

Tetrodotoxin Desensitization in Aggregates of Embryonic Chick Heart Cells

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ABSTRACT Spontaneous beating of heart-cell aggregates from 4-day chick embryos was initially blocked by 10^{-5} g/ml tetrodotoxin (TTX). With continued exposure to the drug, the fraction of blocked aggregates decreased from about 80% at 15 min to about 25% at 2–3 h, at which time, beating aggregates had become desensitized to the toxin, showing no response to a fresh dose. Aggregates from 5-day hearts were more sensitive to TTX, but fewer became desensitized in its presence. Desensitization to TTX was not seen in 6- and 7-day aggregates. Inhibition of protein synthesis by cycloheximide did not affect beating or initial sensitivity to TTX of 4-day aggregates, but desensitization failed to occur. Before TTX, the mean value of maximal upstroke velocity (\dot{V}_{\max}) of the action potentials in 4-day aggregates was 33 V/s. After desensitization \dot{V}_{\max} was 12 V/s. Activity of desensitized aggregates in the presence of TTX was augmented by elevated calcium levels, and suppressed by presumed inhibitors of slow inward current (manganese, D600). Desensitization was reversible; upon removal of TTX 10^{-5} g/ml, aggregates regained their responsiveness to a fresh dose of the drug with a 2–3 h time-course similar to that of desensitization. This was prevented by continued exposure to TTX at concentrations as low as 10^{-8} g/ml. These data suggest that (a) desensitization involves a change in the mode of action-potential generating from one involving Na-specific, TTX-sensitive channels to one utilizing slower Mn-sensitive channels; (b) the process of desensitization occurs over a period of 2–3 h and is dependent upon the products of protein synthesis; and (c) desensitization is reversible after removal of TTX over a 2–3 h time-course similar to its onset.

INTRODUCTION

Tetrodotoxin (TTX) specifically blocks the fast inward sodium current in many excitable tissues, including heart (Narahashi et al., 1964; Dudel et al.,

1967; Rougier et al., 1969). In addition, it abolishes electrical activity in those pacemaker cells whose action-potential generation is dependent upon a transient increase in sodium conductance (Brown and Noble, 1969; Mathieu and Roberge, 1971). In other tissues, for example barnacle muscle (Hagiwara and Nakajima, 1966) and guinea pig taenia coli (Bulbring and Tomita, 1968), the rising phase of the action potential is dependent on a calcium current instead of sodium. These action potentials have a slower onset and are insensitive to TTX.

The inward current during an action potential in adult cardiac muscle is composed of these same two components: a TTX-sensitive fast current carried by sodium, and a TTX-insensitive slow inward current carried by sodium and/or calcium (Rougier et al., 1969; Beeler and Reuter, 1970; Tarr, 1971; New and Trautwein, 1972). The initial rapid phase of the action potential is thought to be due to the fast inward current, while the slow sodium/calcium component occurs during the overshoot and plateau phases.

Unlike the adult heart, cardiac tissue of the early chick embryo was found to be virtually insensitive to TTX (Shigenobu and Sperelakis, 1971; McDonald et al., 1972; Sperelakis and Shigenobu, 1972). Intact hearts from 2- to 4-day old embryos beat spontaneously in TTX at concentrations up to 10^{-5} g/ml. TTX sensitivity increased progressively with development, however. Hearts from 7-day embryos were blocked at a concentration of 10^{-7} g/ml TTX. Spheroidal aggregates formed from reassociated heart cells behaved in similar fashion, increasing in TTX sensitivity with embryonic age (McDonald et al., 1972).

In the present study, we report the finding that the fraction of such aggregates that is blocked by TTX also changes with time after exposure to the drug. Those aggregates, prepared from cells of 4-day hearts whose spontaneous beat is suppressed upon treatment with TTX, gradually lose their sensitivity to the drug over a 2-3 h period of exposure, resume beating in its presence, and are not blocked by a fresh dose at the same concentration (10^{-5} g/ml). The experiments reported here were designed to test whether this loss of TTX response: (a) resulted from desensitization of the aggregate cells or from an inactivation of the drug in their presence, (b) was associated with changes in electrophysiological parameters of the treated cells, and (c) was prevented by inhibitors of protein synthesis.

METHODS

Heart cell aggregates were prepared as previously described (McDonald et al., 1972; Sachs and DeHaan, 1973). After dissociation of the hearts into their component cells with trypsin by techniques now standard in this laboratory (DeHaan, 1967), an inoculum of 5×10^5 cells was added to 3 ml of culture medium contained in a 25 ml Erlenmeyer flask. The flask was gassed with 5% CO₂, 10% O₂, 85% N₂ and sealed with a silicone rubber stopper. Aggregation occurred during an 18-24 h period

in a gyratory shaker bath (37°C) at 70 rpm. The contents of each flask were transferred to a Falcon plastic tissue culture dish (35 mm diameter) (Falcon Plastics, Oxnard, Calif.), and the aggregates were allowed to attach to the bottom of the dish for 2 h in the culture incubator (water-saturated atmosphere of 5% CO₂, 10% O₂, 85% N₂ at 37.5°C). The plates were washed with fresh medium and placed on the constant temperature stage (37°C) of an inverted phase-contrast microscope in an atmosphere identical to that in the incubator.

As illustrated previously, the aggregates were usually of spheroidal shape (DeHaan and Sachs, 1972) and ranged in volume between 5×10^4 and $5 \times 10^6 \mu\text{m}^3$; an aggregate of $1 \times 10^5 \mu\text{m}^3$ contains approximately 200 cells.

Beating Activity

100–200 aggregates were observed individually on each plate and scored as beating or nonbeating after a 20 s observation period at either 63 or 160 \times . 95–100% of the aggregates beat spontaneously at a mean rate of about 120 beats/min in control plates containing 1.3 or 4.3 mM/liter potassium.

Electrophysiology

The tissue culture dishes were placed on the warm stage (37°C) of an inverted microscope, and a gassing ring directed a stream of 10% O₂, 5% CO₂, 85% N₂ over the dish. Mineral oil was layered over the medium to prevent evaporation (DeHaan and Gottlieb, 1968).

Transmembrane potentials were recorded between intra- and extracellular micro-pipette electrodes. Both electrodes were prepared by the procedure of Tasaki et al. (1968); the intracellular high-impedance pipette was filled with 2 M KCl, and the extracellular low-impedance pipette was filled with an isotonic balanced salt solution. The resistance of the intracellular microelectrodes was 30–100 M Ω ; the tip potentials of these electrodes, measured by breaking the tip, were between 0 and 6 mV. The intracellular electrode was connected to a capacitance-compensated amplifier (Instrumentation Laboratory, Inc., Lexington, Mass., Model IL-181 Picometric) and the indifferent (extracellular) to ground through a 50 mV calibrator. Potentials arising at the electrode tips or at the interfaces with the Ag/AgCl electrode holders were balanced out before each cell impalement. The maximum rate of rise of the action potential was measured from fast-sweep records of the action-potential upstroke. Aggregates were stimulated extracellularly with 5-ms pulses at 1 Hz using tungsten microelectrodes (Microtrode, Transidyne General Corp., Ann Arbor, Mich.).

Amino Acid Incorporation

After 18 h of gyration, the contents of two aggregation flasks were pooled. Aggregates were pulse labeled during 15 min of gyratory incubation with a mixture of [¹⁴C]amino acids (International Chemical and Nuclear Corporation, Burbank, Calif., sp act 1.1 mCi/mg) at 0.25 $\mu\text{Ci/ml}$. After centrifugation at 1,200 g for 5 min at 4°C, the supernatant was aspirated, the aggregate pellet was resuspended in a balanced salt solution and recentrifuged. After two additional wash cycles, the pellet was suspended in 1 ml of 5% TCA and incubated at 85°C for 10 min. The acid-insoluble material was col-

lected on Whatman GF/C filters, washed three times with 5% TCA, dried, and counted in toluene fluor in a scintillation counter.

Medium

Aggregates were cultured in medium 818A (DeHaan, 1970). The medium contains 20% M 199 (Grand Island Biological Co., Grand Island, N. Y.), 2% heat-inactivated horse serum (Colorado Serum Co., Denver, Col.), 4% fetal calf serum (Grand Island Biological Co.), penicillin G (100 U/ml), and streptomycin sulfate (50 μ g/ml) in potassium-free Earle's balanced salt solution, (millimolar): NaCl, 116.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.8; $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.9; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.8; NaHCO_3 , 26.2; and glucose, 5.5. The fetal calf serum was previously dialyzed against potassium-free balanced salt solution. The potassium concentration of the final medium was adjusted to 1.3 mM or 4.3 mM. Low-sodium medium for testing the effects of sodium depletion contained 9 mM NaCl plus 214 mM sucrose to maintain osmolarity. Experiments in which 0.5 mM manganese was tested were performed in medium made with salt solution which contained no bicarbonate or phosphate ions, but was instead buffered with 10 mM Tricine (*N*-Tris-(hydroxymethyl) methylglycine (California Biochemical Corp., Los Angeles, Calif.) (Gardner, 1969).

Drugs

The volume of medium in the tissue culture dish was 2 ml, and the volume of drug solution added was usually 20 μ l. Cycloheximide (Acti-dione, Nutritional Biochemicals Corporation, Cleveland, Ohio) and tetrodotoxin (Sigma Chemical Co., St. Louis, Mo.) were dissolved in water to make stock solutions of 10^{-3} and 10^{-5} g/ml, respectively. TTX was further diluted in 10-fold steps as needed. Compound D600-hydrochloride (No. 536-A-1; Knoll AG, Ludwigshafen am Rhein, West Germany) was diluted to 0.1 mg/ml with water.

RESULTS

The response of aggregates to TTX was dependent on the age of the donor embryo. Fig. 1 shows the effect of TTX on the beating activity of aggregates from hearts aged 4–7 days. 15 min after the addition of the drug, 26% of the 4-day aggregates were beating while less than 10% of the 6- and 7-day aggregates remained active. During the next 2 h, many of the 4- and 5-day aggregates resumed beating while there was little or no increase in the number of active 6- and 7-day aggregates.

This pattern of suppression and recovery in 4- and 5-day aggregates treated with TTX was apparent whether aggregates were formed from ventricular cells, atrial cells, or cells from the whole heart. The response was also essentially the same in medium containing 1.3 or 4.3 mM/1 K^+ . The percentage of 4-day aggregates beating after 15 min in TTX varied from culture to culture but was usually 15–35%. Within the next 2–3 h, the percentage of beating aggregates increased to between 50 and 80%. During prolonged incubation

with TTX, the beating percentage after 6 h was usually the same as after 3 h. Aggregates from 7 day heart failed to show a similar suppression-recovery response even at lower doses of TTX. For example, at 2.5×10^{-8} g/ml 40% of 7-day aggregates continued beating. Over a 4 h incubation at that concentration none of the suppressed aggregates resumed activity.

To confirm that the toxin was not inactivated in the culture plates, TTX was added to one of a pair of plates containing 4- or 5-day aggregates. After 3 h, the TTX medium was transferred to the companion plates. Such an experiment is shown in Fig. 2. The two plates had essentially identical suppres-

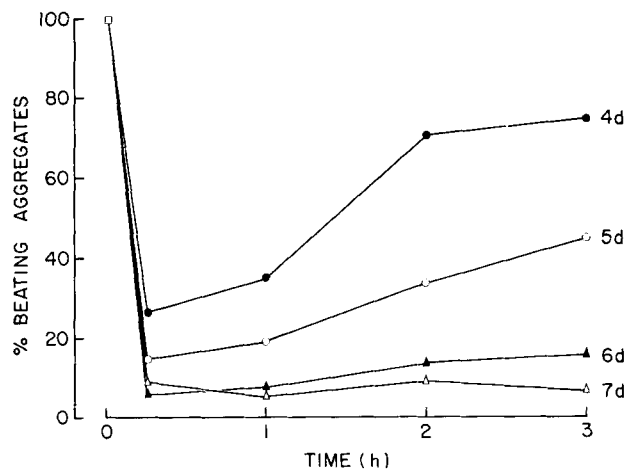


FIGURE 1. The response of heart cell aggregates to TTX (10^{-8} g/ml). Aggregates were formed from dissociated heart cells of chick embryos aged 4–7 days and maintained in medium 818A containing 1.3 mM/liter K^+ . Data for 4- and 7-day aggregates are mean values from six to eight plates in four different cultures, data for 5- and 6-day aggregates are mean values of two plates each.

sion-recovery responses, indicating that the toxin remained active. Further, when the companion plate (Fig. 2 B) was washed and incubated for another 45 min with fresh TTX the percentage of beating aggregates did not substantially decline.

The electrical activity of 4-day aggregates was monitored at intervals during the 3 h incubation with TTX (Fig. 3). Normal pacemaker activity was blocked about 3 min after the addition of the drug, resulting in a stable resting potential (Fig. 3 A, arrow). Approximately an hour later, in those aggregates that resumed beating, the stable resting potential gave way to small oscillations and subsequent resumption of full-scale pacemaker action potentials (Fig. 3 B). Since all the cells in an aggregate appear to be electrically coupled (Sachs and DeHaan, 1973), it was not surprising that resumption of electrical activity in a cell was always accompanied by a contractile event in the whole

aggregate. Although not shown in the example illustrated (Fig. 3), aggregates that resumed beating often displayed irregular rhythms during the first few minutes, but usually stabilized thereafter.

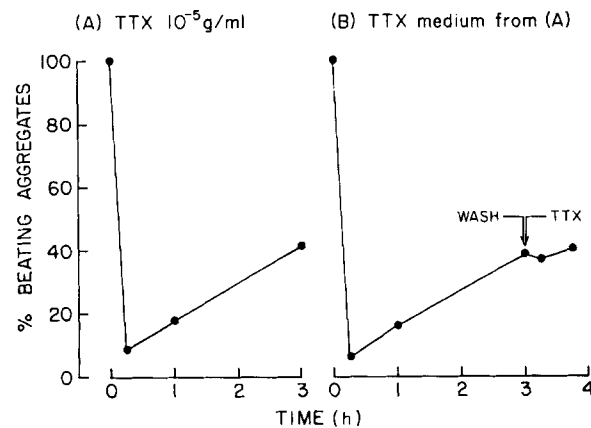


FIGURE 2. The response of 5-day aggregates to TTX (10^{-5} g/ml). (A) One plate of aggregates was incubated for 3 h with medium containing TTX. (B) After the 3 h incubation, the medium from plate A was transferred to duplicate plate B for 3 h. Plate B was subsequently washed three times and immediately treated with fresh TTX.

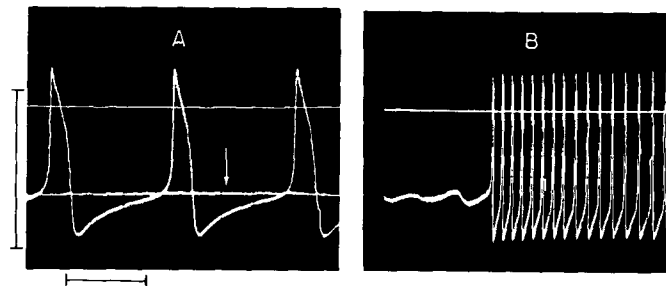


FIGURE 3. Electrical activity in a 4 day heart aggregate before and during incubation with TTX (10^{-3} g/ml). (A) Superimposed records showing control action potentials before TTX, and a stable resting potential without action potentials (arrow) 3 min after treatment with the drug. (B) Resumption of pacemaker activity during TTX incubation. Record B was obtained about 1 h after record A. Voltage calibration is 100 mV. The upper horizontal line in both records indicates 0 mV. Time calibration is 400 ms in A and 4 s in B.

To assess the changes in electrical properties of cells that might be correlated with desensitization, action potentials were recorded in some 60 aggregates (in three experiments) before treatment with TTX and during the second and third hours of incubation with the drug. The majority of impalements during the TTX incubation were from aggregates which had stopped

and then resumed beating. The results are shown in Table I. There was no important difference in the maximum diastolic potential (MDP) or overshoot (OS) of aggregates before and during TTX. However, in the course of TTX desensitization, the maximum rate of rise of the action potential (\dot{V}_{\max}) was reduced on the average by more than 50%. Before TTX, \dot{V}_{\max} ranged from 8 to 45 V/s and exceeded 30 V/s in about one-third of the impalements, while in the presence of the drug the highest \dot{V}_{\max} was 18 V/s.

The appearance of TTX-insensitive action potentials in skeletal muscle after denervation was found to be dependent on a protein-synthetic process, and was sensitive to inhibition by cycloheximide (Grampp et al., 1972). Accordingly, we investigated the effect of cycloheximide on the TTX response

TABLE I
ACTION-POTENTIAL PARAMETERS OF BEATING 4-DAY
AGGREGATES BEFORE AND DURING
INCUBATION WITH TTX

| | Control | | Experimental | |
|---------------------------|----------|----------|--------------------|--------------------|
| | 0 | 0 | 1×10^{-5} | 1×10^{-5} |
| TTX (g/ml) | 0 | 0 | 1×10^{-5} | 1×10^{-5} |
| K ⁺ (mM/liter) | 1.3 | 4.3 | 1.3 | 4.3 |
| MDP (-mV) | 67.8±2.4 | 66.4±1.9 | 72.2±1.5 | 70.9±1.6 |
| OS (mV) | 16.8±1.0 | 21.2±1.3 | 20.3±1.0 | 23.2±1.4 |
| \dot{V}_{\max} (V/s) | 22.8±1.5 | 33.4±2.1 | 12.1±0.9 | 12.4±1.1 |
| N | 20 | 18 | 20 | 19 |

The maximum diastolic potential (MDP), overshoot (OS), and maximum rate of rise of the action potential (\dot{V}_{\max}) was measured in aggregates before TTX and during the second and third hours of incubation with the drug in medium containing 1.3 or 4.3 mM/liter potassium. Data represent mean \pm SE for N number of impalements.

of 4-day aggregates. The incorporation of [¹⁴C]amino acids into the acid-insoluble fraction of 4-day aggregates was inhibited by cycloheximide (5 μ g/ml); after 15, 30, and 75 min of incubation with cycloheximide, the inhibition was 86%, 95%, and 92%, respectively. Despite this suppression of incorporation, 99% of the aggregates were still beating after 24 h in the inhibitor. Although mean beating rate had declined to 62% of control, other electrical properties of these aggregates had not changed substantially. In nine impalements of 24-h cycloheximide-treated aggregates (drug dissolved at 5 μ g/ml in medium containing 1.3 mM K⁺), maximal diastolic potential averaged $-75.1 \pm$ mV, overshoot was 24.3 ± 0.9 mV, and mean \dot{V}_{\max} was 24.7 ± 4.2 V/s. These values compare favorably with those shown for untreated controls in Table I, column 1.

When 4-day aggregates were pretreated with cycloheximide for 15 min, 1 h, or 4 h, and then incubated for a further 3½ h with both cycloheximide

and TTX, the results were as shown in Fig. 4. The control aggregates (filled circles) rebounded from 16% beating after 15 min with TTX to about 75% beating after 3 h. Aggregates pretreated for 15 min with cycloheximide (open triangles) or 1 h (not shown) exhibited a similar pattern of desensitization. In marked contrast, aggregates pretreated for 4 h with cycloheximide (filled triangles) did not become desensitized. After 3½ h the percentage of beating aggregates was still only 17%. 15 min after washing out the TTX, the percentages of beating aggregates in the control and 4-h cycloheximide plates returned to 100% and 98%, respectively.

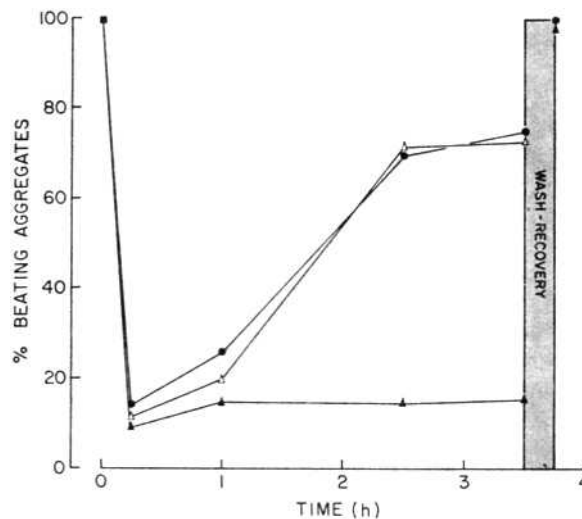


FIGURE 4. Inhibition of TTX-desensitization in 4-day heart aggregates by treatment with cycloheximide (5 $\mu\text{g}/\text{ml}$). Control aggregates treated with TTX (10^{-5} g/ml) (filled circles); 15 min pretreatment with cycloheximide, followed by cycloheximide and TTX (open triangles); 4 h pretreatment with cycloheximide, followed by cycloheximide and TTX (filled triangles). Aggregates were washed three times after 3½ h in TTX. Values are the means of two plates each.

The ionic requirements of electrical activity in TTX-treated aggregates were investigated by varying the external sodium and calcium concentrations. 10 aggregates were impaled in low-sodium, sucrose-substituted medium containing 1.8 mM or 5 mM calcium. Potassium concentration was held at 1.3 mM to increase excitability. Nonetheless, these aggregates were electrically quiescent at either calcium concentration, and were inexcitable when stimulated extracellularly at $5 \times$ threshold. In plates of aggregates with medium containing normal sodium, those aggregates which were still suppressed after 3 h with TTX resumed spontaneous beating upon raising the calcium from 1.8 to 5 mM (two plates). In normal sodium medium with 5 mM calcium, nearly 100% of the aggregates remained beating after 15 min in TTX (three

plates). The action-potential overshoot was measured on four desensitized aggregates beating in the presence of TTX, by repeated impalements. As the external calcium concentration was raised stepwise from 1.8 to 7.2 mM, the overshoot increased 20–25 mV/decade.

The slow inward current of adult cardiac muscle is selectively blocked by low doses of manganese (Rougier et al., 1969; Tarr, 1971) or by D600 (Kohlhardt et al., 1972). To test whether 4-day aggregates responded to these agents in similar manner, preparations in Tricine-buffered medium were treated with TTX, allowed to undergo desensitization for 2.5 h, and then exposed to manganese ion (Fig. 5). At a concentration of 0.5 mM, which had no appreciable effect on the activity of control aggregates, the percentage of

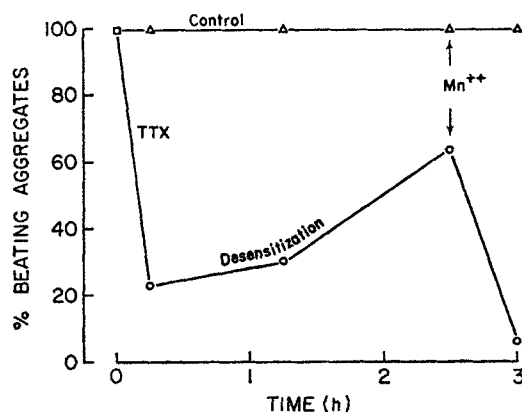


FIGURE 5. Response of 4-day aggregates to manganese. Aggregates were treated with 10^{-6} g/ml TTX (circles) or an equal volume of diluent (triangles) and the percentage of beating aggregates was determined 15, 75, and 150 min later. At that time, $20 \mu\text{l}$ of 50 mM MnCl_2 was added to each dish, bringing the final concentration to 0.5 mM. 30 min later % BA was again determined. Each point represents the mean of four plates.

desensitized aggregates that remained beating decreased from 64 to 6%. In one experiment, D600 ($1 \mu\text{g}/\text{ml}$) was found to have a similar effect. Impalement of nonbeating aggregates in the presence of either agent showed them to be electrically inactive.

To determine whether desensitization is a time-dependent process that might be triggered by even a brief exposure to TTX, the experiment illustrated in Fig. 6 was performed. Aggregates were treated with TTX for 15 min, washed, incubated with drug-free medium for $2\frac{3}{4}$ h, and then exposed to TTX again (Fig. 6 A). The percentage of suppressed aggregates was similar after both doses. When companion aggregates were desensitized by a 3 h exposure to TTX, washed, and again treated with the toxin, the percentage of beating aggregates counted 15 min later was similar to that present at the end of the 3 h TTX desensitization period (Fig. 6 B). The response of de-

sensitized aggregates to the second dose of TTX was in marked contrast to that of control aggregates, indicating that a brief exposure to TTX does not produce desensitization, and that desensitized aggregates remain in that state after the removal of TTX.

To determine how long the desensitization lasts after removal of TTX, we employed the following experimental protocol: aggregates were incubated with TTX for 3 h, washed, and then incubated for 0–4 h in drug-free medium

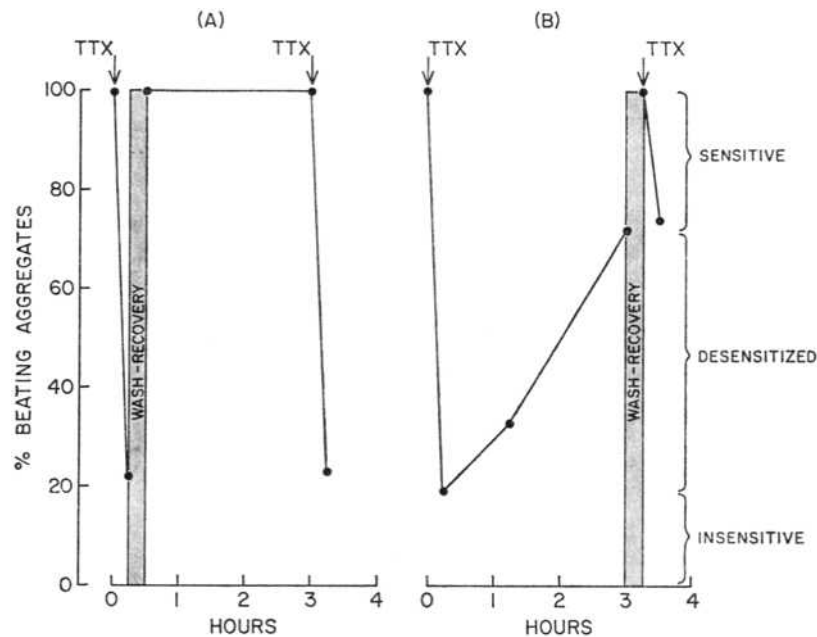


FIGURE 6. The response of 4-day heart aggregates to successive doses of TTX (10^{-5} g/ml). (A) Aggregates were incubated with TTX for 15 min, washed three times and then incubated in drug-free medium for $2\frac{3}{4}$ h before the second dose of TTX. (B) Aggregates were incubated for 3 h with TTX, washed three times, and then treated with TTX 15 min later. Values are the means of two plates each. Also illustrated is the method used to classify aggregates into three groups—sensitive, desensitized, and insensitive—for the experiments described in Fig. 7.

before exposure to a second dose of TTX. After the initial 3 h incubation with TTX, the aggregate population was divided into three groups (see Fig. 6 B): (a) Sensitive aggregates—those not beating after 3 h in TTX. (b) Desensitized aggregates—aggregates which resumed beating between 15 min and 3 h in TTX. (c) Insensitive aggregates—those which had continued beating after the initial 15 min in TTX. In the cycloheximide experiment (Fig. 4, filled triangles) the percentage of insensitive aggregates remained nearly constant throughout the 3 h incubation period with TTX. Assuming that the percentage of sensitive aggregates also remained constant during experiments

similar to that depicted in Fig. 6 B, any decrease in the total percentage of beating aggregates after the second dose of TTX may be attributed to a decline in the number of desensitized aggregates.

The percentage of desensitized aggregates after the second dose of TTX was expressed as a fraction of the percentage of desensitized aggregates after the initial 3 h incubation with TTX. This fraction was plotted against elapsed time between washing and the second dose of TTX (Fig. 7). The fraction of desensitized aggregates remained nearly constant for 1 h, declined sharply during the next hour, and stabilized thereafter at about zero. That is, 4 h after removal of TTX, just as many aggregates were sensitive to the drug as had been before the first exposure.

The curve in Fig. 7 illustrates the reappearance of response to TTX after withdrawal from a prolonged exposure to the drug at 10^{-5} g/ml. In earlier experiments (McDonald et al., 1972) during which the dose of TTX was

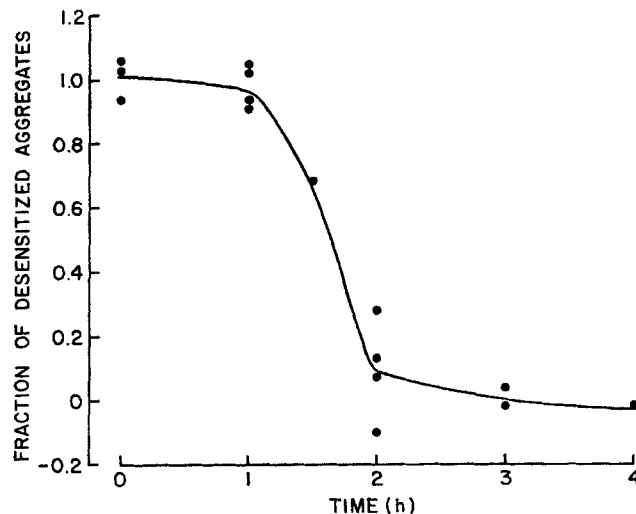


FIGURE 7. Time-course of the reappearance of response to TTX by 4-day aggregates. Aggregates were incubated with TTX (10^{-5} g/ml) for 3 h, washed three times, and incubated in drug-free medium for a specified period of time before receiving a second dose of TTX. The abscissa denotes the time in drug-free medium before the second dose. The percentage of desensitized aggregates beating 15 min after the second dose of TTX was compared to the percentage of desensitized aggregates at the end of the initial 3 h TTX incubation and expressed as a fraction thereof. For example, the fraction 0.2 indicates that only one-fifth of the original population of desensitized aggregates were beating after the second dose of TTX. Fractions greater than 1.0 or less than 0 occurred when the calculated percentage of desensitized aggregates after the second dose of TTX either exceeded the original 3 h percentage or was lower than the 15 min count of the 3 h incubation. (For further details refer to the text and Fig. 6 B.) Each point represents one plate containing 100–200 aggregates taken from six different cultures.

raised in increments gradually from 10^{-8} g/ml to 10^{-5} g/ml, desensitization was sometimes apparent by the time (about $1\frac{1}{2}$ h) the final dose of 10^{-5} g/ml was administered, i.e., the large dose did not provoke as great a depression of beating as had a smaller dose earlier. Since desensitization occurred upon exposure to lower doses of TTX, it seemed possible that the reappearance of sensitivity might be prevented by concentrations of the drug below 10^{-5} g/ml. After the standard 3 h desensitization with 10^{-5} g/ml TTX, aggregates washed and incubated for 4 h with 10^{-8} g/ml TTX were still found to be desensitized, as shown by a second challenging dose of 10^{-5} g/ml; in two experiments the mean fraction of desensitized aggregates was 0.93, as compared with 0.05 in the control plates. Thus, once cells are desensitized to TTX, they do not regain the ability to respond as long as they are exposed to the drug, even at low doses.

DISCUSSION

The embryonic chick heart begins to beat during the second day of incubation as a primitive tubular structure, and attains an adult-like four-chambered configuration at about 7 days. During this period, the heart also undergoes a series of developmental changes in its electrophysiological parameters. These include dramatic alterations in (a) size and shape of the action potential (Lieberman and Paes de Carvalho, 1965; McDonald and DeHaan, 1973), (b) intracellular ion levels (McDonald and DeHaan, 1973), (c) sensitivity of pacemaker activity to suppression by elevated extracellular potassium (DeHaan, 1970), and (d) responsiveness of the heart to TTX and other current-blocking agents (McDonald et al., 1972; Pappano, 1972; Shigenobu and Sperelakis, 1971; Sperelakis and Shigenobu, 1972). Intact hearts from embryos aged 2–4 days continue beating in TTX at 10^{-5} g/ml, while by day 7 spontaneous activity is blocked at a toxin concentration of 10^{-7} g/ml (McDonald et al., 1972). Concomitantly \dot{V}_{\max} increases from 10–20 V/s at the early stages to 100 V/s or more beyond 7 days (Shigenobu and Sperelakis, 1972; Sachs, McDonald and DeHaan, in preparation). A similar rise in \dot{V}_{\max} has been reported in the developing rat heart between 11 days gestation and birth (Couch, 1969).

The marked correlation between \dot{V}_{\max} and TTX sensitivity seems not to be restricted to embryonic cardiac tissue. Paes de Carvalho et al. (1969) reported \dot{V}_{\max} values of 10–13 V/s for action potentials from adult rabbit nodal and nodal-His tissues, which are known to be insensitive to 10^{-5} g/ml TTX (Yamagishi and Sano, 1966), and suppressible by manganese (Zipes and Mendez, 1973).

The rapidly rising initial phase of the action potential in a variety of heart, muscle, and nerve preparations results from an early rapid increase in sodium conductance. By determining the fluxes of several organic cations across the

frog myelinated sciatic nerve membrane, Hille (1971) was able to deduce certain properties of the ion-selective channel that carries this fast inward current. The model he proposed for the sodium channel is of a small pore $3 \times 5 \text{ \AA}$ wide, lined with oxygens able to form hydrogen bonds, and having one anionic site capable of interacting with cations. The known structure of TTX (Narahashi, 1972), with a positively charged guanidinium group and hydroxyls capable of forming hydrogen bonds, is consistent with the hypothesis that the drug acts by binding specifically in the opening of the fast sodium channel. This binding has been measured (Keynes et al., 1971; Hafeemann, 1972), the density of binding sites on the membrane has been estimated (Colquhoun et al., 1972), and the site has been isolated and partially characterized (Henderson and Wang, 1972; Benzer and Raftery, 1972)—all attesting to the reality of the channel as a definable structure and the specificity of its association with TTX. The role of TTX as a specific blocker of the fast sodium channel in heart muscle is also well documented (Rougier et al., 1969; Brown and Noble, 1969; Tarr, 1971).

The second component of the inward action current, often referred to as the slow inward current, occurs during the overshoot and plateau phases, and seems to be carried by either sodium and/or calcium ions (Beeler and Reuter, 1970; Tarr, 1971; New and Trautwein, 1972). These slow cation channels are unaffected by TTX but are blocked by manganese (Coraboeuf and Vassort, 1968; Rougier et al., 1969; Tarr, 1971) or by D600 (Kohlhardt et al., 1972). It has been previously suggested that the TTX-insensitive slowly rising action potentials of the early embryonic heart are produced by currents carried exclusively through these slow cation channels, and that the transition with development to rapidly rising TTX-sensitive action potentials represents the appearance in the heart cell membrane of a fast-channel mechanism (McDonald et al., 1972; Pappano, 1972; Sperelakis and Shigenobu, 1972).

In the present work, we have shown that aggregates of 4-day heart cells respond to TTX in one of three ways. Some (20–25%) stop beating immediately and remain electrically inactive as long as they are exposed to the drug. In a second group (usually about 50%), electrical activity ceases upon exposure to the toxin, but beating is gradually resumed over the next 2–3 h in its continued presence. These aggregates can be shown to have been rendered unresponsive to a second dose of TTX (i.e., desensitized). The third group of aggregates appear to be unaffected by TTX (up to 10^{-5} g/ml) and continue beating throughout the period of exposure. With increasing embryonic age a progressively greater fraction of such aggregates are sensitive to the drug, and fewer undergo desensitization in its presence. A reasonable hypothesis to explain these differences is that action-potential generation in sensitive aggregates involves a “fast” sodium-specific channel, while those aggregates which remain active in the presence of TTX, as well as those which become desensitized, utilize a slow-channel mechanism.

Alternative hypotheses can also be suggested. Cessation of spontaneous beating in an aggregate exposed to TTX could result from failure of the pacemaker mechanism to bring the membrane to a normal threshold, from an alteration of threshold to levels beyond the reach of the pacemaker process, or from an effect on sodium conductance other than simple blockage, such as a shift in the sodium current activation curve (h factor) in a hyperpolarizing direction. In any of these cases, an explanation of the resumption of beating with desensitization would not necessarily invoke a shift from fast to slow channels. Definitive tests of these possibilities would require application of voltage-clamp techniques. However, existing evidence that the membrane potential of TTX-blocked aggregates is stable (does not oscillate) and lies near the normal threshold level (Fig. 3 A), and that maximum diastolic potential and overshoot values show only small changes (Table I), fails to point strongly towards any of these alternatives.

Desensitization was blocked by inhibition of protein synthesis with cycloheximide, as long as the aggregates were exposed to the inhibitor for a long enough time (4 h) before being treated with TTX. This suggests that a pool of protein precursors are somehow involved in the TTX desensitization, and must be exhausted before the consequences of the inhibition of amino acid uptake can be observed. Hartzell and Fambrough (1973) have reported that the incorporation of acetylcholine receptors into tissue-cultured skeletal muscle myotubes is also dependent on protein synthesis, and that receptor incorporation or activation is blocked only after inhibition of protein synthesis for 2–3 h by cycloheximide.

It is unlikely that the prevention of desensitization by cycloheximide resulted from general deleterious effects of that inhibitor. It is known that cycloheximide does not interfere with oxygen consumption or oxidative phosphorylation in chick cardiac cells (Gazzola et al., 1972). Moreover, aggregates continued to beat vigorously even after 24 h in cycloheximide with no significant changes in action-potential parameters. It is also unlikely that cycloheximide prevented TTX desensitization by increasing the responsiveness of the membrane to TTX. On the contrary, we have found that the normal increase in sensitivity to TTX between 4 and 7 days can be inhibited *in vitro* by cycloheximide (Sachs, McDonald, and DeHaan, in preparation). Whether desensitization of cardiac aggregates involves the modification of existing fast channels, activation of dormant slow channels, synthesis and/or insertion of new slow channels into the membrane, or some unrelated mechanism remains to be resolved.

The appearance of a slowly rising, manganese-sensitive, TTX-insensitive action potential that we have observed in desensitization seems superficially to be related to the catecholamine-induced excitability of TTX-blocked adult heart tissue. In frog atrium (Aceves and Erlj, 1967; Vassort et al., 1969), beef Purkinje fiber (Carmeliet and Vereecke, 1969), and guinea pig atrium (Pap-

pano, 1970) it has been reported that epinephrine or norepinephrine restores spontaneous activity in tissues made quiescent by TTX or elevated K^+ , but that the resultant action potentials have low \dot{V}_{max} values, are dependent on Ca^{++} in the medium, and are blocked by manganese. Shigenobu and Sperelakis (1972) have recently observed the same phenomenon in "old" embryonic chick ventricle (9–19 days of incubation). Obvious differences exist, however, between the catecholamine-induced phenomenon and the recovery of activity in our desensitized aggregates. Catecholamine-induced action potentials appear immediately upon treatment of the tissue with epinephrine or norepinephrine without a 2–3 h period of onset. Although the effects of inhibition of protein synthesis on catecholamine-induced excitability has not been tested, the immediacy of its onset argues against any such requirement as in desensitization. Finally, desensitized aggregates continue beating in the presence of TTX indefinitely. In contrast, catecholamine-induced excitability is a transient phenomenon. The characteristic slow action potentials continue for only a few minutes after a dose of catecholamine. It is not clear whether a second exposure to hormone reinitiates the phenomenon.

The cells that comprise an embryonic heart do not represent a homogeneous population. When isolated in tissue culture, 22% of the cells from 4-day hearts beat spontaneously in the presence of 10^{-5} g/ml TTX (McDonald et al., 1972, Table II). When reassociated into aggregates, however, the properties of the component cells appear to change as a result of cellular interactions (Sachs et al., 1973). Moreover, the aggregate does not respond as if it were a composite of heterogeneous individual cells. It behaves instead as a single large cell. When tested by impalement with two or more electrodes, all of the cells of an aggregate are found to be electrically coupled and the system is isopotential (Sachs and DeHaan, 1973; DeHaan and Fozzard, in preparation). It is presumably this coupling that permits each aggregate to respond to TTX as an integrated entity, whether sensitive, insensitive, or able to undergo desensitization.

4-day aggregates become desensitized only in the continued presence of TTX, and the process is dependent upon products of protein synthesis. There is a growing body of literature (Pardee, 1971) suggesting that events at the cell membrane can influence intracellular synthetic mechanisms. For example, Vaughan and Cook (1972) have found that ouabain-treated HeLa cells generate new Na-K pump sites which restore ion transport within 3 h without significant loss of the initially titratable ouabain binding sites. This response was blocked by cycloheximide and appeared to result from active synthesis due to the altered concentration of cell electrolytes rather than from normal turnover. Analogous logic applied to our present results raises the possibility that embryonic heart cells respond to TTX blockade by active conversion of their electrogenic mechanism from one which involves sodium-specific fast channels to one dependent exclusively on slow channels.

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