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Effects of Oxytocin on Beating Properties, Myosin ATPase Activity and Macromolecular Synthesis of Rat Myocardial Cells in Culture[1]

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The effects of oxytocin on the beating properties of rat myocardial cells in cultures supplemented with serum, and on the spreading functions in serum-free culture were investigated.

Oxytocin significantly increased the beating rates of single cells and cell clusters, and made quiescent single cells beat in a 2-day culture with Eagle's minimum essential medium supplemented with 10% bovine serum. The spontaneous activity of myocardial cells increased by oxytocin was not inhibited by the addition of propranolol, contrary to the case with epinephrine. Oxytocin improved the arrhythmia of myocardial cells which showed partial or continuous cellular fibrillation and mild irregular beating induced by lowering the potassium concentration of culture media.

As for the spreading functions, oxytocin increased myosin ATPase activity and protein synthesis, increasing the spreading phenomenon, but did not affect the amounts of ATP and phosphocreatine, or the nucleic acid synthesis of myocardial cells cultured with the sample in serum-free medium for 2 days.

Keywords——oxytocin; cultured myocardial cell; beating; spreading; arrhythmia; myosin ATPase activity; macromolecular synthesis

It has been reported that some hormones, *i.e.*, thyroxine,[3] growth hormone,[3] angiotensin,[4] glucagon,[5] bradykinin[6] and oxytocin,[7] affect heart functions in various ways. These findings reflect the complexity of humoral control of heart functions, in addition to nervous system control. Although reports have appeared on the cardiac effects of oxytocin in a variety of experimental situations, *i.e.*, on antiarrhythmia,[7b,8] positive[7a] or negative[9] chronotropism, positive[7a] or negative[9] inotropism, electrocardiographic change[10] and vasodilation,[7a,11] the physiological significance of oxytocin action on heart functions is still obscure. Recently, Harary *et al.*,[12] Goshima,[13] Wollenberger[14] and Aonuma *et al.*[15] reported that cultured

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3) M. Beznak, *Circulation Res.*, 15, Supple. II, 1964, 14.
4) W. Farr and G. Grapp, *J. Pharmacol. Exp. Ther.*, **156**, 528 (1967).
5) K. Inoue, H. Smulyan, E. Mucha, and R.H. Eich, *Jap. Heart J.*, **14**, 239 (1973).
6) H. Oya, J. Yamamoto, K. Maeda, M. Nakajima, and J. Sekiya, *Nippon Yakurigaku Zasshi*, **67**, 49 (1971).
7) *a*) J. Nakano and R.D. Fisher, *J. Pharmacol. Exp. Ther.*, **142**, 207 (1963); *b*) B.G. Covino, *Am. Heart J.*, **66**, 627 (1963).
8) A. Beaulnes, J-C. Panisset, J. Brodeur, E. Beltrami, and G. Gariepy, *Circulation Res.*, 15, Supple. 11, 1964, 210.
9) R.A. Woodbery and B. Abreu, *Amer. J. Physiol.*, **142**, 14 (1944).
10) *a*) K.I. Melvill and D.R. Varma, *Brit. J. Pharmacol.*, **17**, 218 (1961); *b*) B. Lipton, S.G. Herskey, and S. Baez, *J. Amer. Med. Ass.*, **179**, 410 (1962).
11) A.H. Kichin, S.M. Lloyd, and M. Pickford, *Clin. Sci.*, **18**, 399 (1959).
12) I. Harary and F. Farley, *Exp. Cell Res.*, **29**, 451 (1963).
13) K. Goshima, *Exp. Cell Res.*, **80**, 432 (1973); K. Goshima, *ibid.*, **92**, 339 (1975).
14) A. Wollenberger, *Circulation Res.*, 15, Supple. 11, 1964, 184.
15) *a*) S. Aonuma, Y. Kohama, K. Akai, and M. Fujioka, *Chem. Pharm. Bull.* (Tokyo), **26**, 709 (1978); *b*) S. Aonuma, Y. Kohama, K. Akai, Y. Muramoto, and S. Nakajima, *ibid.*, **26**, 2957 (1978).

myocardial cells retain various functions of differentiation, beating rhythmicity and drug-induced beating properties, essentially similar to those of an intact heart *in vivo*. In order to clarify the direct action of oxytocin on the heart, we therefore investigated the effects of oxytocin on rat myocardial cell functions as regards beating properties, arrhythmic movements, myosin ATPase activity and macromolecular synthesis in culture. Further, we investigated the effects of oxytocin on ^{32}P incorporation and cyclic AMP level in the intact heart of rats.

Experimental

Materials——Synthetic oxytocin (potency with *in vitro* rat uteri, 400 IU/mg) was purified by successive chromatographies on Sephadex G-15 and Amberlite CG-50 columns[16] before use. Quinidine sulfate was purchased from Wako Pure Chemical Ind. Ltd. $H_3{}^{32}PO_4$ (carrier-free) was a product of New England Nuclear. Leucine-4,5-^3H, uridine-5-^3H, thymidine methyl-^3H and cyclic AMP assay kit were products of the Radiochemical Centre.

Measurement of ^{32}P Incorporation and Cyclic AMP Level in the Rat Myocardium——The measurements were carried out as described by the authors.[17]

Culturing, Beating and Spreading Assays of Rat Myocardial Cells——The procedures for culturing, beating and spreading assays of rat myocardial cells were described previously.[15] Briefly, a sample was added to the culture after cultivation for 2 days in the standard medium, and then the relative beating rate and the relative beating % of myocardial cells were measured by microscopic observation. The standard medium was Eagle's minimum essential medium (MEM) buffered with 15 mM $NaHCO_3$ and supplemented with 10% bovine serum, 0.013% penicillin G potassium and 0.02% dihydrostreptomycin sulfate. For spreading assay in serum-free culture, the cells were cultured with a sample in an albumin medium, consisting of MEM buffered at pH 7.3 with 10 mM $NaHCO_3$ and 10 mM N,N'-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES), supplemented with 0.5% bovine serum albumin (BSA) and antibiotics. For microscopic observation of the beating properties of cells, the medium was buffered at pH 7.3 with 10 mM BES instead of $NaHCO_3$ to avoid an increase of pH. Arrhythmic movements of myocardial cell clusters were induced by low potassium concentrations in the culture medium.[18]

Biochemical Assay——The cells (5×10^5) were cultured with the sample in albumin medium for 48 hr. The extraction procedures were carried out at 0°. After cultivation for 48 hr, the medium was decanted off and the plates were washed twice with 3 ml of ice-cold 0.05 M Tris-HCl buffer (pH 7.5) by decantation. The cells were scraped off and homogenized in 4 ml of the same buffer. Myosin ATPase activity of homogenate was measured by the method of Desmond *et al.*[19] For the determinations of ATP and phosphocreatine (PC), a tenth volume of 3.3 M perchloric acid was added to the homogenate and after mixing, centrifugation was carried out at $950 \times g$ for 5 min. In the supernatant, ATP and PC were measured by enzymatic methods.[20] Macromolecular synthesis was investigated as follows. The cells (10^5) were cultivated with the sample and 1 μCi of tritiated leucine, uridine or thymidine in albumin medium. After 48 hr, the cells were washed with phosphate-buffered saline (PBS, pH 7.4) and lysed by homogenization in PBS. Trichloroacetic acid (15%, 2 volumes) was added to the homogenate. After centrifugation at $1500 \times g$ for 10 min, the acid-soluble radioactivity in the supernatant and the radioactivity incorporated in the precipitate were determined using the standard liquid scintillation procedure. The acid-soluble radioactivity represents the intracellular precursor pool.

Results

Effect of Oxytocin on ^{32}P Incorporation and Cyclic AMP Level in Rat Myocardium

We previously reported that when substances affecting cardiac movement were injected into rats together with phosphoric acid-^{32}P, the incorporation of ^{32}P into myocardium was

16) *a*) S. Aonuma, K. Tanaka, and H. Akamatsu, *Nippon Naibunpi Gakkai Zasshi*, **45**, 387 (1970); *b*) S. Aonuma and Y. Kohama, *ibid.*, **47**, 1008 (1972).
17) S. Aonuma and T. Hama, *Yakugaku Zasshi*, **79**, 1436 (1959); T. Hama, *Endocrinol. Japan.*, **7**, 57 (1960); S. Aonuma, Y. Kohama, S. Nakajin, S. Yashiki, H. Egawa, M. Wada, and Y. Komiyama, *Nippon Naibunpi Gakkai Zasshi*, **52**, 93 (1976).
18) K. Goshima, *J. Mol. Cell. Cardiol.*, **8**, 217 (1976).
19) W. Desmond, Jr. and I. Harary, *Arch. Biochem. Biophys.*, **151**, 285 (1972); S.V. Perry, *Methods Enzymol.*, **2**, 582 (1955).
20) W. Lamprecht, P. Stein, F. Heinz, and H. Weisser, "Methods of Enzymatic Analysis," ed. by H.V. Bergmeyer, Academic Press Inc., New York, 1974, pp. 1777—1785, 2101—2111.

increased by stimulatory substances and decreased by inhibitory substances as compared with the control.[17] As shown in Fig. 1, oxytocin significantly increased the incorporation of ^{32}P per total phosphorus into the myocardium of male and female rats at 30 and 120 min, as well as that into the uterus. As regards the myocardium, the incorporation of ^{32}P at 120 min was more than at 30 min, but the incorporation of ^{32}P into the uterus was slower at 120 min. Thus, the time course of incorporation of ^{32}P into the myocardium was different from that into the uterus, suggesting a continuous action of oxytocin on the heart. Table I shows cyclic AMP levels in the myocardium of rats administered oxytocin. At 5 min after the injection of oxytocin, the cyclic AMP level increased to about twice the level at 0 time and then decreased gradually. At 120 min the increase was still significant, as in the case of ^{32}P incorporation mentioned above.

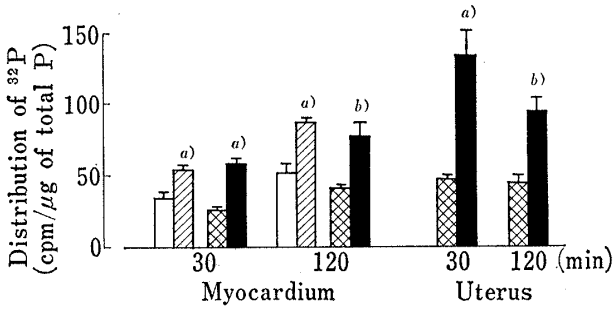

Fig. 1. Incorporation of ^{32}P into the Myocardium of Rats Administered Oxytocin

Each rat ($n=5$) was injected with oxytocin (50 μg/100 g, *i.p.*) and ^{32}P (100 μCi/100 g, *i.p.*) and sacrificed 30 or 120 min later.
□ male control, ▨ male treated with oxytocin,
▩ female control, ■ female treated with oxytocin.
a) $p<0.01$: significantly different from control value.
b) $p<0.05$: significantly different from control value.

TABLE I. Cyclic AMP Level in the Myocardium of Rats Administered Oxytocin

Cyclic AMP level (pmol/mg tissue)[a] Time after injection (min)			
0	5	30	120
0.72±0.07	1.39±0.11[b]	1.19±0.06[b]	1.04±0.04[c]

Rats were each injected with oxytocin (50 μg/100 g, *i.p.*).
a) Mean ± s. e. ($n=4$).
b) $p<0.01$: significantly different from 0 time.
c) $p<0.05$: significantly different from 0 time.

Effect of Oxytocin on the Beating Properties of Myocardial Cells in Culture

Table II shows the effect of oxytocin on the beating rate and beating % of single cells in culture. Fifty ng/ml of oxytocin increased relative beating %, which corresponds to a capacity to stimulate quiescent cells lacking spontaneous activity, but did not affect the relative beating rate. Five hundred ng/ml significantly increased not only the relative beating

TABLE II. Effect of Oxytocin on the Beating of Myocardial Single Cells in Culture

Sample	Relative beating rate[a]	Relative beating %[a]
Solvent	100±2.1	101± 3.4
Oxytocin (50 ng/ml)	95±1.9	131± 6.0[b]
Oxytocin (500 ng/ml)	138±2.7[b]	137± 6.3[b]
Epinephrine (7 ng/ml)	146±5.0[b]	148±10.6[b]

a) Relative beating rate or $\% = \frac{\text{sample value}}{\text{control value}} \times 100$, mean ± s.e. ($n=4$).
b) $p<0.01$: significantly different from solvent value.

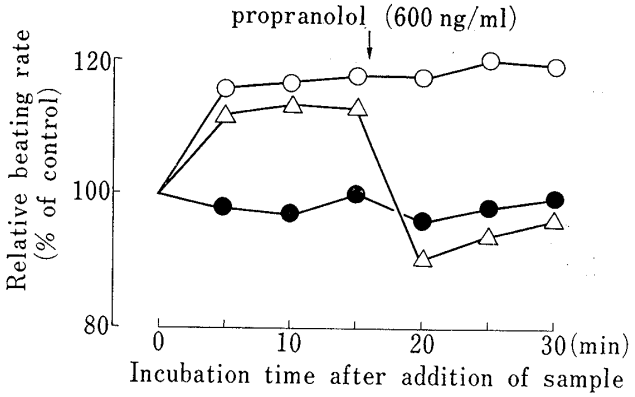

Fig. 2. Effect of Oxytocin on the beating of Myocardial Cell Clusters in Culture

Each point represents the mean of 2 dishes. Propranolol (600 ng/ml final concentration) was added to each dish 16 min after incubation.
(○) oxytocin (500 ng/ml), (△) epinephrine (7 ng/ml), (●) solvent.

% but also relative beating rate, as did epinephrine. The effect on beating cell clusters is illustrated in Fig. 2. Oxytocin also increased the relative beating rate of cell clusters at 500 ng/ml, and this effect was not inhibited by the addition of propranolol, unlike that of epinephrine.

Arrhythmic movements of myocardial cells, such as cellular fibrillation and irregular beating, appeared on lowering the potassium concentration as described by Goshima.[18] A 2-day cell cluster culture (2×10^6), which exhibited rhythmic spontaneous activity in the standard medium (5.4 mM potassium), was placed in KCl-free MEM buffered with 10 mM BES supplemented with 2.5% bovine serum, and adjusted to potassium concentrations of 0.7, 0.6 and 0.5 mM, by addition of 50 mM KCl. The effects of oxytocin on 3 types of arrhythmic movements are shown in Figs. 3 and 4. When the potassium concentration of the

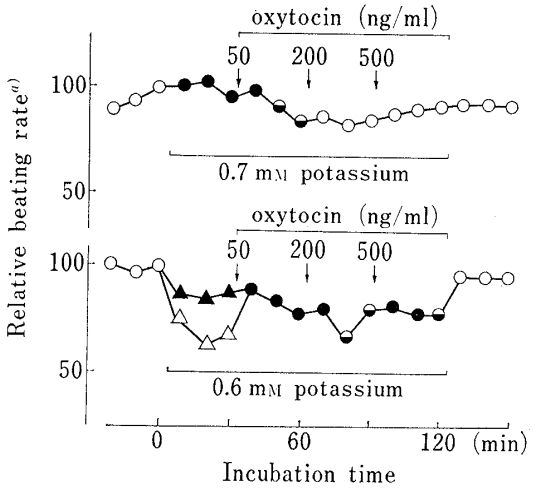

Fig. 3. Effect of Oxytocin on Arrhythmic Movements of Myocardial Cell Clusters Induced by Low Potassium (I)

a) % of beating rate at 0 time in the standard medium.
(○) rhythmic beating, (◒) rhythmic beating with partial cellular fibrillation, (●) rhythmic beating with continuous cellular fibrillation, (▲, △) irregular strong and weak beating with continuous cellular fibrillation; (▲) strong and weak beating counted, (△) only strong beating counted.

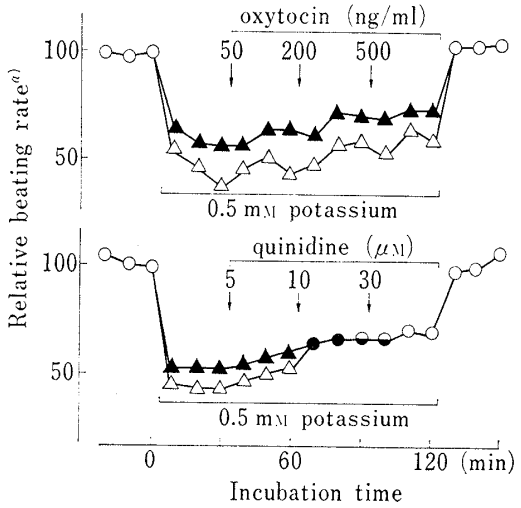

Fig. 4. Effect of Oxytocin on Arrhythmic Movements of Myocardial Cell Clusters Induced by Low Potassium (II)

a) % of beating rate at 0 time in the standard medium.
(○) rhythmic beating, (◒) rhythmic beating with partial cellular fibrillation, (●) rhythmic beating with continuous cellular fibrillation, (▲, △) irregular strong and weak beating with continuous cellular fibrillation; (▲) strong and weak beating counted, (△) only strong beating counted.

medium was 0.7 mM, the cells showed rhythmic beating with continuous cellular fibrillation at more than 90% relative beating rate. This arrhythmia was improved to partial fibrillation and then to normal beating by 50 and 200 ng/ml of oxytocin (Fig. 3). Irregular beating with continuous cellular fibrillation at 70—90% relative beating rate at 0.6 mM potassium, was improved to incompletely rhythmic beating with partial or continuous fibrillation by 200 and 500 ng/ml of oxytocin (Fig. 3). At 0.5 mM potassium, the cells showed irregular beating with

continuous cellular fibrillation at 40—60% relative beating rate, and this degree of arrhythmia was not influenced by oxytocin, though the beating rate increased gradually (Fig. 4). Quinidine sulfate improved such arrhythmia under these experimental conditions (Fig. 4).

Effect of Oxytocin on Myosin ATPase Activity, and ATP, PC and Macromolecular Syntheses in Serum-free Culture

As shown in Table III, addition of oxytocin enhanced myosin ATPase activity to 2.58 μmol Pi/dish/5 min from 1.24 μmol Pi/dish/5 min (solvent value). Serum also increased this

Table III. Effects of Oxytocin on Myosin ATPase Activity, and ATP and Phosphocreatine Levels of Myocardial Cells in Serum-free Culture

Sample	Myosin ATPase activity[a] (μmol Pi/dish/5 min)	ATP[a] (nmol/dish)	Phosphocreatine[a] (nmol/dish)
Solvent	1.24±0.19	9.54±0.62	13.90±0.94
Oxytocin (500 ng/ml)	2.58±0.51[b]	11.22±0.67	15.34±1.96
Serum (100 μl/ml)	2.83±0.16[c]	18.40±1.47[c]	15.10±0.30

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

activity, in agreement with the results of Lewis *et al.*[21] However, oxytocin had no effect on the amounts of ATP and PC in this serum-free culture (Table III). The effect of oxytocin on macromolecular synthesis in myocardial cells in serum-free culture is summarized in terms of the attached cell number and the results of spreading assay in the same experiment in Tables IV and V. As recognized previously,[15b] oxytocin did not change the cell number

Table IV. Effect of Oxytocin on the Macromolecular Synthesis of Myocardial Cells in Serum-free Culture (I)

Sample	Cell No. ($\times 10^5$)	Spreading %	Incorporation of ^{3}H-leucine into cells (cpm/10^5 cells)	
			Protein precursor	Protein
Solvent	1.08±0.08	13.7±1.2	374± 42	118±14
Oxytocin (500 ng/ml)	0.95±0.10	26.1±3.7[a]	920±221[a]	238±23[b]
Serum (100μl/ml)	1.04±0.07	72.9±2.7[b]	760±162[a]	555±35[b]

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

Table V. Effect of Oxytocin on the Macromolecular Synthesis of Myocardial Cells in Serum-free Culture (II)

Sample	Incorporation of ^{3}H-compound into cells (cpm/10^5 cells)			
	RNA precursor	RNA	DNA precursor	DNA
Solvent	7586±426	3002± 309	856±117	1199±119
Oxytocin (500 ng/ml)	9038±405[a]	3425± 204	1025±130	912±114
Serum (100 μl/ml)	26421±626[b]	7259±1613[a]	1868± 54[b]	6749±495[b]

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

21) H. Lewis and I. Harary, *Arch. Biochem. Biophys.*, **142**, 501 (1971).

and significantly increased spreading % (Table IV). Oxytocin increased the incorporation of ^{3}H-leucine in the intracellular precursor pool and protein of myocardial cells as well as in serum (Table IV). Thus, oxytocin stimulated protein synthesis in myocardial cells in serum-free culture. Serum also increased the incorporation of ^{3}H-uridine and ^{3}H-thymidine in RNA and DNA and their precursor pools, while oxytocin increased the incorporation of ^{3}H-uridine in RNA precursor, but did not affect RNA synthesis. The incorporation of ^{3}H-thymidine was not changed by oxytocin.

Discussion

It was reported that oxytocin suppresses the cardiac arrhythmia induced during cyclopropane anesthesia for parturition[22] and also has antiarrhythmic effects in experimental animals.[7b,8] Further, its effects on the coronary artery,[10a] O_2 consumption of myocardium[10a] and electrocardiogram have been described, but the results were not always in agreement. These discrepancies might be due to varieties of purity, dose, administration route of oxytocin and animals. Nakano *et al.*[7a] suggested that the effects of oxytocin on the cardiovascular system were due to an action on peripheral vasculatures. Therefore, we investigated the effects of oxytocin on cultured myocardial cells directly, separated from vasculature, connective tissues and nervous control. It was found that oxytocin had direct effects on myocardial cells, *i.e.*, this hormone stimulated the spontaneous beating of cells in the standard culture. Some investigators[23] noted that a β-adrenoceptor system exists in trypsin-dissociated myocardial cells and may play an important role in the beating function. Since the stimulation by oxytocin was not inhibited by the addition of propranolol, unlike that due to epinephrine, it appears that the β-adrenoceptor system is not involved with the activity of oxytocin. Myocardial cells in culture showed arrhythmic movements under some conditions[18] of low potassium, high calcium concentrations or addition of ouabain, which are known to induce arrhythmia in the whole heart. The present results imply that the antiarrhythmic effect of oxytocin in the whole heart is due to the improvement of arrhythmia at the cellular level by oxytocin.

Previously, we reported that oxytocin promoted the spreading phenomenon of myocardial cells in serum-free culture, and that this phenomenon was indicative of the health and differentiation of cells.[15b] In this work, we studied the spreading properties in more detail. Lewis *et al.*[21] showed that myosin ATPase activity was related to the growth and division of myocardial cells, and that the presence of serum in the medium was important for the maintenance of myosin ATPase activity. Generally, as in research on cell growth, more accurate results could be obtained by the measurement of macromolecular synthesis. DNA varies with the state of cell division and the changes of RNA and protein are associated with cell conditions or functions. Oxytocin stimulated myosin ATPase activity and protein synthesis and increased spreading, but did not affect RNA and DNA syntheses at 48 hr after the start of cultivation. In other words, it seemed that oxytocin accelerated the differentiation of myocardial cells and enhanced their functions.

The effective dose (50—500 ng/ml) of oxytocin for myocardial cells in culture was higher than the normal plasma level of oxytocin, which was determined by means of assays with the uterus or mammary gland, and the immunological specificity paralleled the biological activity.[16b,24] However, under the present experimental conditions, especially in the case

22) L.E. Moris, M.J. Thornton, and J.W. Harris, *Am. J. Obst. Gynec.*, **63**, 171 (1952).
23) R.J. Ertel, D.E. Clarke, J.C. Chao, and F.R. Franke, *J. Pharmacol. Exp. Ther.*, **178**, 73 (1971); K. Goshima, *Exp. Cell Res.*, **84**, 223 (1974).
24) R.C. Barcia and H. Heller, "Oxytocin," Pergamon Press, Oxford, 1961, pp. 358—377, 412—436; J.A. Coch, C. Fielitz, J. Brovetto, H.M. Cabot, H. Coda, and A. Faga, *J. Endocr.*, **40**, 137 (1968); J.S. Robert and L. Share, *Endocrinology*, **84**, 1076 (1969).

of spreading assay after cultivation for 48 hr at pH 7.3 at 37°, oxytocic activity on the uterus or mammary gland must be decreased to a considerable extent by dimerization and inactivation of the molecule. That is to say, it is possible to consider that a lower dose of oxytocin might influence myocardial cell functions, or that activity on the uterus or mammary gland might not reflect the activity on myocardial cells.