[Chem. Pharm. Bull. 28(11)3340—3346(1980)]

Studies on Heart. XX.[1] Further Effects of Bovine Ventricle Protein (BVP) and Antiarrhythmic Peptide (AAP) on Myocardial Cells in Culture[2]

Shigeru Aonuma, Yasuhiro Kohama, Kunihisa Akai, and Setsuko Iwasaki

Faculty of Pharmaceutical Sciences, Osaka University[3]

(Received June 6, 1980)

In order to elucidate the physiological significance of bovine ventricle protein (BVP) and antiarrhythmic peptide (AAP) obtained from bovine heart, we investigated their effects on spreading, beating, arrhythmic movements, macromolecular synthesis, continuous cultivation and calcium incorporation of cultured rat myocardial cells in Eagle's minimum essential medium (MEM) supplemented with or without serum. Both BVP and AAP at 10^{-7} M stimulated the spreading phenomenon and the protein synthesis of myocardial cells in serum-free culture, and promoted prolonged survival with spontaneous beating in culture with 1% serum. As regards the beating properties of myocardial cells, they had different effects. AAP, which improved the arrhythmic movements induced by low potaasium, high calcium or addition of ouabain, depressed the beating rate and the ratio of beating cells at 10^{-7}—10^{-6} M. However, BVP promoted beating even in continuous cultivation over 10 days, but did not affect the arrhythmic movements induced by low potassium or high calcium at 10^{-7}—10^{-6} M. When myocardial cells were cultured in modified Eagle's MEM-0.5% bovine serum albumin at 0.5 mM potassium, 5 mM calcium or 0.2 mM ouabain (which induced arrhythmic movements of cultured cells), their spreading was significantly suppressed. Quinidine, oxytocin and insulin, which improve arrhythmia in the intact heart, stimulated the spreading phenomena and improved the arrhythmic movements induced by low potassium as well as AAP. AAP also decreased the incorporation of ^{45}Ca into myocardial cells, but BVP did not have this effect. It is concluded that AAP shows antiarrhythmic action as a result of depressions of calcium incorporation and potassium effusion, with low excitation and prolonged survival of myocardial cells. BVP shows prolonged survival with spontaneous and strong beating in continuous culture for 10 days.

Keywords——bovine ventricle protein; antiarrhythmic peptide; cultured myocardial cell; beating;, spreading; prolonged survival; macromolecular synthesis; calcium incorporation

During studies on the humoral factors affecting heart function, we previously isolated from bovine heart two different active principles, bovine ventricle protein (BVP), which maintains the spreading and beating phenomena of myocardial cells in serum-free culture,[4] and antiarrhythmic peptide (AAP), which improves the rhythmicity of myocardial cells affected by low potassium, high calcium or addition of ouabain.[1] In order to aid in elucidating the physiological roles of BVP and AAP, this paper describes their effects on spreading, beating, arrhythmic movements, continuous cultivation, macromolecular synthesis and calcium incorporation of myocardial cells in culture.

Experimental

Materials——BVP and AAP were prepared from bovine hearts as described previously.[1,4] Synthetic oxytocin was purified by the method of Aonuma *et al.*[5] Insulin, propranolol and ouabain octahydrate were

1) Part XIX: S. Aonuma, Y. Kohama, K. Akai, Y. Komiyama, S. Nakajima, M. Wakabayashi, and T. Makino, *Chem. Pharm. Bull.*, **28**, 3332 (1980).
2) A part of this work was presented at the 100th Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April, 1980.
3) Location: *133-1, Yamadakami, Suita, Osaka, 565, Japan.*
4) S. Aonuma, Y. Kohama, K. Akai, Y. Muramoto, and S. Nakajima, *Chem. Pharm. Bull.*, **26**, 2957 (1978).
5) S. Aonuma, K. Tanaka, and H. Akamatsu, *Nippon Naibunpi Gakkai Zasshi*, **45**, 387 (1970); S. Aonuma and Y. Kohama, *ibid.*, **47**, 1008 (1972).

products of Sigma Chemical Co. Quinidine sulfate was obtained from Wako Pure Chemicals Ltd., ajmaline (crystals) from Nakarai Chemical Ltd., $^{45}CaCl_2$ from New England Nuclear and leucine-4,5-^{3}H, uridine-5-^{3}H and thymidine methyl-^{3}H from the Radiochemical Centre.

Culture of Rat Myocardial Cells——The procedures for preparation, measurements of macromolecular synthesis and antiarrhythmic movements, and beating and spreading assays of rat myocardial cells in 2-day culture were described previously.[4,6] Briefly, for spreading assay in serum-free culture, myocardial single cells (10^5 cells/dish) were cultivated in a CO_2-incubator for 2 days with the test sample in Eagle's minimum essential medium (MEM) buffered with 10 mM N,N'-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) and 10 mM $NaHCO_3$ and supplemented with 0.5% bovine serum albumin, 0.013% penicillin G potassium and 0.02% dihydrostreptomycin sulfate at 37°. Attached cells and spreading cells in 6 mm^2 on the bottom surface of the dish were counted with an inverted phase-contrast microscope (Nikon MD), and the ratio of spreading cells to attached cells (spreading %) was calculated. The spreading % of myocardial single cells cultured in low or high potassium and low or high calcium media was also investigated. In the assay system for beating, the cells (10^5 cells/dish) were cultivated in Eagle's MEM supplemented with 10% bovine serum, 15 mM $NaHCO_3$ and antibiotics for 2 days. The medium was then changed to Eagle's MEM buffered with 10 mM BES or the medium supplemented with 10% serum, and the test sample was added. The number of beating cells among more than 50 myocardial single cells and the beating rate of more than 10 cells were counted just before and 1 hr after sample addition, and the percentage of beating cells and the mean beating rate were calculated. For continuous cultivation for 10 days, the cells (1.5×10^6 cells/dish) were cultivated with a test sample in Eagle's MEM buffered with 10 mM BES and 10 mM $NaHCO_3$ and supplemented with 1% serum and antibiotics. The culture medium containing the test sample was changed every 2 days, and the cells were observed at the first, 2nd, 4th, 6th, 8th and 10th days under a microscope. The viability of cells was tested by neutral red staining. The measurement of ^{45}Ca incorporation into cells was carried out as described by Goshima *et al.*[7] Briefly, single cells (10^5 cells/dish) were cultivated for 2 days in Eagle's MEM supplemented with 10% serum and washed four times with a solution that consisted of 125 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 5.5 mM glucose and 10 mM BES (pH 7.3). After further equilibration with the solution for 5 min, the cells were incubated with 3 ml of the solution containing the test sample and 2 μCi of $^{45}CaCl_2$. The cells were incubated for 20 min, then washed four times with the solution. The washed cells were suspended in 2 ml of 0.1 N NaOH and a half of the NaOH solution was used for measurement of radioactivity by a standard liquid scintillation procedure. The other half was used for determination of protein content by a micro biuret method.[8]

Results and Discussion

Effects on Spreading, Survival, Organization and Macromolecular Synthesis

Table I shows the effects of AAP on the phenomena of attachment and spreading of myocardial cells in serum-free culture for 2 days. AAP significantly increased the spreading %, and showed the maximum effect at 10^{-7} M. As described previously, BVP did not influence the attached cell number, but significantly increased the spreading % in serum-free

Table I. Effect of AAP on the Attachment and Spreading of Myocardial Single Cells in Serum-free Culture

Sample	Final concentration (M)	Attachment (cell No./6 mm^2 mean ±s.e., $n=5$)	Spreading %[a] (mean ±s.e., $n=5$)
Solvent	0	302± 9	22.8±1.0
AAP	10^{-9}	307± 9	24.9±2.4
AAP	10^{-8}	287± 7	30.4±1.3[b]
AAP	10^{-7}	298±11	39.7±1.2[b]
AAP	10^{-6}	296±21	38.8±1.2[b]

a) Spreading $\% = \frac{\text{spreading cells}}{\text{attached cells}} \times 100$ in an area of 6 mm^2.
b) $p<0.01$: significantly different from solvent value.

6) a) S. Aonuma, Y. Kohama, K. Akai, and M. Fujioka, *Chem. Pharm. Bull.*, **26**, 709 (1978); b) S. Aonuma, Y. Kohama, K. Akai, T. Morita, S. Nakajima, N. Maeda, and M. Sakamoto, *ibid.*, **27**, 1008 (1979).
7) K. Goshima, K. Owaribe, H. Yamanaka, and S. Yoshino, *Infect. Immun.*, **22**, 821 (1978).
8) R.F. Itzhaki and D.M. Gill, *Anal. Biochem.*, **9**, 401 (1964).

culture at a concentration of 10^{-7} M.[4] The spreading phenomenon of myocardial cells in culture is thought to be an indication of their health, differentiation, survival, organization and growth,[9] and it is known that the prolonged survival and growth of somatic cells in culture are affected by humoral factors such as hormones[10] and proliferation-stimulating substances.[11] Single, separate myocardial cells in culture with 10% serum grow to beating fiber-like masses upon appropriate continuous cultivation.[11] The effects of BVP and AAP, which influence the functions of myocardial cells in short-term culture, on the survival and the organization of myocardial cells in continuous culture with 1% serum were observed. Myocardial single cells cultured in Eagle's MEM supplemented with 10% serum became attached to the surface of the dish within the first day, then spread out and put out long protoplasmic extensions with spontaneous beating to form a network of connected cells. The individual cells were connected to each other with spontaneous synchronous beating at the 2—8th days, as shown in Fig. 1. Subsequently, the myocardial cells migrated to form beating centers which consisted of many

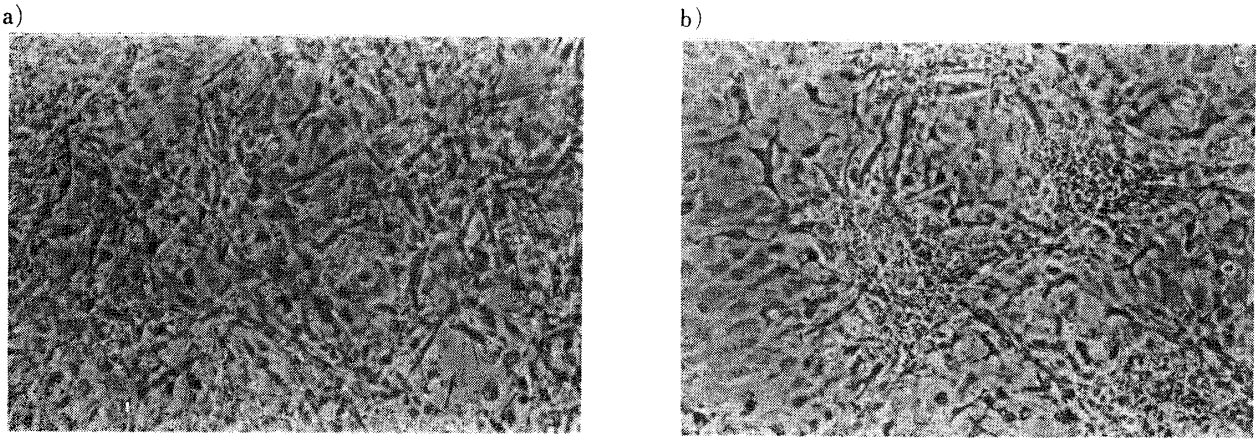

Fig. 1. Typical Morphologies of Myocardial Cells in Continuous Culture with 10% Serum (×160)

a) At the 6 th day. b) At the 10 th day.

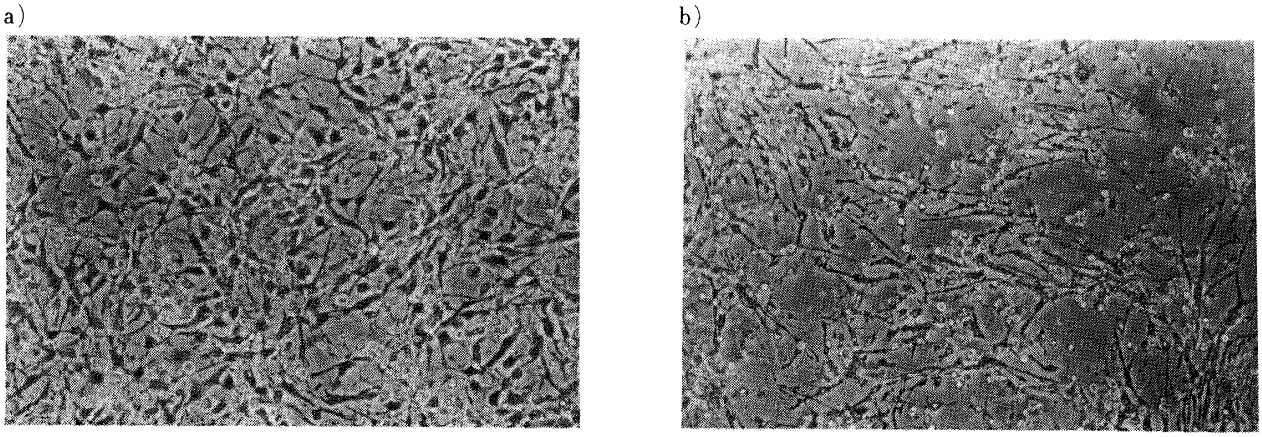

Fig. 2. Typical Morphologies of Myocardial Cells in Continuous Culture with 1% Serum (×160)

a) At the 6 th day. b) At the 10 th day.

9) H.P. Gordon and M.C. Brice, *Exp. Cell Res.*, **85**, 303 (1974).
10) I. Hayashi, J. Larner, and G.H. Sato, *In Vitro*, **14**, 23 (1978); P. Schelling, D. Ganten, G. Speck, and H. Fisher, *J. Cell. Physiol.*, **98**, 503 (1979); J.B. Backer, R.L. Simmer, K.C. Glenn, and D.D. Cunningham, *ibid.*, **98**, 561 (1979).
11) D. Gospodarowicz and J.S. Moran, *Ann. Rev. Biochem.*, **45**, 531 (1976).

closely crowded cells, and developed fiber-like masses at the 10th day, as reported by Harary and Farley.[12] As shown in Fig. 2, in the case of the culture in Eagle's MEM—1% serum, myocardial cells grew and beat till the 4th day in the same way as in 10% serum, but at the 6—8th days, the elongated parts of some cells began to shrink and most of the cells were not stained by neutral red, indicating cell death, by the 10th day. The spontaneous beating stopped by the 6th day. Upon culture with BVP (10^{-7} M) or AAP (10^{-7} M) in the medium supplemented with 1% serum, myocardial cells grew and beat until the 6th day as well as they did in the culture with 10% serum and showed a tendency to form beating centers from the 6th to 10th day, without shrinkage or death even at the 10th day, as shown in Fig. 3. The cells with BVP continued to beat as synchronously and strongly over the 10-day period as those with 10% serum. The cells with AAP also continued to beat synchronously over the period, but their beating rate and intensity were clearly lower than those with BVP and 10% serum. Thus, BVP and AAP clearly prolonged the survival of myocardial cells in continuous culture with 1% serum.

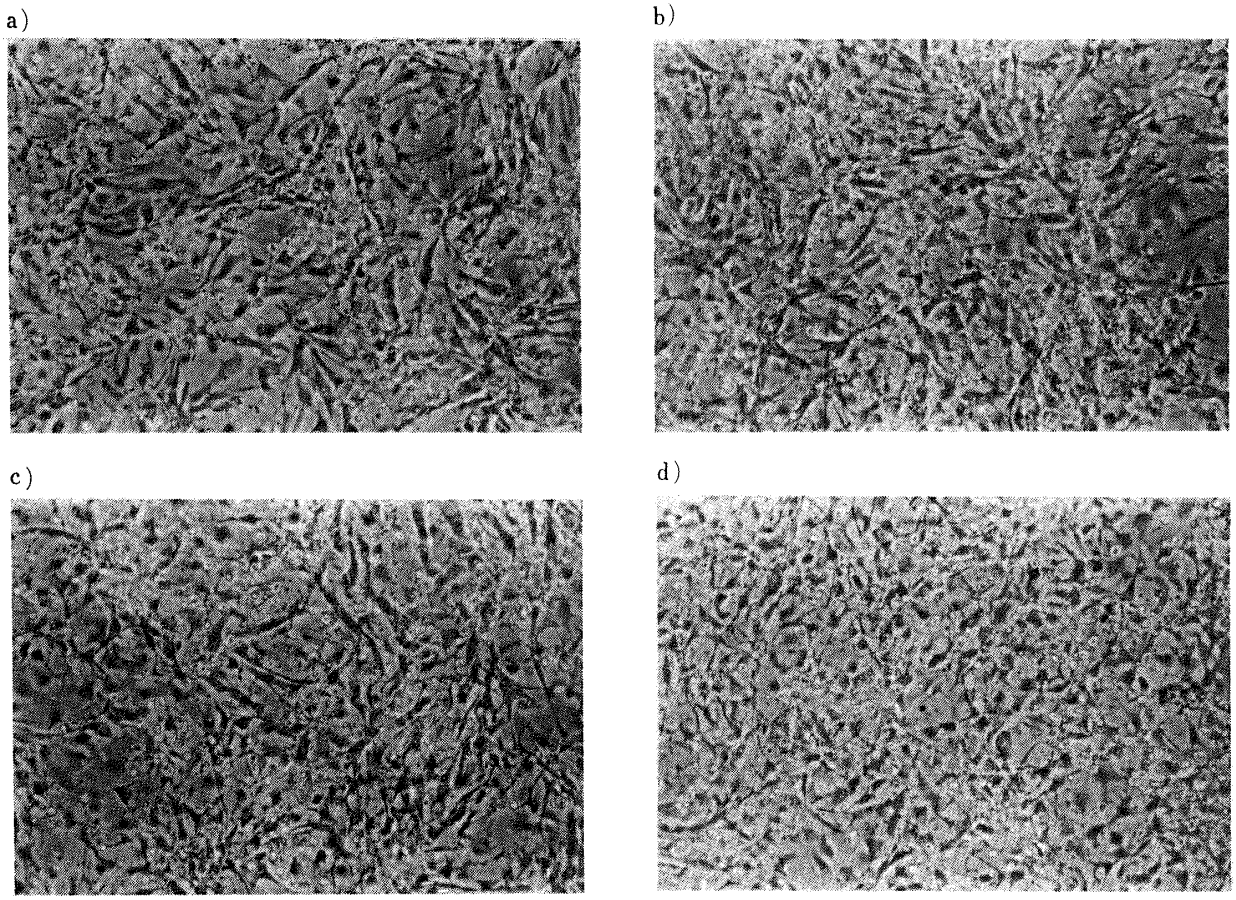

Fig. 3. Typical Morphologies of Myocardial Cells in Continuous Culture with 1% Serum and BVP or AAP (×160)

a) At the 6 th day with BVP (10^{-7} M). b) At the 10 th day with BVP (10^{-7} M). c) At the 6 th day with AAP (10^{-7} M). d) At the 10 th day with AAP (10^{-7} M).

We previously reported that myocardial cells cultured for 2 days with 10% serum showed healthy morphology and increased macromolecular synthesis.[6b] The effects of BVP and AAP on macromolecular synthesis by myocardial cells were investigated in the culture system

12) I. Harary and B. Farley, *Science*, **132**, 1839 (1960).

Table II. Effects of BVP and AAP on the Macromolecular Synthesis of Myocardial Cells in Serum-free Culture

Sample	Final concentration (M)	Incorporation of ^{3}H-compound into cells (cpm/10^5 cells)					
		Protein precursor	Protein	RNA precursor	RNA	DNA precursor	DNA
Solvent	0	260±24	137±22	9630±376	4563±591	697± 58	1215±172
BVP	10^{-7}	270±37	281± 9[a]	10564±268	4050±514	842±116	1572±268
AAP	10^{-7}	307±29	345±61[a]	8815±923	3252±438	1251±213[b]	1388±304

Cells (10^5 cells/dish) were incubated for 48 hr with ^{3}H-leucine (1 μCi), ^{3}H-uridine (1 μCi) or ^{3}H-thymidine (1 μCi). Data are means ±s.e., $n=5$.
a) $p<0.01$: significantly different from solvent value.
b) $p<0.05$: significantly different from solvent value.

used for the spreading assay. When single myocardial cells were cultured with BVP or AAP and tritiated protein, RNA or DNA precursors in serum-free medium for 48 hr, both samples showed significantly increased protein synthesis (Table II). It seems that the promoting effects of BVP and AAP on the growth of myocardial cells in culture are at least in part due to their stimulating effects on the protein synthesis of the cells.

Effects on Beating Properties

As mentioned above, BVP and AAP showed slightly different effects on the beating properties of myocardial cells in continuous culture with 1% serum. In previous experiments, BVP increased the percentage of beating cells and the beating rate of myocardial cells in serum-free culture at a concentration of 10^{-7} M.[4] However, in this experiment it was found that BVP did not influence the percentage of beating cells or the beating rate of myocardial cells in culture with 10% serum, and also had no effect on the arrhythmic movements of myocardial cells induced by low potassium or high calcium in the presence of 2.5% serum in the concentration range of 10^{-8}—10^{-6} M. As shown in Table III, AAP decreased the relative beating % in serum-free culture at 10^{-6} M and the relative beating % and rate in culture with 10% serum at 10^{-7}—10^{-6} M. Thus, the effects of BVP and AAP on the beating properties with arrhythmic movements were different.

Table III. Effect of AAP on the Beating of Myocardial Single Cells in Culture

Sample	Final concentration (M)	Relative beating %[a] (mean ±s.e., $n=5$)		Relative beating rate[b] (%, mean ±s.e., $n=3$)
		Serum-free culture	Culture with serum	Culture with serum
Solvent	0	43.1±2.2	100.0±2.1	98.8±1.6
AAP	10^{-8}	38.5±2.9	103.5±8.0	
AAP	10^{-7}	38.0±3.6	74.3±9.4[c]	87.3±3.0[c]
AAP	10^{-6}	33.2±2.6[c]	64.3±6.7[c]	77.8±4.6[d]

a) Relative beating $\% = \frac{\%\text{ of beating cells 1 hr after sample addition}}{\%\text{ of beating cells before sample addition}} \times 100$ in more than 50 cells.
b) Relative beating rate $= \frac{\text{mean beating rate 1 hr after sample addition}}{\text{mean beating rate before sample addition}} \times 100$ in more than 10 cells.
c) $p<0.05$: significantly different from solvent value.
d) $p<0.01$: significantly different from solvent value.

Relation of AAP to Spreading and Arrhythmic Movements

To aid in understanding the effect of AAP on myocardial cells in culture, we investigated the mechanism of arrhythmic movements of the cells. The myocardial cells showed arrhythmic movements in a medium with low potassium or high calcium, or upon addition of

Table IV. Effects of Potassium, Calcium and Ouabain on the Spreading of Myocardial Single Cells in Serum-free Culture

Medium	Final concentration (mM)			Spreading %[a] (mean ±s.e., $n=5$)
	Potassium	Calcium	Ouabain	
Normal	5.4	1.8	0	21.3±1.1
Low potassium	0.5	1.8	0	15.1±1.1[b]
High potassium	54	1.8	0	22.7±1.3
Low calcium	5.4	0.2	0	22.9±0.8
High calcium	5.4	5.0	0	13.8±0.9[b]
Ouabain	5.4	1.8	0.2	17.0±0.7[b]

a) Spreading $\% = \frac{\text{spreading cells}}{\text{attached cells}} \times 100$ in the area of 6 mm².
b) $p<0.01$: significantly different from normal value.

ouabain.[1,13] Table IV shows the spreading % of myocardial cells when the potassium or calcium concentration was changed or when ouabain was added to the culture medium. The media with low potassium, high calcium and added ouabain all significantly decreased the spreading % of cells, but the media with high potassium and low calcium did not induce any arrhythmic movements or change the spreading %. The intracellular ion concentrations are strongly influenced by the extracellular ion concentrations. Furthermore, the movements of calcium and potassium through the membrane of myocardial cells seem to be inversely related; for example, when the intracellular potassium concentration decreases, the intracellular calcium concentration increases.[14] Ouabain inhibits the Na–K ATPase of the myocardial cell membrane and leads to a decrease of intracellular potassium concentration and an increase of intracellular calcium concentration at high doses.[15] It is considered that increase of calcium concentration and decrease of potassium concentration in the myocardial cells induce poor spreading, and that the maintenance of spreading may be very important for spontaneous rhythmic beating of myocardial cells in culture. The relationship between spreading phenomena and arrhythmic movements was studied further and the effects of some compounds which are known to improve arrhythmia in the intact heart on the spreading phenomenon and the arrhythmic movement induced at 0.7 mM potassium are summarized

Table V. Effects of Antiarrhythmic Drugs on the Spreading of Myocardial Single Cells in Serum-free Culture and on the Arrhythmic Movement of Myocardial Cell Clusters

Sample	Spreading %[a] (mean ±s.e., $n=5$)	Effect on arrhythmic movement induced by 0.7 mM potassium
Solvent	22.8±1.0 (0)[c]	—
Quinidine	31.5±1.9[b] (1×10^{-6})	Improvement (10^{-5}—3×10^{-5})[c]
Oxytocin	34.7±3.8[b] (5×10^{-7})	Improvement (5×10^{-8}—5×10^{-7})
Insulin	32.0±1.8[b] (2×10^{-7})	Improvement (2×10^{-4}—4×10^{-4})
Ajmaline	22.5±1.9 (5×10^{-6})	No improvement (5×10^{-8}—5×10^{-3})[d]
Propranolol	20.1±1.6 (5×10^{-6})	No improvement (5×10^{-8}—10^{-4})[d]

a) Spreading $\% = \frac{\text{spreading cells}}{\text{attached cells}} \times 100$ in the area of 6 mm².
b) $p<0.01$: significantly different from solvent value.
c) The final concentration (M) is given in parentheses.
d) Beatings stopped at the maximum dose.

13) K. Goshima, *J. Mol. Cell. Cardiol.*, **8**, 217 (1976).
14) D. Noble, "The Initiation of the Heartbeat," Gakkai Shuppan Center, Tokyo, 1977, p. 148.
15) M. Tanaka and S. Fujino, "Kyoshinyaku no Yakuri," Asakurashoten, Tokyo, 1971, p. 171.

in Table V. Quinidine, oxytocin and insulin caused significant increases of spereading % and improvement of rhythmicity of myocardial cells in culture, as did AAP.[1] However, ajmaline and propranolol, which show antiarrhythmic effects mainly because of their antiadrenergic action instead of by direct action, caused neither an increase of spreading % nor an improvement of rhythmicity of myocardial cells in culture. It is widely known that insulin together with glucose stimulates the incorporation of potassium into somatic cells,[16] that oxytocin acts on the transport of potassium and calcium through the uterine cell membrane[17] and that quinidine suppresses potassium effusion from heart cells.[18]

Table VI. Effects of BVP and AAP on the Incorporation of ^{45}Ca in Cultured Myocardial Cells

Sample	Final concentration (M)	Incorporation of ^{45}Ca[a] (cpm/mg protein, mean ±s.e., $n=5$)
Solvent	0	1462±262
BVP	10^{-7}	1222±323
AAP	10^{-7}	613±323[b]

a) See "Experimental" for details.
b) $p<0.01$: significantly different from solvent value.

Table VI shows the effects of BVP and AAP on the incorporation of ^{45}Ca into myocardial cells. AAP significantly depressed the incorporation of ^{45}Ca at 10^{-7} M, which is a concentration sufficient to influence the phenomena of spreading and beating, whereas BVP had no effect.

Judging from these results, we consider that the antiarrhythmic effect of AAP arises at least partly because AAP depresses the incorporation of calcium into myocardial cells and the effusion of potassium from the cells, in addition to its effect of reducing the excitation of myocardial cells (Tables III and VI). The reason why BVP did not improve the rhythmicity may be that it could not depress the excitation of the cells or modify the transport of calcium through the cell membrane. The action of BVP in cultures with more than 2.5% serum might be masked by factors in the serum.

16) D. Sodi-Pallares, M.R. Testelli, B.L. Fisher, A. Bisteni, G.A. Medrano, C. Friedland, and De Micheli, *Am. J. Cardiol.*, 9, 166 (1962).
17) A. Csapo, "Oxytocin," ed. by R. Caldeyro-Baracia and H. Heller, Pergamon Press, Inc., New York, 1961, p. 100.
18) Y. Hashimoto, "Rinshoyakurigaku," ed. by H. Yoshida, Asakurashoten, Tokyo, 1977, p. 39.