res. 56, 387 (1969)