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Studies on Heart. XVIII.[1] Heart Component Influencing the Maintenances of Spreading and Beating of Rat Myocardial Cells in Serum-free Culture[2]

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Heart component influencing the maintenances of spreading and beating of rat myocardial cells in serum-free culture was investigated. Spreading and well-spreading % of single cells cultured with sample in Eagle minimum essential medium (MEM)-0.5% bovine serum albumin for 2 days at 37°, and relative beating % and rate of single well-spreading cells incubated with sample in Eagle MEM for 1 hr at 37° were used as parameters of the maintenance activities. Culture filtrates of heart ventricle fragments cultured in Eagle MEM for 4 days at 37° significantly promoted all of 4 parameters, and precipitates salted out with 30—50% saturated ammonium sulfate from aqueous alkaline extracts of rat, bovine and rabbit ventricles had the promoting effects like culture filtrate. But extracts prepared from brain, liver, spleen, pancreas, small intestine, kidney and skeletal muscle in the same manner as done in ventricles did not affect or inhibited spreading and beating. Desalted culture filtrates and extracts of ventricles did not possess trypsin inhibitory activity.

Bovine ventricle extract was fractionated for an active principle using successive chromatographies on DEAE-cellulose, Sephadex G-100 and CM-cellulose column. The isolated principle, Fr. BVP (bovine ventricle protein), was a protein possessing molecular weight of 100000 and 18 kinds of amino acids. Fr. BVP significantly maintained spreading and beating of myocardial cells in serum-free culture in a concentration of 100 μg/dish. This protein differed biologically and chemically from Fr. A, inotropic protein of bovine heart, which was effective for promoting the beating behaviors of myocardial cells cultured with serum.

Keywords——cultured myocardial cell; spreading and well-spreading cell; beating percentage and rate; ventricle culture filtrate; bovine ventricle protein

In early chick embryo, myocardial cells synthesize the extracellular materials which may play some roles in various functions of heart.[4] Gordon *et al.*[5] demonstrated that spreading and beating of myocardial single cells in chick embryo were improved in the defined medium which conditioned by culture filtrate of embryonic heart fragments and that the proportions of spreading and beating cells were increased in higher single cell density in culture. These facts suggest that myocardial cells secrete the materials which improve functions of the cells own. We previously reported that Fr. A, protein obtained from bovine heart, promoted the percentage and the rate of beating of rat myocardial cells cultured in Eagle minium essential medium (MEM) containing 10% serum but did not increase the proportion of spreading cell cultured in Eagle MEM containing 0.5% bovine serum albumin (BSA)

1) Part XVII: S. Aonuma, Y. Kohama, K. Akai, and M. Fujioka, *Chem. Pharm. Bull.* (Tokyo), **26**, 709 (1978).
2) Presented at the 27th Annual Meeting of the Kinki Branch Pharmaceutical Society of Japan, Kobe, Nov., 1977.
3) Location: *133-1, Yamadakami, Suita, Osaka.*
4) F.J. Manasek, *J. Exp. Zool.*, **174**, 415 (1970).
5) *a*) H.P. Gordon and M.C. Brice, *Exp. Cell Res.*, **85**, 303 (1974); *b*) H.P. Gordon and M.C. Brice, *ibid.*, **85**, 311 (1974).

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without serum.[1] The present paper describes heart component influencing the maintenances of spreading and beating of rat myocardial single cells in serum-free culture.

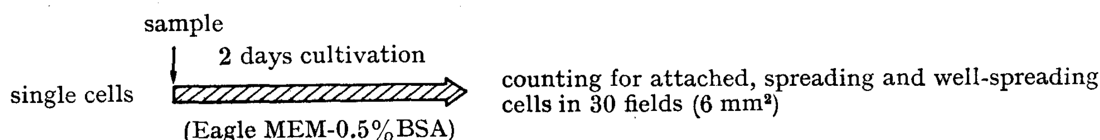
Experimental

Preparation of Ventricle Culture Filtrate——Culture filtrate was prepared in the following manner. Heart ventricles of neonatal (1 to 3 day old) or adult (5 to 8 week old) rats (Wistar strain) were minced into 0.5—1.0 mm fragments in phosphate-buffered saline (PBS). After washed 2 times with PBS and then with Eagle MEM buffered at pH 7.3 with 15 mM $NaHCO_3$, fragments of neonatal and adult rat ventricles were cultured in 2.0 and 20 ml per one organ, respectively, of Eagle MEM buffered with 15 mM $NaHCO_3$ and added with 0.013% penicillin G potassium and 0.02% dihydrostreptomycin sulfate. Culture were gassed with 5% CO_2 in air at 37° using CO_2-Incubator (Ikemoto Rika Kogyo). At the conclusion of cultivation, culture filtrates were obtained by filtration with Toyo filter paper No. 2 and stored at −25° before use.

Preparations of Organ Extracts——Sources of extracts were heart ventricle of adult rat, bovine and rabbit, brain, liver, spleen, pancreas, small intestine, kidney and skeletal muscle of adult rat and serum of bovine. Organs removed just after decapitation were sufficiently washed by cold saline to exclude a contamination of blood, and especially, liver was perfused with saline through its blood vessels. Organs were minced and dried with acetone, and the dried powder was extracted with 8 volumes of distilled water at pH 8.0. Following centrifugation at $15000 \times g$ for 10 min, the supernatant was fractionated with ammonium sulfate. Precipitate with ammonium sulfate was dialyzed and lyophilized. In a case of serum, serum was diluted with equal volume of PBS and fractionated with ammonium sulfate in the same manner as in organs.

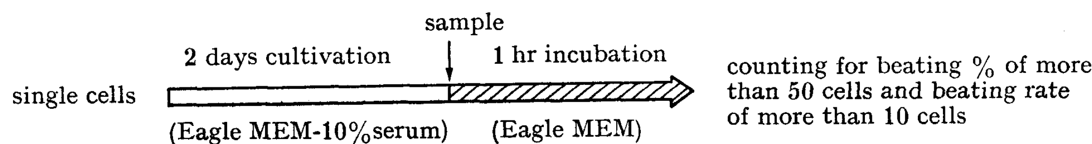
Assay Systems for Spreading and Beating of Rat Myocardial Cells in Serum-free Culture——The techniques for the culturing of rat myocardial cells are essentially those described previously,[1] except a beating assay. The assay for spreading of single cells was performed as shown in Fig. 1a). Trypsin dissociated single cells were incubated in Eagle MEM containing 2% BSA, 10 mM N,N′-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) and 10 mM $NaHCO_3$ for 10 min at 37° to remove an effect of trypsin. The cells were collected by

a) Spreading assay



$$\text{spreading } \% = \frac{\text{spreading cells}}{\text{attached cells}} \times 100, \quad \text{well-spreading } \% = \frac{\text{well-spreading cells}}{\text{spreading cells}} \times 100$$

b) Beating assay



$$\text{relative beating } \% \text{ of rate} = \frac{\text{sample value}}{\text{control value}} \times 100$$

Fig. 1. Assay Systems for Spreading and Beating of Rat Myocardial Cells in Serum-free Culture

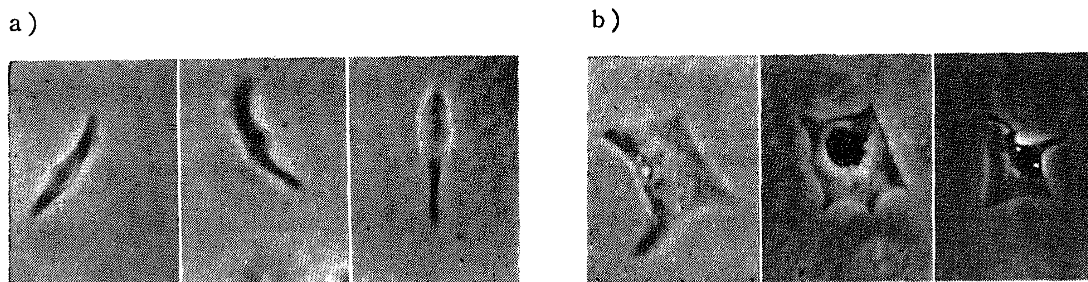


Fig. 2. Typical Morphologies of Myocardial Spreading and Well-spreading Cells (×160)

a) Spreading cell. b) Well-spreading cell.

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centrifugation for 5 min at $350\times g$ and resuspended in Eagle MEM containing 0.5% BSA, 10 mM BES and 10 mM $NaHCO_3$ (albumin-medium). The single cells (10^5/dish) were cultivated in 2.5 ml of albumin-medium containing sample for 2 days at 37° under an atomosphere of 5% CO_2 in air. After cultivation, culture medium was carefully exchanged with the fresh medium of the same composition as added before cultivation. Myocardial cells attached to a bottom of dish were observed using an inverted phase contrast microscope (Nikon MD, magnification: $\times 160$) in different 30 fields (a field size: 0.2 mm^2) at 37°. Attached, spreading and well-spreading myocardial cells were counted, and spreading % (% spreading cells/attached cells) and well-spreading % (% well-spreading cells/spreading cells) were calculated. As shown in Fig. 2, a cell was scored spreading if it was non-refractile in phase contrast and if its periphery was ditorted from circular shape. If a cell spread out with irregular stellar and flattened configurations, it was called well-spreading cell.

In an assay system for beating, trypsin dissociated single cells were poured into a centrifugal tube containing cold Eagle MEM buffered with 15 mM $NaHCO_3$ and supplemented with 10% bovine serum, 0.007% penicillin G potassium and 0.01% dihydrostreptomycin sulfate and centrifuged at $350\times g$ for 5 min. The single cells (10^5/dish) were cultured in the above medium containing serum at 37° for 2 days as shown in Fig. 1b), to prepare well-spreading cells, about a half of which gave regular and stable beating. Then, the cultured cells were washed and exchanged with Eagle MEM buffered with 10 mM BES and supplemented with 10% serum, and the control values of beating rate and beating % were taken at 37° under the inverted phase contrast microscope (magnification: $\times 160$). The dishes were washed 4 times with Eagle MEM buffered with 10 mM BES without serum (BES-medium), filled with 2.5 ml of BES-medium containing sample at 37° for 1 hr, followed by counting of the sample values. A beating % of single cells was investigated on more than 50 cells per each dish and average of spontaneous beating rate (beat/min) was calculated from the values of more than 10 cells per each dish counted for 20 to 60 sec per each cell. The cells which exhibit the beating rate of less than 10 beat/min, more than 150 beat/min and the arrhythmic beating phenomena were excluded from the assay because they give indefinite results. In the counting of the sample values, the same cells as in the control values were investigated. Assay result was given as relative beating rate and relative beating % which were the percentages of sample values to control values, as shown in Fig. 1b).

Samples were dissolved or diluted in albumin-medium for spreading assay and in BES-medium for beating assay. An effect of sample was given by comparing with the solvent value tested in only medium. The culture dish used was Petri glass dish (P2, 45 mm in diameter, Miharu Seisakusho, Tokyo). All of media and solutions were sterilized by filtration through a Milipore filter (Type HA).

The viability of cultured cell was tested using 0.01% neutral red just after the assay of spreading and beating and the cultivation.

Column Chromatography——For gel filtration Sephadex G-100 column (3.5×125 cm) equilibrated with 50 mM phosphate buffer (pH 8.0) was used. DEAE-cellulose column (3.5×30 cm) was equilibrated with 5 mM phosphate buffer (pH 8.0) and CM-cellulose column (1.7×30 cm) was equilibrated with 10 mM sodium phosphate buffer (pH 6.0). Bovine serum γ-globulin, BSA and ovalbumin were used as marker in a determination of molecular weight using Sephadex G-100. Sephadex G-25 column (2.5×92 cm) was used for desalting.

Determination of Trypsin Inhibitory Activity——Proteolytic activity was examined by casein digestion method.[6]

Physico-chemical Analyses——The analyses were performed as described previously.[1]

Results

Spreading and Beating Activities of Culture Filtrate and Extract of Ventricle

Spreading activities in culture filtrates obtained from ventricle fragments of neonatal rats cultured without serum for various periods were shown in Fig. 3. When myocardial cells were cultured for 2 days with 1.0 ml each of culture filtrates of 2nd to 8th day, both spreading % and well-spreading % of the cells were clearly increased, compared with solvent values of the cells cultured in albumin-medium. The 4th day filtrate had the most promoting effect on the spreading phenomena. The longer cultivation resulted in a gradual decrease of spreading activity. The fragments cultured for various periods were recognized to be viable by staining their cross-sections with neutral red. Simultaneously, protein contents in culture filtrates gradually increased up to the 4th day and then kept to be plateau (Fig. 4). These results suggested that protein-like material which influenced on the spreading behaviors

6) M. Laskowski, *Methods Enzymol.*, 2, 26 (1955).

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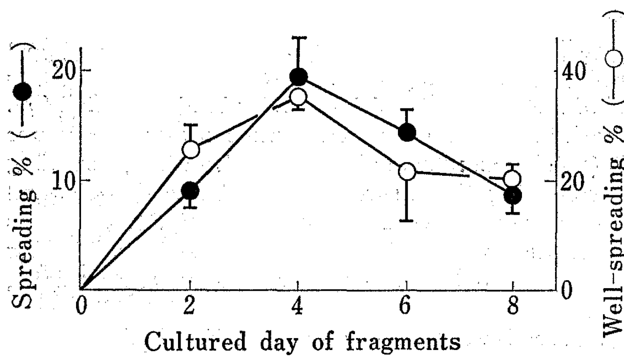

Fig. 3. Spreading Activities in Culture Filtrates of Neonatal Rat Ventricle Fragments Cultured for Various Periods

Each point represents mean±s.e. (n=4) which corrected by subtracting the solvent value, which was 16.5±0.6 in spreading % and 17.2±1.8 in well-spreading %.
dose: 1.0 ml/dish.

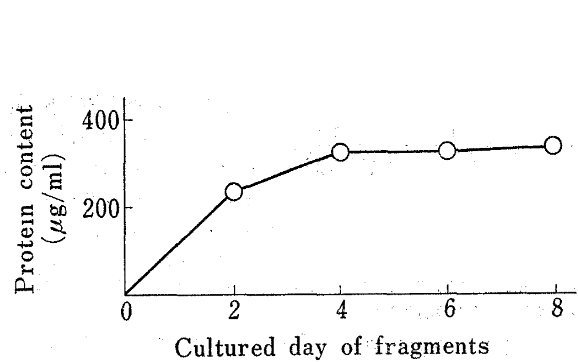

Fig. 4. Protein Contents in Culture Filtrates of Neonatal Rat Ventricle Fragments Cultured for Various Periods

of myocardial cells, was liberated in culture filtrate of ventricle fragments cultured for the first 4 days.

Table I showed spreading and beating activities in the 4th day culture filtrates of neonatal and adult rat ventricles. The culture filtrate from neonatal rat significantly increased not only spreading % and well-spreading % but also relative beating rate and relative beating % of myocardial cells. That from adult rat also promoted the behaviors of spreading and beating in a dose of 0.3 ml per dish.

TABLE I. Spreading and Beating Activities of Culture Filtrates of Rat Ventricle Fragments

Filtrate[a]	Spreading[b]		Beating[b]	
	Spreading %	Well-spreading %	Relative beating %	Relative beating rate
Solvent	17.0±0.4	16.1±1.4	29.9±2.5	13.4±3.5
Neonatal	28.8±3.7[c]	50.3±3.7[c]	147.7±6.6[d]	58.0±7.4[d]
Adult	42.8±9.2[c]	69.0±4.6[d]	43.6±4.7[c]	34.7±5.9[c]

a) Added 1 ml for neonatal and 0.3 ml for adult into each dish.
b) Mean ±s.e. (n=5).
c) $p<0.05$: significantly different from solvent value.
d) $p<0.01$: significantly different from solvent value.

TABLE II. Spreading and Beating Activities of Ventricle Extracts

Sample[a]	Spreading[b]		Beating[b]	
	Spreading %	Well-spreading %	Relative beating %	Relative beating rate
Solvent	16.5±1.6	19.8± 2.7	32.1±11.0	19.1±6.2
Rat	38.4±2.3[c]	51.5±10.5[d]	100.3±21.4[d]	39.3±4.9[d]
Bovine	28.2±0.8[c]	67.3± 5.0[c]	82.6±15.7[d]	75.7±1.2[c]
Rabbit	24.3±0.6[d]	40.6± 1.7[c]	75.4±10.7[d]	56.2±9.7[d]

a) Extract: precipitate salted out with 30—50% saturated ammonium sulfate, added 0.55 mg for spreading and 11 mg for beating into each dish.
b) Mean±s.e. (n=5).
c) $p<0.01$: significantly different from solvent value.
d) $p<0.05$: significantly different from solvent value.

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Aqueous extract from adult rat ventricles were fractionated into 0—30%, 30—50% and 50—100% precipitates and supernatant of saturated ammonium sulfate, respectively. Each fraction was assayed for spreading and beating activities. The activities of 0—30% precipitate, 50—100% precipitate and supernatant were 25.4, 25.3 and 21.7 in spreading %, 19.1, 19.7 and 19.1 in well-spreading %, 0, 26.0 and 5.1 in relative beating %, and 0, 9.1 and 0.8 in relative beating rate, respectively. As shown in Table II, only the 30—50% precipitate significantly increased all of spreading %, well-spreading %, relative beating rate and relative beating % as well as culture filtrate. Also, 30—50% precipitates obtained from rabbit and bovine ventricles apparently promoted spreading and beating of myocardial cells (Table II).

The myocardial cells added with culture filtrate or ventricle extract were live more than 95 %, their configurations were almost similar to those of cells cultured at the presence of serum rather than in albumin-medium, the photograph of which reported previously.[1)]

Spreading and Beating Activities of Various Organ Extracts

Precipitates salted out by 30—50% saturated ammonium sulfate were prepared from adult rat organs and bovine serum in the same manner as done in ventricle. The assay results were summarized in Table III. In the spreading assay, 30—50% precipitates of

TABLE III. Spreading and Beating Activities of Extracts Obtained from Rat Various Organs and Bovine Serum

Sample[a)]	Spreading %[b)]	Relative beating %[b)]
Solvent	15.5±2.4	36.2± 5.6
Brain	22.0±1.1	7.5± 1.7[c)]
Liver	25.7±3.6	4.4± 4.4[d)]
Spleen	17.5±0.6	4.9± 2.5[c)]
Pancreas	24.2±8.6	0
Small intestine	25.7±9.7	1.0± 1.0[c)]
Kidney	15.3±4.6	15.6± 3.5[d)]
Skeletal muscle	8.6±1.7[c)]	27.0±15.6
Serum	39.3±1.3[c)]	73.0± 2.2[c)]

a) Added 0.55 mg for spreading and 11 mg for beating into each dish for organ extracts, added 3.8 mg for both assays into each dish for serum extract.
b) Mean±s.e. ($n=3$).
c) $p<0.01$: significantly different from solvent value.
d) $p<0.05$: significantly different from solvent value.

brain, pancreas, small intestine and skeletal muscle showed the significant decrease of attached cell number. Extract of liver, spleen, kidney and serum did not change the number of attached cells from the value in the solvent experiment, as well as culture filtrate and ventricle extract. Spleen and kidney extracts did not affect spreading % in the same dose concentrations as those of ventricle extract. Brain, liver, pancreas and small intestine extracts gave a little increase without a significant difference, and that of skeletal muscle significantly decreased spreading %. In all dishes added with organ extracts, there were seen few complete well-spreading cells, in other words, there were many degenerated spreading cells which become to shortening to poor spreading cells rather than going to well spreading cells. In the experiment of these organ extracts, such cells were scored only spreading but not well-spreading. The organ extracts except skeletal muscle, appearently decreased beating %. Also, there were observed the degenerated cells, a part of which were still beating, in the dishes incubated with these samples. On the other hand, serum extract significantly increased spreading % and relative beating % like culture filtrate and ventricle extract. Thus, the effects of culture filtrate and ventricle extract on spreading and beating phenomena of myocardial single cells in serum-free culture, entirely differed from the other organ extracts, and they were similar to only serum extract.

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Spreading and Beating Activities of Fr. A

Fr. A, inotropic protein of bovine heart, which was effective for promoting the beating behaviors of myocardial cells cultured with serum,[1] was applied to the present assay. As shown in Table IV, Fr. A was ineffective for the maintenances of spreading and beating of the cells cultured without serum. Consequently, it was considered that an active principle in ventricle extract was different from Fr. A.

Table IV. Spreading and Beating Activities of Fr. A

Sample	Final concentration (M)	Spreading %[a]	Relative beating %[a]
Solvent	0	16.8±1.8	45.3± 9.2
Fr. A	3×10^{-6}	13.8±2.7	0
Fr. I-B	2×10^{-5}	0.5±0.3[b]	0
Epinephrine	4×10^{-8}	12.7±1.7	64.2±16.0
Oxytocin	5×10^{-7}	25.6±2.8[c]	34.6± 9.5

a) Mean±s.e. (n=4).
b) $p<0.01$: significantly different from solvent value.
c) $p<0.05$: significantly different from solvent value.

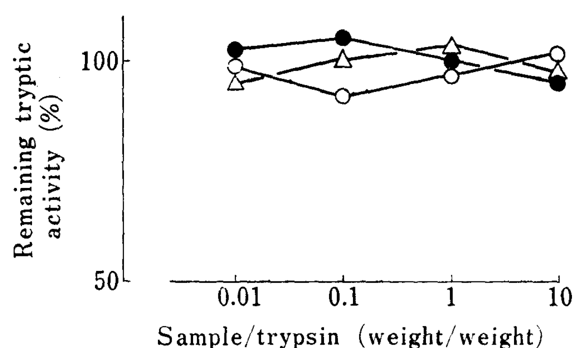

Fig. 5. Reaction of Trypsin with Desalted Culture Filtrates and Bovine Ventricle Extract

○: desalted culture filtrate of neonatal rat ventricle.
●: desalted culture filtrate of adult rat ventricle.
△: bovine ventricle extract.

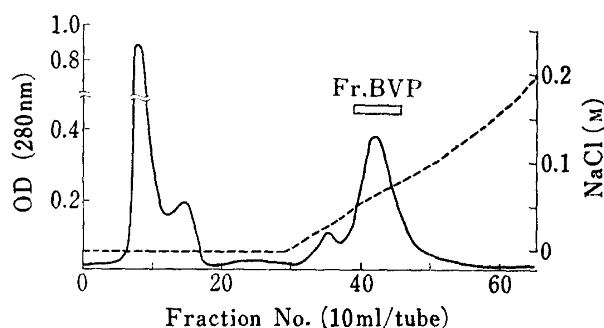

Fig. 6. Final Purification of Active Principle on CM-Cellulose Column

The active fraction obtained from Sephadex G-100 column chromatography was dissolved in 20 ml of 10 mM sodium phosphate buffer (pH 6.0) and applied on column (1.7×30 cm). At fraction No. 21 the gradient elution by 225 ml each of buffer and 0.2 M NaCl in buffer was started. The fraction of No. 38—47 named Fr. BVP.

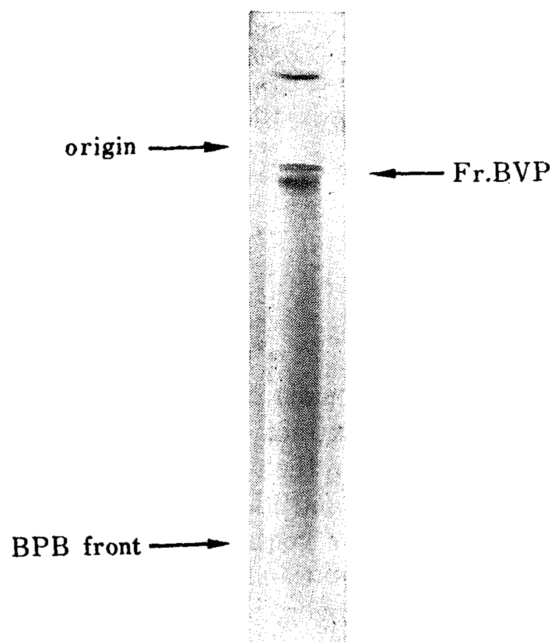

Fig. 7. Electrophoretic Pattern of Fr. BVP

Sample: Fr. BVP (40 μg) treated with 2% sodium dodecyl sulfate (SDS) in 50% glycerol-0.02 M phosphate buffer (pH 7.2) at 50° for 2 hr.
Condition: 10% polyacrylamide gel in 0.1% SDS, 8 mA/tube, 4 hr.
Staining: Amino Black 10B.
Lower: anode.

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Fr. I–B of bovine thymus lipoprotein and epinephrine which possessed inotropic and chronotropic actions,[1] also did not improve spreading and beating. Oxytocin possessing the similar actions showed a significant promoting effect on spreading but not beating.

Trypsin Inhibitory Activities in Culture Filtrates and Extracts of Ventricles

For determination of trypsin inhibitory activities in culture filtrates, filtrates were lyophilized, desalted with Sephadex G-25 column and lyophilized. The yields of high molecular weight fractions were 46 mg and 104 mg from 100 ml of neonatal and adult ventricle culture filtrates, respectively. Needless to say, the desalted fractions were effective on spreading and beating assays in doses of 50 μg/dish for neonatal and 30 μg/dish for adult. As shown in Fig. 5, the desalted fractions of culture filtrates and the bovine ventricle extract did not inhibit tryptic activity in the range of 10 to 0.01 of sample/trypsin ratio. The extracts of rat and rabbit ventricles did not react with trypsin, too.

Fractionation of Active Principle in Bovine Ventricle Extract

In order to isolate an active principle in ventricle, 30—50% precipitate of bovine ventricle which was accessible in a large amount, was fractionated as follows. Two g of precipitate dissolved in 5 mM phosphate buffer (pH 8.0) was filtrated through DEAE-cellulose column using the same buffer, and the filtrate which showed a high absorption peak at 280 nm was dialyzed against deionized water and lyophilized. The dried powder (310 mg) was dissolved in 50 mM phosphate buffer (pH 8.0), chromatographed on Sephadex G-100 column and collected in 12 ml fraction. A fraction which eluted at a position of fraction No. 34—38 was dialyzed and lyophilized. Finally, the active principle was purified by the chromatography on CM-cellulose column as illustrated in Fig. 6, dialyzed, lyophilized and named Fr. BVP (bovine ventricle protein). The yield was 28 mg and that from native ventricle was about 0.002%. A homogeneity of this fraction was demonstrated in a symmetric peak in the gradient elution on CM-cellulose column (Fig. 6) and in a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 7). As shown in Table V, Fr. BVP was a protein which possessed a molecular weight of 100000, and did not contain carbohydrate. Its amino acid composition was summarized in Table V and there existed 18 kinds of standard amino acids.

TABLE V. Molecular Weight and Chemical Composition of Fr. BVP

Molecular weight	100000 (gel filtration method using Sephadex G-100)					
Protein content	99.8% (Lowry-Folin metod)					
Amino acid composition	Asp (8.0)[a]	Thr (9.1)	Ser (13.8)	Glu (13.2)	Pro (4.4)	Gly (7.5)
	Ala (4.2)	Cys/2 (1.9)	Val (12.2)	Met (0.7)	Ile (2.2)	Leu (7.2)
	Tyr (3.9)	Phe (2.1)	Lys (3.6)	His (1.4)	Arg (2.7)	Trp2.0)
Carbohydrate content	Negligible (phenol-H_2SO_4 method)					

a) Molar ratio (%) of standard amino acid by hydrolysis with 6 N HCl at 110° for 24 hr and by ultraviolet absorption method for Trp.

TABLE VI. Spreading and Beating Activities of Fr. BVP

Sample	Final concentration (μg/dish)	Spreading[a]		Beating[a]	
		Spreading %	Well-spreading %	Relative beating %	Relative beating rate
Solvent	0	21.6±0.2	15.9±1.8	43.6± 5.0	23.0±0.9
Fr. BVP	10	27.0±0.9[b]	18.9±1.7	64.0±10.4	24.3±2.0
Fr. BVP	100	29.9±1.0[b]	28.5±0.8[b]	70.5± 3.6[b]	36.6±4.2[c]

a) Mean±s.e. ($n=3$).
b) $p<0.01$: significantly different from solvent value.
c) $p<0.05$: significantly different from solvent value.

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Fr. BVP significantly maintained spreading and beating of myocardial cells in serum-free culture in a concentration of 100 μg/dish as shown in Table VI. A concentration of 10 μg/dish resulted in significant increase of spreading % and slight increase of well-spreading and relative beating %, but in no action on beating rate.

Discussion

Beating phenomenon of myocardial single cells in culture is a basis of beating of a whole heart *in vitro* and *in vivo*. It was reported that well-spreading cell is a healthy and well differentiated[7] and that myocardial single cells cultured in Eagle MEM supplemented with serum spread well and beat regularly,[1,8] *i.e.* the supplement of serum to culture medium stimulated the functions of spreading and beating. While, fractions which promote spreading or division of some strain cells were partially fractionated from serum.[9] In the present paper, we attempted the assay system for the factor influencing the maintenances of spreading and beating myocardial cells in serum free culture to avoid the direct action of serum.

It is reasonable to regard that myocardial cells secrete the material which promote the spreading behaviors of themselves in culture, as shown in Fig. 3. In spite of the constancy of protein content, the spreading activity decreased from 4th day to 8th day. It is a valid interpretation that active component was inactivated or much of nutrients in medium were used up with time in culture. Culture filtrates of neonatal and also adult rat ventricle fragments and in addition, extracts from rat, bovine and rabbit ventricles promoted spreading and beating of myocardial cells, but extracts of other various organs did not, suggesting a organ specificity. Then, bovine ventricle extract was fractionated for the active principle in heart. The isolated active principle, Fr. BVP, was a protein possessing molecular weight of 100000. It had been reported that extracts of chick embryo[10] and tissues,[11] stimulated growth of some strain cells and tissues in culture, and that the crude nucleoprotein fractions prepared from various embryo tissues stimulated growth of chick heart fibroblasts.[12] Furthermore, Gordon *et al.*[5b] had assumed that there were two different materials in chick embryonic heart-conditioned medium which promote the functions of heart cells in culture, and that one is protein-carbohydrate complex for beating and the other is a protein or protein containing compound for spreading. However, direct chemical analyses of heart conditioned medium might give obscure informations because the medium contained numerous nutrients, heart consisting materials and wastes which could contribute to the functions of heart cells. In fact, in our experiment of rat ventricle culture filtrate, there was optimal concentration of filtrate for the promoting activities of spreading and beating of myocardial cells. The excess concentration of filtrate resulted in apparent inhibitions of both spreading and beating. Fr.BVP was seemed to be a new material differing from substances reported.[5b,10–12]

Since mammalian cells are known to be strongly proteolytic when grown in vitro, the promoting mechanism of spreading of cells might involve inhibition of the cellular tryptic action.[10b] The active fractions were not active as inhibitors of proteolysis by agents like trypsin because the fractions did not inhibit the proteolytic activity of trypsin.

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