The Effect of Acetylcholine on Single Embryonic Chick Ventricle Cells[1] (36101)

ALLEN J. SINCLAIR, WILLIAM H. BARRY, AND DONALD C. HARRISON
Cardiology Division, Stanford University School of Medicine, Stanford, California 94305

In addition to the intact heart, several non-innervated preparations have been used to ascertain direct effects of acetylcholine on myocardial tissue. These include isolated whole hearts (1–8), cardiac fragments (6, 8), trypsin-dissociated cellular clusters (9–11), and single cells (12). Studies of isolated whole hearts (2, 5–8) and ventricular fragments (6, 8) demonstrate the negative chronotropic effect of acetylcholine which has also been established for the innervated intact heart (13). Harary and Farley (12) found a negative chronotropic action on cultured neonatal rat heart cells, while other studies utilizing cultured chick heart cells showed either no effect (10) or a small, inconsistent effect (9, 11) of acetylcholine on the beating rate of ventricular cells. This discrepancy in results provided an opportunity to apply a new, non-invasive monitoring technique developed by us (15) for studying cardiac pharmacology. We considered that it was important to establish whether or not cultured ventricular cells were indeed insensitive to acetylcholine, while ventricular fragments from embryos of the same age are sensitive, because of the implications such a discrepancy would have on the use of the single cultured heart cell model for studying cardioactive agents. Such an alteration in responsiveness would imply significant changes in receptor–membrane interaction induced by the trypsin dissociation or the culturing methods.

Methods. The cultures were prepared by de Haan's method with minor modifications (14). The ventricles of 8 to 9 day chick embryos were removed, cut into pieces of 0.5 mm^3 and washed in calcium-magnesium free Hank's balanced salt solution. The tissue pieces from 5–6 ventricles were presoaked in a Ca^+–Mg^+ free Hank's solution containing 0.5% trypsin for 10 min without stirring at 37°. The supernatant trypsin solution was discarded. The pieces were then incubated with 3.3 ml of 0.05% trypsin solution for 8 min with stirring at 37°. The supernatant suspension was then placed in 20 ml of cold trypsin inhibitor medium composed of 20% M199 nutrient medium, 10% heat-inactivated fetal calf serum, 1% antibiotic solution (10,000 units/ml penicillin and 10,000 μg streptomycin/ml), and 69% K-free balanced salt solution. This trypsinizing procedure was repeated twice more, putting the supernatant suspension into the cold trypsin inhibitor medium each time. After the final dissociation cycle, the cell-containing solution was centrifuged at 1000 rpm for 10 min. The liquid was discarded and the cells were resuspended in a final maintenance medium consisting of 6% heat-inactivated fetal calf serum, 20% M199 nutrient medium, 1% penicillin–streptomycin antibiotic solution, and 73% K-free balanced salt solution. This contained 147 meq/liter sodium and 1.8 meq/liter potassium. It maintained a pH of 7.3 in a 5% carbon dioxide atmosphere. The suspension of cells was diluted with maintenance medium to give a final concentration of about 2×10^5 cells/ml and placed in plastic tissue culture dishes. The cultures were kept for 1–2 days in a humidified 5% carbon dioxide, 95% air atmosphere at 37°. At 1 and 2 days single, spontaneously beating cells were observed.

Experimental observation on the heart cells were made with an electro-optical system described previously (15). Briefly, the tissue culture dish containing the cells was placed

[1] This work was supported in part by N.I.H. Grants Nos. HE-5709 and HE-05866. Mr. Sinclair was supported in part by U.S. Public Health Service Grant No. 5-SO-1-FR-05353.

on the stage of an enclosed, inverted stage phase contrast microscope in a humidified, 5% carbon dioxide, 37° atmosphere. The image of a single cell was viewed from a side arm adapter of the microscope by a TV camera and monitor. A photocell was positioned on the face of the monitor in such a way that the moving cell wall of the beating heart cell traversed the photocell aperture. The photocell was one arm of a Wheatstone resistor bridge in circuit with a power source. The change in voltage across the bridge was recorded by a multichannel oscillographic pen recorder and this primary signal is differentiated to be recorded on a second channel. A cardiotachometer with beat-by-beat response receives input from the amplifier of the primary channel and a beat rate is displayed on a third channel. Using this system rate of beating, cell wall movement amplitude and cell wall velocity can be obtained.

In this study one cell per culture dish was monitored. After recording a two-minute control period, the medium in the dish was withdrawn and replaced with fresh, warm maintenance medium containing the desired dose of acetylcholine or no drug at all for control experiments. Measurements were then made for 2 min intervals at 1½, 5, 9, and 13 min after drug administration. Doses of 10^{-5}, 10^{-7}, 10^{-6}, and 10^{-5} g/ml of acetylcholine as acetylcholine chloride were used. At least five cells were monitored at each dose and five cells with medium containing no acetylcholine. Another series of studies was performed in which drug-containing medium was introduced as above and then washed out with fresh medium at 10 min after drug addition. These cells were monitored immediately prior to drug administration, 5 min after acetylcholine was given and 5 min after washing with fresh medium. Five cells using no drug and 10^{-6} g/ml acetylcholine were studied.

Results. Acetylcholine produced a decrease in the rate of beating of single, cultured ventricular cells (Fig. 1). By unpaired test the 10^{-8} g/ml dose of acetylcholine gave no significant difference from the control rates, but the 10^{-7} g/ml dose showed a significant

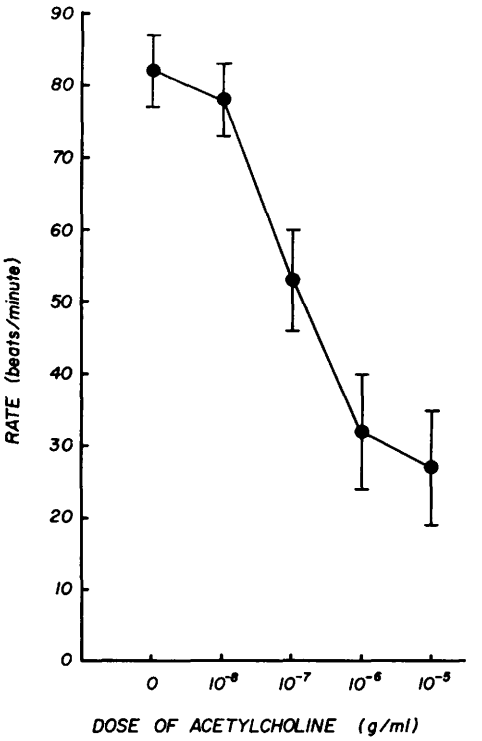

FIG. 1. Effect of acetylcholine on beating rate of single cultured chick embryo heart cells. The circles indicate the means, and brackets the S.E.M. Beating rates were determined 5 to 7 min after drug administration.

($p<.05$) slowing compared to controls. The 10^{-6} and 10^{-5} g/ml doses showed highly significant ($p<.01$) slowing. At the highest dose, 10^{-5} g/ml, 2 of 7 cells stopped beating immediately after the acetylcholine was introduced. Washing out acetylcholine with fresh medium caused an increase in beat rate to a level near the rate measured before adding the drug (Fig. 2). The time course of drug action indicated that most of the rate decrement had occurred before the 1½ – 3½ min interval (Fig. 3). There were no significant differences in pre-drug beating rates between any of the dose groups and the control group.

The amplitude of wall motion and the cell wall velocity were measured prior to drug addition and 5 min after addition for all the control and 10^{-6} g/ml acetylcholine cells (Table I). No significant change in either of these variables was seen with administration

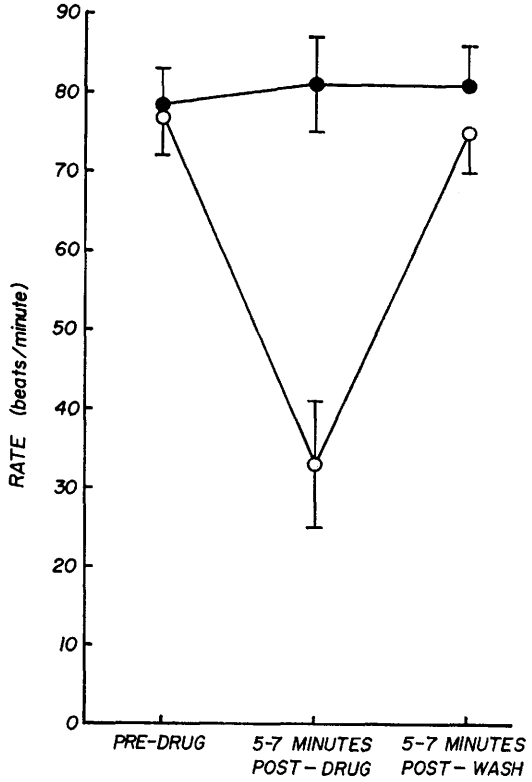

FIG. 2. Effect of 10^{-6} g/ml acetylcholine on beating rate of single cultured embryo heart cells, followed by removal of acetylcholine (open circles) compared to effects on rate of changing medium without addition of acetylcholine (closed circles). The circles indicate the means, the brackets the S.E.M.

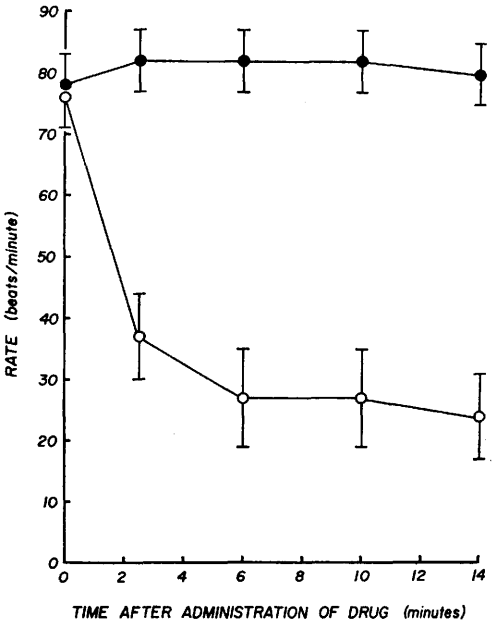

FIG. 3. Time course of negative chronologic effect of 10^{-6} g/ml acetylcholine on eating rate of cultured embryonic heart cells (open circles). The time course of the beating rate of an equal group of cells in which the medium was changed but no acetylcholine was added is indicated by closed circles. The circles indicate the means, the brackets the S.E.M.

of acetylcholine.

Discussion. These data clearly show a negative chronotropic effect of acetylcholine on cultured, single, ventricular cells. Moreover, the effect is long-lasting and reversible. These facts imply that cultured embryonic ventricular cells, as well as embryonic ventricle fragments, are directly affected by acetylcholine. These findings contrast with those of Sperelakis and Kehmkuhl (10) and Nakanishi and Takeda (11) who found insensitivity to acetylcholine in cultured chick embryonic ventricle cells at doses above those used in the present study. There are several differences between these studies and the present one which may account for the discrepancy. First, the previous investigations were done using penetrating microelectrodes to obtain electrophysiological data, as well as to monitor rate of beating. This intervention may have altered the responsiveness of the cultured cell membrane to acetylcholine. Another possible factor is that these investigators worked with large clusters of cells, while in this study the effect of acetylcholine on single isolated beating cells was determined. The mechanics of excitation and im-

TABLE I. Inotropic Effects of Acetylcholine on Cultured Heart Cells.

	Pre-drug control	5–7 min. after 10^{-7} gm acetylcholine
Cell Wall Motion Amplitude ($\mu \pm$ SEM)	3.7 ± 1.5	3.7 ± 1.5
Cell Wall Motion Velocity ($\mu \pm$ SEM)	52 ± 13	51 ± 11

pulse generation in clusters of cultured myocardial cells are not well understood, but pacemaker activity by one or several cells in a cluster seems to take place (16). These pacemakers may have different sensitivities to acetylcholine than do single, spontaneously beating cells. A final possible factor is the culturing technique. Higher concentrations of trypsin, with longer incubation periods, different media, etc., may damage or destroy receptors for neurotransmitter substance which are on cell surfaces. These previous studies used different culturing techniques from those described in the present study.

The data on the amplitude of cell wall motion and cell wall velocity do not suggest a major inotropic effect of acetylcholine on those cells. In studies on myocardial mechanics the maximum velocity extrapolation and the position of the force–velocity curve have been proposed as indicators in the inotropic state of the muscle (17). On the single cell level, a change in contraction velocity would be expected to produce a change in cell wall velocity. The force against which the cell is contracting or afterload will not change as long as the cell maintains the same attachments and configuration, and therefore an increase in contractile force should be indicated by an increase in amplitude of contraction. No change in any of these variables was observed in the experimental cells, thus no change in the inotropic state with acetylcholine was demonstrated.

Questions concerning atrial cell sensitivity compared with ventricular sensitivity and the changes in sensitivity to acetylcholine with increasing embryonic age and development are not well defined (18), but since age variation and atrial cells were not studied in this experiment, we cannot comment on these questions at the present time. It would appear, however, that with the present study there is good evidence that both cultured and non-cultured post-innervation age chick embryo ventricle cells are sensitive to acetylcholine. This system may be of further use in pharmacologic studies involving non-innervated ventricular cells.

Summary. The effect of acetylcholine on the beating rate of single chick embryo heart cells cultured from 8 to 10 day ventricles was determined using a new television monitor optical system. Acetylcholine had a clear negative chronotropic effect on these cells, with a threshold at 10^{-8}–10^{-7} g/ml. The slowing in beating rate after the addition of acetylcholine was reversible, and was not associated with any detectable change in the inotropic state of the cells. These findings are consistent with the effect of acetylcholine on non-cultural chick embryo ventricular tissue of the same age.

1. Armstrong, P. B., J. Physiol. (London) **84**, 20 (1935).

2. Fingl, E., Woodbury, L. A., and Hecht, H. H., J. Pharmacol. Exp. Ther. **104**, 103 (1952).

3. Jones, D. S., Anat. Rec. **130**, 253 (1958).

4. Markowitz, C., Amer. J. Physiol. **97**, 271 (1931).

5. McCarty, L., Lee, W. C., and Shideman, F. E., J. Pharmacol. Exp. Ther. **129**, 315 (1960).

6. Dufour, J. J., and Posternak, J. M., Helv. Physiol. Acta **18**, 563 (1960).

7. Roberts, C. M., Gimeno, M. A., and Webb, J. L., J. Cell. Comp. Physiol. **66**, 267 (1965).

8. Alving, B., Nature (London) **214**, 394 (1967).

9. Fange, R., Persson, H., and Thesleff, S., Acta Physiol. Scand. **38**, 173 (1957).

10. Sperelakis, N., and Lehmkuhl, D., Amer. J. Physiol. **209**, 693 (1965).

11. Nakanishi, H., and Takeda, H., Jap. J. Pharmacol. **19**, 543 (1969).

12. Harary, I., and Farley, B., Science **131**, 1674 1960).

13. Blumenthal, M. R., Wang, H. H., Markee, S., and Wang, S. C., Amer. J. Physiol. **214**, 1280 (1968).

14. DeHaan, R. L., and Gottlieb, S. H., J. Gen. Physiol. **52**, 643 (1968).

15. Sinclair, A. J., Miller, H., and Harrison, D. C., J. Appl. Physiol. **29**, 747 (1970).

16. Lehmkuhl, D., and Sperelakis, N., Amer. J. Physiol. **205**, 1213 (1963).

17. Braunwald, E., Ross, J., Jr., and Sonnenblick, E. H., N. Eng. J. Med. **277**, 794 (1967).

18. Coraboeuf, E., Obrecht-Coutris, G., and Le Douarin, G., Amer. J. Cardiol. **25**, 285 (1970).

Received Sept. 1, 1971. P.S.E.B.M., 1972, Vol. 139.