TETRODOTOXIN SENSITIVITY OF CULTURED EMBRYONIC HEART CELLS DEPENDS ON CELL INTERACTIONS

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INTRODUCTION

Tetrodotoxin (TTX) is a potent and specific inhibitor of the sodium current in nerve and muscle (3, 4, 10) This current is responsible for the rapid rising phase of the action potential in many excitable cells (5) During development of the chick heart the action potentials (12) and pulsatile activity (6) undergo a transition from a TTX-insensitive state at day 4 of incubation to a TTX-sensitive state by day 7: Isolated embryonic myocardial cells and small monolayer sheets are relatively insensitive to TTX at all ages, whereas aggregates of such cells reflect the differentiation taking place in the parent hearts (6).

We here demonstrate that aggregates formed from isolated cells are TTX-sensitive, whereas single cells derived by trypsin-dissociation of such aggregates are TTX-insensitive Further, exposure to dilute trypsin renders intact aggregates TTX-insensitive. The process of resensitization is dependent on protein synthesis.

MATERIALS AND METHODS

Primary monolayer cultures were prepared after the dissociation of 7-day hearts into their component cells by the multiple-cycle trypsinization method (2). Plates were seeded at 2×10^5 cells/35 mm plate in medium 818A and incubated at 37 5°C under a water-saturated atmosphere of 5% CO_2, 10% O_2, and 85% N_2. After 20 h of incubation, plates were washed and placed on the constant-temperature stage (37°C) of an inverted microscope, under an atmosphere identical to that in the culture incubator. Pulsatile activity was determined as previously described (2).

Primary aggregates were prepared by the technique of Moscona (8) after dissociation of hearts into their component cells. Erlenmeyer flasks (25 ml) containing 5×10^5 cells in 3 ml of medium 818A were gassed with 5% CO_2, 10% O_2, and 85% N_2, stoppered, and placed in a gyratory shaker bath (37°C) at 70 rpm After 20 h of gyration, the contents of each flask were transferred to a Falcon tissue culture dish (Falcon Plastics, Division of B-D Laboratories, Inc , Los Angeles, Calif) and allowed to attach to the bottom of the dish during a 2-h period in the culture incubator. The plates were washed and placed on the microscope warm stage. Aggregates ranged in volume from about 5×10^4 μm^3 to 5×10^6 μm^3, an aggregate of 10^5 μm^3 contains approximately 200 cells.

Secondary monolayer cultures were prepared by dissociating the aggregates into single cells by treatment with 0.1% trypsin (1:300, Nutritional Biochemicals Corporation, Cleveland, Ohio) for 15 min. Secondary aggregates were prepared from monolayer cells by the same techniques as primary aggregates after removal of these cells from the dish surface with 0 1% trypsin Such aggregates were comparable to primary aggregates in size and beating rates.

RESULTS AND DISCUSSION

Fig. 1 compares the normalized percentages of aggregates and single cells beating in the presence of 10^{-8} g/ml and 10^{-7} g/ml TTX. The filled circles are values determined for primary aggregates and cells, while the columns are values for secondary aggregates and cells. As reported previously (6), more than 80% of the primary aggregates stopped beating in 10^{-7} g,ml TTX In contrast, primary monolayer cells were relatively insensitive to TTX; 68% of these isolated cells continued beating at this drug concentration. When these cells were formed into secondary aggreates, however, the aggregates (first pair of columns) were as sensitive as primary aggregates (second pair of filled circles). Secondary monolayer

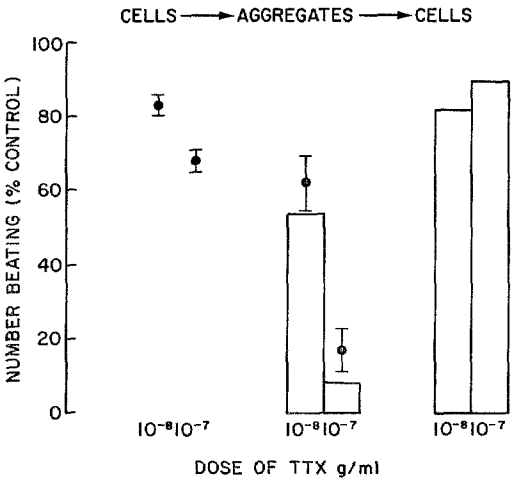

FIGURE 1 Response of primary and secondary cells and aggregates to tetrodotoxin. Primary cells and aggregates (filled circles, mean ± SE, n = 6–10 experiments) were tested for TTX response 20 h after culturing. The absolute values for control percent beating were 37% and 99% for primary cells and aggregates, respectively. Secondary cells and aggregates (columns) (mean values, n = 400 cells, 200 aggregates) were tested about 20 h after subculturing from primary aggregates and cells, respectively. The absolute values for percent beating were 38% and 98% for secondary cells and aggregates, respectively Data were normalized (to 100%) as percent of control beating. TTX responses were measured 15 min after addition of drug to culture dish, and the two doses were tested sequentially.

cells (second pair of columns) cultured from primary aggregates, displayed the same TTX-insensitivity as primary monolayer cells. Thus the expression of TTX-sensitivity would appear to reflect the degree of cellular organization

Since trypsin is known to alter the cell surface (9), we asked whether aggregates exposed to trypsin but not dissociated by the treatment would lose TTX-sensitivity but retain the capacity to restore it

The normal serum-containing medium in the culture dish was replaced with a serum-free medium (60% balanced salt solution, 40% medium 199) Control experiments indicated that more than 95% of the aggregates beat at normal rates during a 4-h incubation in this medium Aggregates were subject to a 20-min treatment with 0 005% trypsin Many aggregates lost their attachment to the culture dish and became rough-surfaced as a result of this treatment, but the aggregates did not become dissociated,

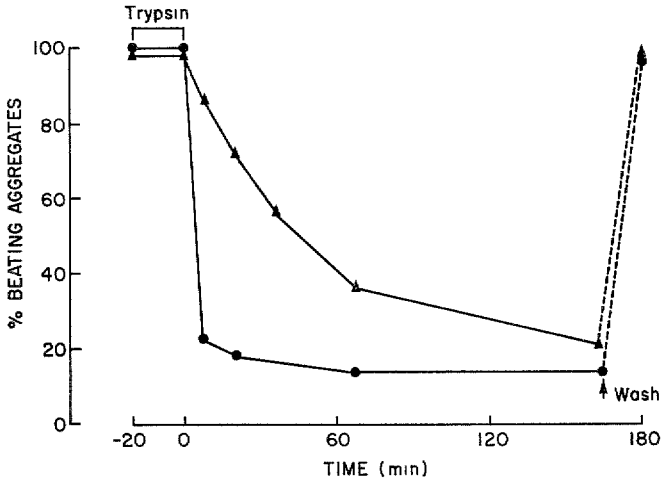

FIGURE 2 Response of heart cell aggregates to TTX 2 5 × 10^{-8} g/ml. Control (●) aggregates in serum-free medium, TTX, and fetal calf serum (10%) added at time 0. Trypsin-treated (▲) aggregates were exposed to 0.005% trypsin in serum-free medium for 20 min, fetal calf serum (10%) and TTX were added at time 0. At 170 min (arrow), medium in the dishes was changed three times with normal serum-containing medium without tetrodotoxin. Data are the means of two experiments.

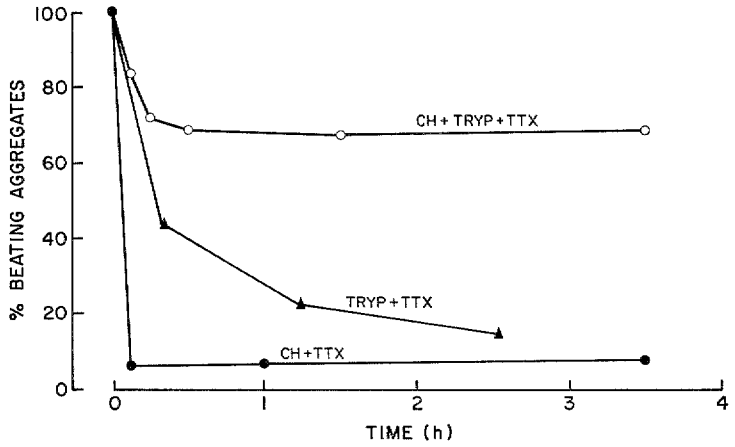

FIGURE 3 Response of cycloheximide (CH) pretreated aggregates to tetrodotoxin 1 × 10^{-6} g/ml. Control (●) aggregates were incubated for 23 h with cycloheximide 5 μg/ml before addition of TTX at time 0. Trypsin-treated (TRYP) (○) aggregates were incubated for 23 h with cycloheximide 5 μg/ml, and then exposed to trypsin 0.01% in serum-free, cycloheximide-containing medium for 30 min Fetal calf serum (10%) and TTX were added at time 0. Trypsin control (▲) aggregates were not preincubated with cycloheximide, but exposed to trypsin (0.01%) in serum-free medium for 30 min. Fetal calf serum (10%) and TTX were added at time 0. Data are the means of two experiments.

and the cellular mass continued to beat synchronously The trypsin was inactivated by the addition of fetal calf serum (10% in the medium), and the TTX-sensitivity was compared with control aggregates in the same medium, but not treated with trypsin. As shown in Fig 2, 80% of the aggregates not exposed to trypsin stopped beating in TTX (2 5 × 10^{-8} g/ml) within minutes after addition of the drug. In contrast, the pretreatment with trypsin rendered aggregates initially insensitive to the drug. After 10 min in TTX, for example, 90% of the enzyme-treated aggregates

were still beating. Over the next 90 min there was a gradual increase in the number of aggregates blocked by the toxin Even after 170 min, however, more trypsin-treated aggregates were beating than control aggregates

To investigate whether protein synthesis was involved in the resensitization to TTX after mild trypsinization, primary aggregates were preincubated with cycloheximide (5 μg/ml) for 23 h, exposed to 0 01% trypsin for 30 min, and tested for TTX-sensitivity, in the continued presence of cycloheximide. This cycloheximide concentration inhibited [^{14}C]amino acid incorporation by 92% after 1 h; 98% of the aggregates continued beating even after a 24-h exposure (although the mean beating rate declined by 43%). Fig. 3 shows, first, that cycloheximide alone does not reduce the sensitivity of aggregates to TTX, immediately after exposure to the toxin (1×10^{-6} g/ml) only 6% of the aggregates continued beating Aggregates treated with trypsin were initially relatively insensitive to TTX, but regained sensitivity over the subsequent 2.5 h, as also shown at the lower TTX concentration in Fig 1 In contrast, treatment with both trypsin and cycloheximide significantly blocked the slow recovery of sensitivity Thus it would appear that protein synthesis is required for TTX-resensitization of heart tissue made insensitive by trypsin.

The finding that multucellular structure is needed for expression of the differentiated state is not peculiar to embryonic myocardial cells. Neural retinal aggregates show a greatly increased induction of glutamine synthetase by hydrocortisone when compared to monolayer cultured cells (7) and specific enzyme activities of acetylcholine transferase and acetylcholine esterase are considerably higher in embryonic brain reaggregates than in monolayer cultured cells (11). Similarly, blastula cells of *Xenopus* isolated in culture fail to synthesize alkaline phosphatase, but cell aggregates do so (1).

It has been suggested (12) that isolated myocardial cells in culture lose TTX-sensitivity because of denervation during the culture procedure, rather than exposure to trypsin The present results demonstrate that cells can regain sensitivity to the toxin on reaggregation, and lose that sensitivity upon reisolation Therefore, denervation does not appear to play a role in the expression of TTX-sensitivity.

Strickholm and Clark have reported that TTX acts on excitable cells by interaction with a protein component of the surface membrane (13). Our finding that intact aggregates lose TTX-sensitivity upon mild trypsinization, but regain sensitivity by a protein-synthetic pathway, suggests that it is enzyme action at the cell surface, rather than physical separation of cells during dissociation, that causes them to become insensitive to TTX It seems probable that trypsin treatment alters the nature of either the TTX binding site on the heart cell surface, or the sodium channel itself, and thus prevents the drug from blocking action potentials A cell-to-cell interaction which apparently occurs in aggregates but not in isolated cells, is required for the synthetic pathway by which cells regain their sensitivity to TTX.

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