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Studies on Heart. XVII.[1] Effects of Frog Heart Inotropic Substances on Beating of Rat Myocardial Cells in Culture[2]

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The effects of frog heart inotropic substances on beating phenomena in cultured myocardial cells of neonatal rats were investigated. The beating rate and percentage of trypsin-dissociated cells were determined in Eagle minimum essential medium (MEM) supplemented with 10% bovine serum at 2 days in culture. The attachment and the spreading of cells were observed in Eagle MEM supplemented with 0.5% bovine albumin.

The positive inotropic glycoprotein, Fr. A from calf heart, and its cyanogen bromide peptide, Fr. A-1, significantly increased the beating rate and percentage of single cells in their dosages to be effective on frog heart assay as well as epinephrine. Another positive inotropic lipoprotein, Fr. I-B from calf thymus, also increased the rate, but did not effect on the percentage like acetylcholine. Negative inotropic dipeptide, anserine, decreased the rate but not the percentage of single cells. In the beating rate of cell-cluster, Fr. A, Fr. A-1 and Fr. I-B showed accelerating effects in their concentrations of 10^{-8} M, 10^{-7} M and 10^{-9} M, respectively. The effect of Fr. I-B on cell-cluster was transitory 5 to 20 min after sample addition, and those of Fr. A and Fr. A-1 were relatively continuous up to 40 min. The chronotropic actions on cell-cluster by these three samples were not affected by the addition of propranolol.

Fr. A significantly increased the attached cells and Fr. I-B greatly decreased the spreading cells, but both of them did not promote the spreading process of myocardial cells in their doses of improving the beating properties.

Keywords——myocardial cells; culture; inotropic substances; beating rate; beating percentage; spreading cells; attached cells

It is well known that when myocardial single cells isolated from fetal or neonatal rat,[4] mouse,[5] hamster[6] or chick[7] heart are cultured in a synthetic medium supplemented with serum, some of them become attached to a bottom of dish, spread and then spontaneously beat. Individual myocardial cell beats independently and rhythmically, but when the single cells come in contact with each other, they grow into synchronous beating cell-clusters. It is interesting that the cultured myocardial cells show various functions of a differentiation, a beating rhythmicity and drug-induced beating properties, essentially similar to those of an adult intact heart *in vivo*.[4–8]

During studies on the humoral factor affecting heart function, we previously isolated frog heart inotropic substances from calf heart and thymus.[1,9] In order to clarify the

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further biological properties of these inotropic substances, this paper describes the effects of them on beating phenomena, attachment and spreading of the cultured rat myocardial cells.

Experimental

Materials——Acetylcholine chloride and N,N′-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) were products of Nakarai Chemicals, Ltd., Kyoto. DL-Propranolol hydrochloride, anserine nitrate and bovine albumin (Fraction V) were products of Sigma Chemical Company, St. Louis, Mo. L-Epinephrine was product of E. Merk, AG. Frog Heart inotropic substances, kallikrein-like glycoprotein[9b] (Fr. A) from calf heart and lipoprotein Fr. I-B[1] from calf thymus, were prepared as described previously. Active fragment Fr. A-1 of Fr. A was isolated as follows. Five hundred mg of cyanogen bromide peptides obtained from Fr. A by the method as described by Gross[10] was fractionated on Sephadex G-50 column equilibrated with 0.2 M NH_4HCO_3 (pH 8.6). A fraction which eluated at a position of K_{av}: 0.40—0.60, was rechromatographed on the same column, lyophilized and named Fr. A-1. The yield was 30 mg.

Culture and Microscopical Observations of Rat Myocardial Cells——The techniques for the culturing of rat myocardial cells are essentially those reported for mouse by Goshima.[5] Ventricles dissected from 1 to 3 day old rats (Wistar strain) were minced into small pieces and soaked for 5 min at 37 ° in phosphate-buffered saline (PBS). After the treatment for 5 min at 37° with 0.06% trypsin (Difco, 1: 250, dissolved in PBS), the tissues were digested with 10 ml of 0.06% trypsin at 37° for 3 succesive periods of 10 min each with gently stirring. The supernatants from 2nd to 4th trypsinizations were poured into a centrifugal tube containing a cold standard medium and centrifuged at $350 \times g$ for 5 min. The pellet was resuspended in the standard medium and the suspension was filtered through 3 layers of lenz paper. The filtered cells were seeded into Petri glass dishes (P2, 45 mm in diameter, Miharu Seisakusho, Tokyo) and incubated at 37° in the standard medium under an atomosphere of 5% CO_2 and 95% air. The standard medium consists of Eagle minimum essential medium (MEM) buffered with 15 mM $NaHCO_3$ and supplemented with 10% bovine serum in a final concentration.

Microscopical observations were performed after the cultivation for 2 days, using an inversed phase-contrast microscope (Nikon MD, magnification: 80 to 160). For microscopical observations the standard medium buffered at pH 7.3 with 10 mM BES instead of $NaHCO_3$ was used. Less than 200 μl of sample dissolved in Eagle MEM buffered at pH 7.3 with BES was added into 2 ml of the culture medium. A beating percentage of single cells was investigated on more than 100 cells per each dish and an average of spontaneous beating rate (beat/min) of the cells was calculated from the values of more than 20 cells per each dish counted for 20 to 60 sec per each cell.

In an experiment to test the behaviors of attachment and spreading of single cells on a bottom of dish, the cultures of trypsin-dissociated cells were prepared as described above using Eagle MEM buffered with 10 mM BES and 10 mM $NaHCO_3$ and supplemented with 0.5% albumin as the medium (albumin-medium). A half ml of sample solution was added into the dish containing 10^5 cells suspended in 2 ml just before the cultivation. Thirty field counts (a field size: 0.2 mm^2) were made of the attached and the spreading myocardial cells in each dish at 2 days in culture without the medium exchange.

All of media and solutions were sterilized by filtration through Milipore filters (Type HA).

Measurements of Inotropic Activity and Physico-Chemical Properties——Inotropic activity on the perfused frog heart was measured as described by West[11] and was expressed in a minimum effective concentration of sample which produced 10% more contraction height on a kymograph than that shown before sample addition.[1] Protein and carbohydrate contents were determined by Biuret reaction[12] and by phenol–sulfuric acid method,[13] respectively. Molecular weight was determined using sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis reported by Weber *et al.*[14] Amino acid analysis was carried out with Hitachi amino acid analyzer (KLA 3, resin: Hitachi Custom Ion-Exchange Resin 2611) according to Spackman[15] and by ultraviolet absorption method.[16] The protein sample was hydrolyzed with 6 N HCl in evacuated, sealed tube at 110° for 24 hr. The molecular weight used in the calculation of amino acid composition was obtained from the above SDS–polyacrylamide gel electrophoresis. N-terminal amino acid was detected by dancyl method as described by Gray.[17]

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Results

Properties of Active Fragment, Fr. A-1

Criterion of purity of Fr.A-1 obtained by the cyanogen bromide degradation of Fr.A was performed by polyacrylamide gel electrophoresis in 0.1% SDS as shown in Fig. 1. The result of electrophoresis revealed a single peak stained with amino black 10B at a position of *Rf*: 0.67 under the given condition. The molecular weight of Fr.A-1 was calculated to be 5500 from the *Rf* value which calibrated with insulin, cytochrome c and myoglobin. This fragment was only one active peptide among the cyanogen bromide peptides of Fr.A, and possessed the positive inotropism in the perfused frog heart assay in a minimum effective concentration of 200 μg/ml. Properties of Fr.A-1 were summarized in Table I. This peptide consisted of 15 kinds of amino acids without carbohydrate which contained in Fr.A, and its N-terminal amino acid was histidine. The positive inotropic activity of Fr.A-1 was a quarter of that of native glycoprotein.

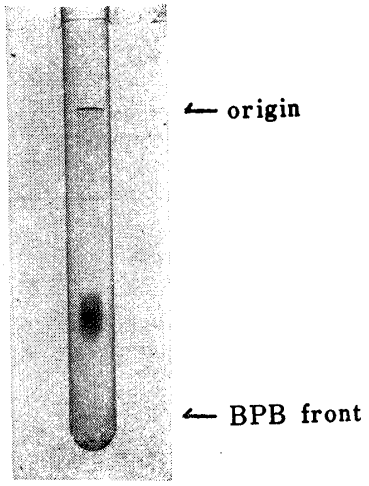

Fig. 1. Electrophoretic Pattern of Fr. A-1

Condition: 15% polyacrylamide gel in 0.1% sodium dodecyl sulfate, 8 mA/tube, 4 hr.
Staining: amino black 10B.
Lower: anode.

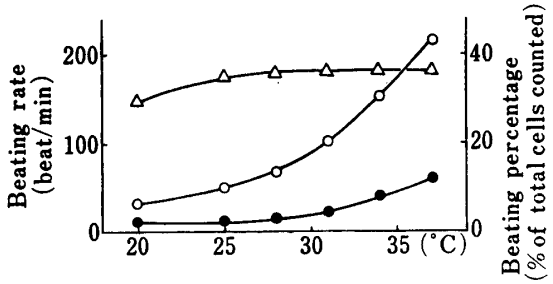

Fig. 2. Beating Rate and Percentage of Myocardial Cells at Various Temperatures

Each point represents the mean of 3 experiments. Beating rate of cell-cluster —○—, beating rate of single cells —●—, beating percentage of single cells —△—.

Table I. Properties of Fr. A-1

Measurement	Data
Inotropic activity of frog heart	Positive inotropism (200 μg/ml)[a]
Molecular weight	5500
Peptide content	95.4%
N-Terminal amino acid	His
Amino acid composition	Asp(5)[b] Gly(5) Phe(2)
	Thr(2) Ala(3) His(1)
	Ser(2) Val(4) Lys(3)
	Glu(8) Ile(2) Arg(2)
	Pro(3) Leu(4) Hse(1)

a) Minimum effective concentration.
b) Nearest integral number/mol.

Effects of Frog Heart Inotropic Substances on Beating Rate and Percentage of Cultured Myocardial cells

The myocardial cells could be easily distinguished from fibroblast-like cells by their configurations on the bottom of dish cultured for 2 days as observed by Harary *et al.*[4] When 2×10^5 cells were inoculated into a dish, a population of single isolated cells was found, and some of them exhibited spontaneous and rhythmical beating at various rate (1 to 220 beat/min at 34°) each other. While, a large cell-cluster was observed in the dish which seeded with 2×10^6 cells and cultured for 2 days, and all myocardial cells beat synchronously. As shown in Fig. 2 the beating rate of single cells and cell-cluster were influenced by the temperature of medium, and decreased with lowering temperature. The former was faster than the latter at temperature between 20° and 37°. But the percentage of single beating cells was found to be almost a constant of 36% at 25° to 37°. By considering the above beating phenomena of the cultured myocardial cells, standard conditions for testing the effects of samples on the beating rate and percentage were defined at 34° for single cells and 31° for cell-cluster in the standard medium buffered with BES at 2 days in culture. The control values under the standard conditions were given in Table II.

Table II. Beating Rate and Percentage of Rat Myocardial Cells under Standard Condition

Group of cell	Beating rate (beat/min, mean ± s.e.)	Beating percentage (% of total cells counted, mean ± s.e.)
Single cell	52±2.6 (n=26 dishes)	36±1.9 (n=21 dishes)
Cell-cluster	115±2.2 (n=16 dishes)	100[a]

Data represent the values in Eagle MEM buffered at pH 7.3 with BES at 34° for single cell and at 31° for cell-cluster after cultivation for 2 days.
a) Beat synchronously.

Table III and IV showed the effects of frog heart inotropic substances on the beating rate and percentage of single cells. The beating cells were first counted in the standard condition and then recounted on the same cells 5 min to 30 min for the rate and 15 min to 40 min for the percentage after sample addition. The results indicated that Fr.I–B, Fr.A and Fr.A-1 significantly increased the rates of single cells in their dosages to be effective on frog heart[1,9b] as occurred by epinephrine. Negative inotropic dipeptide, anserine, apparently decreased

Table III. Effects of Frog Heart Inotropic Substances on Beating Rate of Myocardial Single Cells

Sample	Final concentration (M)	No. of experiments	Beating rate[a] (% of control, mean ± s.e.)
Solvent	0	6	100±2.1
Fr. I–B	2×10^{-5}	5	121±5.3[b]
Fr. A	3×10^{-6}	6	119±6.7[c]
Fr. A-1	8×10^{-5}	6	130±6.4[b]
Anserine	10^{-3}	3	80±8.1[c]
Epinephrine	4×10^{-8}	4	146±5.0[b]
Acetylcholine	10^{-6}	3	80±2.9[b]

a) Counted before and 5 to 30 min after addition of sample.
b) $p<0.01$; significantly different from solvent value.
c) $p<0.05$; significantly different from solvent value.

TABLE IV. Effects of Frog Heart Inotropic Substances on Beating Percentage of Myocardial Single Cells

Sample	Final concentration (M)	No. of experiments	Beating cell[a] (% of control, mean ± s.e.)
Solvent	0	6	101± 3.4
Fr. I-B	2×10^{-5}	5	103±10.5
Fr. A	3×10^{-6}	4	135± 6.5[b]
Fr. A-1	8×10^{-5}	3	126± 8.8[c]
Epinephrine	4×10^{-8}	4	148±10.6[b]
Acetylcholine	10^{-6}	3	98± 0.6

a) Examined before and 15 to 40 min after addition of sample.
b) $p<0.01$; significantly different from solvent value.
c) $p<0.05$; significantly different from solvent value.

the rate like acetylcholine. The same dosages of Fr.A and Fr.A-1 initiated to beat quiescent single cells and significantly increased the beating percentage as well as epinephrine. But Fr.I–B did not effect on the beating of the quiescent cells as shown in anserine and acetylcholine, and these samples did not make the spontaneous beating of cells cease in the tested concentrations of them.

The effects of samples on the beating rate of cell-cluster were shown in Fig. 3. The rate could be counted in a short time because all myocardial cells beat synchronously. The addition of 2×10^{-10} M Fr.I–B resulted in a transitory increasing of the beating rate 5 min to 20 min after sample addition. The rates were continuously accelerated by 3×10^{-9} M Fr.A and 8×10^{-8} M Fr.A-1 up to 40 min after sample additions. The relationships of dose to chronotropisms of cell-cluster by Fr.I–B, Fr.A and Fr.A-1 were given in Fig. 4. These samples accelerated the rates with their increasing concentrations, and sufficient concentrations to accelerate 10% more the rate were 10^{-9} M for Fr.I–B, 10^{-8} M for Fr.A and 10^{-7} M for Fr.A-1. Under the present condition, no sample could elevate the rate of cell-cluster over 30%. On

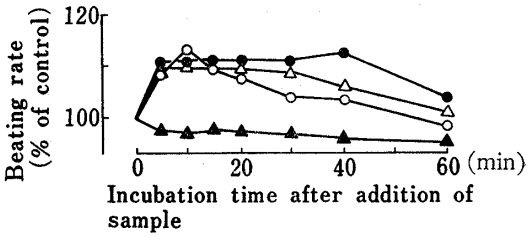

Fig. 3. Effects of Frog Heart Inotropic Substances on Beating Rate of Myocardial Cell-Cluster

Fr. I-B (2×10^{-10} M) —○—, Fr. A (3×10^{-9} M) —●—, Fr. A-1 (8×10^{-7} M) —△—, solvent —▲—.

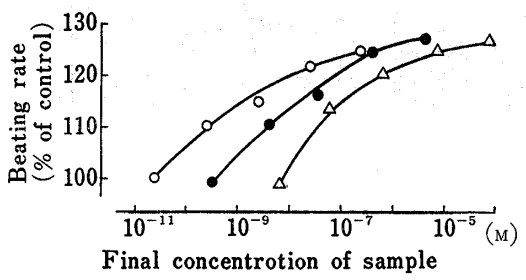

Fig. 4. Dose Response Curves of Frog Heart Inotropic Substances on Beating Rate of Myocardial Cell-Cluster

Fr. I-B —○—, Fr. A —●—, Fr. A-1 —△—.

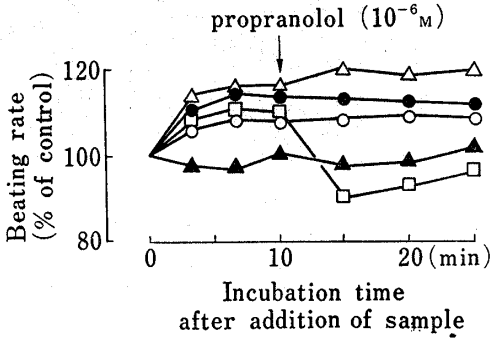

Fig. 5. Effect of Propranolol on Chronotropism of Cell-Cluster by Frog Heart Inotropic Substances

Propranolol was added 10 min after addition of sample.
Fr. I-B (10^{-10} M) —○—, Fr. A (2×10^{-9} M) —●—, Fr. A-1 (8×10^{-7} M) —△—, epinephrine —□—, (4×10^{-8} M), solvent —▲—.

a basis of minimum effective concentration, the order of relative intensity in their rate increase effects was the same as in their inotropic activities on frog heart.[1,9b)]

When 10^{-6} M of propranolol was added into the cell-cluster which beat faster by the additions of positive inotropic substances, their rates were not influenced, differing from the case of epinephrine (Fig. 5).

Effects of Fr.I-B and Fr.A on Attachment and Spreading of Myocardial Single Cells

The results were shown in Table V and Fig. 6. In the control experiment that myocardial cells were cultured in low density monodisperse of 10^5 cells per dish in the albumin-

Table V. Effects of Fr. A and Fr. I-B on Attachment and Spreading of Myocardial Single Cells on Bottom of Cultured Dish

Sample	Final concentration (M)	30 field counts of cells (mean ± s.e., $n=6$)	
		Attachment	Spreading
Solvent	0	293±23	34± 4
Fr. I-B	2×10^{-5}	295±48	2± 1[a)]
Fr. A	3×10^{-6}	437±37[b)]	47± 9
Serum	10[c)]	424±47[b)]	203±16[a)]

10^5 cells were cultured with samples at 37° in albumin-medium for 2 days. See methods for details.
a) $p<0.01$; significantly different from solvent value.
b) $p<0.05$; significantly different from solvent value.
c) Final concentration in percentage.

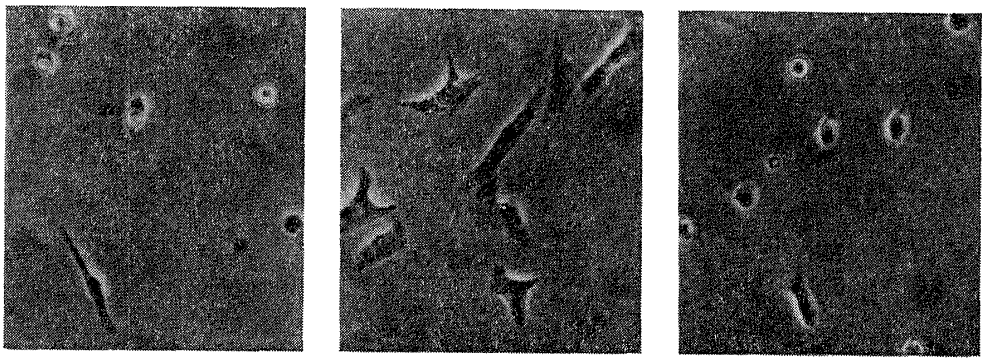

Fig. 6. Morphologies of Myocardial Single Cells Cultured for 2 Days in Different Media at 10^5 Cells per Dish (×160)

a) Albumin-medium.
b) Albumin-medium supplemented with 10% bovine serum.
c) Albumin-medium added with 3×10^{-6} M Fr. A.

medium for 2 days, 293 of them responded to about 10% of total cells became attached to the bottom of dish and 34 of them spread in 30 fields area. The spreading cells mainly exhibited striated or elongated configuration with bulging surface (Fig. 6a)). In the culture dish supplemented with 10% serum, the number of attached and spreading cells significantly increased. As shown in Fig. 6b), most of myocardial cells well spread out with irregular stellar and flattened configurations which apparently distinguished from the spreading cells in the albumin-medium. The addition of Fr.A also increased the attachment of cells up to the level of serum group, but had only an increasing tendency on the spreading number without significant differences from control group, and did not promote the spreading process in the feature of cells as shown in Fig. 6c). Fr.I–B showed no effect on the attachment and greatly inhibited the spreading behavior.

Discussion

Cultured myocardial cells can be used to study a direct action of effective materials on heart cells because they are free of nerves and blood vessels. It has been reported that the beating properties of cells were affected by the environments of temperature, pH and ion concentration in the culture medium, many cardioactive agents and the age of animals.[4–8]

In the present paper the four parameters of beating rate, beating percentage, attachment and spreading were used to evaluate the effects of frog heart inotropic substances on the cultured myocardial cells of neonatal rats. The data showed that the positive inotropic substances, Fr.A and Fr.A-1 from heart, increased the rate and the percentage of single cells under the standard condition, and the negative inotropic dipeptide, anserine, which could be also obtained from heart,[9a] decreased the rate. Both Fr.A and Fr.A-1 also accelerated the spontaneous activities of cell-clusters in a thousandth of the concentrations which needed to promote the functions of intact frog heart.[9b] McDonald *et al.*[18] reported that the sensitivity to tetrodotoxin was higher in aggregates than in single cells of chick embryonic heart, and the former responded to tetrodotoxin in the same manner as intact heart. It is considered that the promoting activities of Fr.A and Fr.A-1 on the rat myocardial cells were functionally identical to their biological effects on intact frog heart. Thymus Fr.I–B accelerated the spontaneous activities of single cells and cell-cluster, but did not influence the quiescent cells which lost only their spontaneous activities as indicated by Goshima.[19] Insensitivity of Fr.I–B to the quiescent cells may be able to explain the transitory accelerating effect on the rate of beating cell-cluster, because Fr.A possessing the increase activity of beating percentage showed relatively continuous accelerating effect.

It has been recognized that there was β-adrenoceptor system in the trypsin-dissociated myocardial cells of chick embryo[8d] and neonatal mouse.[19] In the present experiment, the chronotropism of epinephrine was blocked by propranolol in neonatal rat myocardial cells, but the actions of Fr.A, Fr.A-1 and Fr.I–B were not influenced.

The myocardial cells must be attached to the glass and spread before they will beat, and the behaviors of attachment and spreading were supported by serum component in the culture medium.[8a] Gordon *et al.*[20] suggested that the spreading behavior of heart cells is a regular feature in a primary stage of the development in chick embryonic heart. Fr.A and Fr.I–B did not develop the poor spreading in low density monodisperse of cells in the albumin-medium without serum. It might be concluded that Fr.A and Fr.I–B effect only beating functions of rat myocardial cells.

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