Doxorubicin-induced Automaticity in Cultured Chick Heart Cell Aggregates

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ABSTRACT

The use of doxorubicin, a clinically important antitumor agent, is associated with toxic cardiac side effects, including arrhythmias and, on rare occasions, sudden death. We have used aggregates of cultured chick embryonic heart cells, a preparation free of neuronal and vascular elements, to investigate the direct effects of doxorubicin on automaticity. Under control conditions, normally quiescent aggregates of ventricular origin could be induced to contract spontaneously during a 24- to 48-h incubation with doxorubicin. The percentage of aggregates exhibiting automaticity was concentration dependent between 0.01 and 1 μM doxorubicin. At relatively high drug exposure (e.g., 1 μM doxorubicin for 48 h), beating was shown to be dysrhythmic. Whereas control aggregates exhibited large stable resting potentials on impalement with intracellular microelectrodes, treated aggregates exhibited spontaneous action potentials with Phase 4 depolarization. Doxorubicin, in a dose-dependent manner, increased the rate of Phase 4 depolarization, reduced the maximum diastolic potential, and shortened the action potential duration. In dysrhythmic aggregates, microelectrode recordings also revealed examples of premature depolarizations, extrasystoles, and dropped beats. This study provides the first cellular electrophysiological recordings of doxorubicin-induced automaticity and rhythm disturbances in heart muscle. The results suggest that the induction of automaticity is due to a depolarizing action of doxorubicin on the cardiac membrane.

INTRODUCTION

Doxorubicin, a major chemotherapeutic agent active against certain solid and hematological malignancies, is limited in its clinical use by the development of acute and chronic cardiotoxic effects (1, 2). The most serious manifestation is a chronic dose-related cardiomyopathy that has been shown to increase dramatically above cumulative doses of about 550 mg/m² (3). Less well known is the tendency for doxorubicin to disturb the cardiac rhythm, sometimes resulting in life-threatening arrhythmias and sudden death (4). The drug-induced rhythm disturbances, which can be detected electrocardiographically in humans and animals, have been reported to occur acutely (within the first few hours), after long-term treatment, or within 24 to 48 h following administration of doxorubicin (5–8). The acute dysrhythmias are similar following bolus injections or continuous infusions of doxorubicin. The occurrence of clinically significant cardiac dysrhythmias prior to the development of cardiomyopathy suggests that doxorubicin exerts early and significant effects on the membrane properties of heart cells. Previously, studies of doxorubicin have concentrated on the chronic cardiomyopathy that is characterized by ultrastructural alterations (e.g., myofibrillar loss and cytoplasmic vacuolization) and the destruction of biological membranes, apparently through free radical-dependent lipid damage (9). In general, the same histopathological changes and subcellular effects of doxorubicin that occur in humans develop in animal models (10). Also, in monolayer cultures exposed to doxorubicin, in which the myocardial cells undergo similar ultrastructural changes, spontaneous beating ceases as the cells die (11, 12). With reduced exposure to doxorubicin, arrhythmias could be produced in vitro (11). However, there have been no reports thus far describing the cellular electrophysiological basis of doxorubicin-induced aberrant automaticity in heart muscle at drug concentrations used in a clinical setting. Therefore, in the present study, we analyzed the electrophysiological effects of doxorubicin, making use of an in vitro myocardial preparation, namely, chick embryonic heart cells cultured as spherical clusters, or aggregates.

Aggregates of cultured embryonic heart cells have many advantages for such a study, which have been summarized as follows. (a) Embryonic heart cells remain viable in vitro for days to weeks and exhibit contractile activity typical of the intact embryo heart. (b) The tissue culture approach makes possible the prolonged (e.g., 24–48 h) incubations with doxorubicin that are necessary for cardiotoxic effects to occur. The effects of doxorubicin on the cultured cells are direct without the complications of drug action on neuronal and vascular tissues. (c) Recordings of both transmembrane potentials and contractions can be made in aggregates by using microelectrode and photoelectric techniques. Furthermore, in aggregates of embryonic chick hearts, many of the electrophysiological properties and pharmacological receptors observed are characteristic of the hearts from which the cells are isolated (13). Aggregates cultured from 7-day-old embryonic ventricles, as used in the present study, typically exhibit a quiescent resting membrane potential, tetrodotoxin-inhibitable action potentials, and an absence of automaticity, much as the intact heart (14). These cultured cells also continue to respond in vitro to catecholamines, histamine, acetylcholine, and angiotensin II (15–18). Spherical aggregates retain the tight cell-to-cell electrical coupling characteristic of cardiac muscle (19). The slow calcium inward current and the fast (tetrodotoxin-sensitive) sodium current occur in this preparation and have been directly measured using the two-microelectrode voltage clamp (18, 20). Cell aggregates cultured from 7-day-old chick embryonic hearts are capable, under certain conditions (e.g., in low extracellular K⁺ solutions), of developing pacemaker-like activity, i.e., spontaneously firing action potentials with diastolic (Phase 4) depolarization. The ionic currents underlying this diastolic depolarization have been analyzed and resemble those found in adult cardiac Purkinje fibers (21).

In the present study, an in vitro myocardial preparation has been used as a model system to investigate the possible mechanisms by which doxorubicin produces cardiotoxicity. The major findings are that doxorubicin exposure causes the appearance of spontaneous beating, which is sometimes dysrhythmic, in normally quiescent heart cell aggregates and that the altered behavior is associated with membrane depolarization, a phenomenon not previously described for cardiac cells of ventricular origin.

MATERIALS AND METHODS

Materials

Drugs. Doxorubicin was donated by Dr. Vern Verhoef on behalf of Adria Laboratories (Columbus, OH).

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Culture Supplies. Fertilized eggs were obtained from Sharp Sales (West Chicago, IL). M199, fetal calf serum, and trypsin-EDTA were purchased from Gibco (Grand Island, NY). Trypan blue was obtained from Sigma Chemical Co. (St. Louis, MO). Tissue culture dishes were purchased from Falcon (Oxnard, CA), and bacteriological plates were obtained from Fisher Scientific Co. (Itasca, IL). Glass coverslips (16 mm²) were obtained from Belco Glass Co. (Vineland, NJ), and NU-13 plates to which the heart cells do not adhere. The dissociated cells were separated from fibroblasts on the basis of rapid attachment of fibroblasts to tissue culture dishes (23). From a total of 18 hearts, a suspension of 10⁶ cells/ml was plated into 150-mm² tissue culture dishes (Falcon) to give 400 cell/mm² of surface area. After a 3-h incubation in humidified 95% air:5% CO₂ atmosphere, supernatants containing principally myocytes were collected and centrifuged at 1000 x g for 10 min. A suspension of 10⁴ cells/ml was then resuspended in culture medium without trypsin and treated in all other respects exactly as described above. Four to five additional cycles of alternating trypsin and culture medium were used to completely digest the ventricles. The pellets of each digest were pooled in culture medium with the exception of the first pellet which was discarded. The pellets were washed twice with culture medium, centrifuged, and resuspended in culture medium to which 5 mM HEPES⁴ buffer (pH 7.4) was added. In several cultures examined, viability was greater than 90% by the Trypan blue exclusion test.

Preparation of Chick Cardiac Myocytes. Primary cultures of chick cardiomyocytes were prepared under sterile conditions by a modification of the method of Sachs and DeHaan (22). Hearts from chick embryos were excised on the seventh day in ovo. After discarding the atria, two slits in the apical portion of the ventricle were made to facilitate blood removal. Eighteen ventricles were pooled at room temperature in culture medium composed of M199 with 10% fetal calf serum and containing antibiotics (10,000 units/ml of penicillin and 10 mg/ml of streptomycin). To obtain a suspension of dissociated myocytes, the ventricles were transferred to a 25-ml Ehrlenmeyer flask containing 0.05% trypsin-EDTA, triturated twice using a large-bore pipet, and then gently agitation at 37°C shaker bath for 7 min. The supernatant containing dispersed myocytes was added to an equal volume of M199 with 10% fetal calf serum to terminate the trypsin action. Then, the supernatant was centrifuged at 500 x g for 5 min. Since it was found that mechanical agitation of the tissue without trypsin could produce cell separation to some extent, the undigested ventricular tissue was next resuspended in culture medium without trypsin and treated in all other respects exactly as described above. From a total of 18 hearts, a volume of M199 with 10% fetal calf serum, and trypsin-EDTA were contained in the medium. For contraction studies (see below), aggregates were seeded in 35-mm² tissue culture dishes at a concentration of 1 to 2 aggregates/dish containing aggregates was transferred to an equal volume of M199 with 10% fetal calf serum and to adhere. Drug treatment was initiated 24 h after aggregates were seeded. For contraction studies (see below), aggregates were seeded in 35-mm² tissue culture dishes at a concentration of 1 to 2 aggregates/mm² of surface area. For electrophysiological studies, aggregates were seeded on coated coverslips according to the method of Mains and May (24). Briefly, 16-mm² round coverslips were immersed in protamine sulfate in 0.15 M NaCl. After 30 min, excess protamine solution was removed by aspiration, and the coverslips were immersed in Nutrient supplements were added (50 to 200 μM in diameter). Aggregates were then transferred to new tissue culture dishes or for offline analysis of the cycle length (see below).

Separation of Myocytes from Fibroblasts. Cardiac myocytes were separated from fibroblasts by trypsinization with strongly adhering heart cell aggregates were transferred to a bath (volume of 2 ml) that was attached to the stage of an inverted phase-contrast microscope. The aggregates were continuously superfused at a rate of 6 ml/min with 100 ml of M199 medium, which was recirculated between a reservoir and the bath by means of a peristaltic pump. The fluid was heated to 37°C by means of an interposed condenser. The reservoir fluid was bubbled with 95% O₂-5% CO₂ via a fine polyethylene tube (pH 7.4). At the K⁺ concentration in M199 (about 5.4 mM), the vast majority of aggregates exhibited stable resting membrane potentials and no spontaneous firing, in agreement with the previous findings of Clay and Shrier (21) for aggregates cultured from 7-day-old chick embryo hearts. When desired, the aggregates were paced at a constant frequency (1 Hz) by suprathreshold, rectangular current pulses delivered via a glass pipet (tip diameter, 50 to 100 μM) positioned within 100 μM of the aggregate. To record intracellular potentials, the aggregates were impaled with conventional micropipets which were filled with 3 M KCl and had a resistance of 40 to 60 MΩ. The microelectrode was electrically connected via a silver-silver chloride wire to a high input impedance preamplifier. The bath fluid was grounded via a silver-silver chloride wire, which served as a reference electrode. After amplification, the action potential and a segment of the preceding baseline were digitized using a computer (IBM PC AT) equipped with an analogue-to-digital converter (Tecmar, Inc.) and software to control data acquisition (pCLAMP; Axon Instruments). Data acquisition was triggered by an event detector, much as described in the preceding paragraph. The acquired data were stored on floppy diskettes. Analysis of the action potential waveform was performed offline, using software developed in this laboratory, which extracts various parameters of the stored action potentials (resting potential, maximum diastolic potential, action potential duration at selected levels of repolarization, and the peak overshoot potential). The reader is referred to Vogel and Terzic (25) for a more detailed description of action potential analysis. The slope of the pacemaker potential and the peak overshoot potential was determined for spontaneously firing aggregates. The method for estimating the takeoff potential can be found in the legend to Fig. 5. For quiescent aggregates, the takeoff potential was considered identical to the resting membrane potential just prior to suprathreshold electrical stimulation.

Analysis of Cycle Lengths. To examine the rhythmicity of spontaneous beating in heart cell aggregates treated with doxorubicin, the cycle length intervals between 50 and 200 successive beats were deter-
Electrophysiological effects of doxorubicin in the heart

RESULTS

Induction of Abnormal Automaticity and Dysrhythmia by Doxorubicin in Chick Heart Cell Aggregates

Abnormal Automaticity. Evidence that doxorubicin induces spontaneous beating in quiescent chick cardiac cell aggregates is presented in Fig. 1. The percentage of aggregates contracting spontaneously was low (<10%) in untreated cultures, but was increased markedly following a 24-h incubation with doxorubicin (Fig. 1, Day 1 curve). As can be seen, the effects of doxorubicin on automaticity increased with drug concentration between 0.01 and 1.0 μM; at 1.0 μM, over 80% of the aggregates examined contracted spontaneously. The effects of a 48-h incubation with doxorubicin were also tested (Fig. 1, Day 2 curve). At a doxorubicin concentration of 1.0 μM, a slight decrease in the percentage of aggregates contracting was noted at 48 h (Fig. 1, Day 2 curve), due perhaps to the cytotoxicity of doxorubicin at this concentration. It was therefore tested whether doxorubicin affects cell viability. To do this, a separate series of experiments was performed in which cardiac cell aggregates were exposed to doxorubicin for 48 h and then tested for an ability to exclude the dye, trypan blue. Failure to exclude the dye was observed at 1 and 10 nM doxorubicin only in the noncontracting aggregates that presumably had ceased to function (data not shown). In contrast, the aggregates excluded trypan blue at doxorubicin exposures of less than 1 μM for 48 h (data not shown), indicating that induction of automaticity by doxorubicin was probably not associated with destruction of the cell membrane. Thus, only at relatively high doxorubicin concentrations was there evidence that doxorubicin impaired cell membrane integrity.

Dysrhythmic Beating. To investigate whether doxorubicin causes dysrhythmia in cultured heart cells, the pattern of spontaneous beating was examined in a series of aggregates treated with doxorubicin. Both rhythmic and dysrhythmic beat patterns were observed depending on the doxorubicin concentration. Fig. 2 provides sample photocell recordings of twitch contractions from a rhythmic (Fig. 2A) and dysrhythmic aggregate (Fig. 2B) that had been exposed to 0.3 or 1.0 μM doxorubicin, respectively, for 48 h. The histograms of cycle lengths in Fig. 2 demonstrate a striking difference between rhythmic and dysrhythmic beat patterns. When the pattern of beating is regular, the histogram of the cycle lengths generally has a single dominant peak (Fig. 2A). In comparison, histograms associated with doxorubicin-treated cultures that are beating irregularly have much broader distribution (Fig. 2B). To provide an index of the degree of dysrhythmia caused by doxorubicin treatment, the variance of the interval distributions was calculated. As expected, the variance was larger for the dysrhythmic aggregate (6.7 x 10⁶ ms²) than for the rhythmic aggregate (0.04 x 10⁶ ms²). The measured variances for 92 different treated aggregates are presented in Fig. 3. Aggregates that beat rhythmically (i.e., the interbeat intervals are distributed unimodally) always had variances of less than 1.0 x 10⁶ ms². Whereas the variances were generally well below this value at doxorubicin exposures of 0.1 or 0.3 μM, an exposure to doxorubicin of 1.0 μM (for 48 h) resulted in a mixture of variances 50% of which exceeded (often markedly) a value of 1 x 10⁶ ms². This confirms that this relatively high exposure to doxorubicin can produce dysrhythmic beat patterns in cultured heart cells.

Electrophysiological Actions of Doxorubicin in Cultured Heart Cell Aggregates

Spontaneous Firing and Depolarization of Doxorubicin-treated Heart Cell Aggregates. Representative intracellular microelectrode recordings from control and doxorubicin-treated aggregates are shown in Fig. 4. Control aggregates exhibited stable resting membrane potentials of about —70 mV (Fig. 4A), but could be excited to fire an action potential by a brief electrical stimulus (Fig. 4A, inset). The action potential was accompanied by a twitch contraction that was easily visible under the inverted microscope at a magnification of x200 to x400. Doxorubicin treatment (>10⁻⁷ M for 24 to 30 h) caused the majority of aggregates to beat spontaneously (e.g., see Fig. 1). Spontaneously firing action potentials with diastolic (Phase 4) depolarization were found upon impaling such aggregates. The records in Fig. 4, B to D, illustrate that, for increasing doxorubicin concentrations, there were a progressive depolarization of the maximum diastolic potential and a steeper slope of Phase 4 depolarization.

Concentration-dependent Effects of Doxorubicin on the Cardiac Action Potential. Table 1 summarizes the characteristics of the action potential recorded from a total of 107 aggregates. The control aggregates were paced at a standard frequency of 1 Hz to elicit the action potential, as the vast majority of them did not beat spontaneously. The maximum diastolic potential (i.e., the maximum negative potential during the diastolic interval) had a mean value under control conditions of —67 ± 1 mV (n = 37). The maximum diastolic potential of spontaneously beating aggregates in 0.1, 0.3, and 1.0 μM doxorubicin was —63 ± 3 (n = 8), —57 ± 2 (n = 25), and —51 ± 2 mV (n = 33), respectively, and was significantly depolarized in comparison with control values at the two highest concentrations.

Fig. 1. Effect of doxorubicin to induce automaticity in cultured chick embryo heart cells. The percentage of aggregates contracting spontaneously is plotted as a function of the doxorubicin concentration at exposure times of 24 h (Day 1 curve) and 48 h (Day 2 curve). The experimental protocol was as follows. Aggregates in tissue culture dishes were incubated without drug (control group) or with a single concentration of doxorubicin (range; 10⁻⁸ to 10⁻⁶ mol). For experimental measurements of automaticity, each tissue culture dish was transferred to the thermostatically controlled stage (37°C) of an inverted microscope. The pH of the medium was maintained at 7.4, using a HEPES buffer. The control aggregates were paced at a standard frequency of 1 Hz to elicit the action potential, as the vast majority of them did not beat spontaneously. The maximum diastolic potential (i.e., the maximum negative potential during the diastolic interval) had a mean value under control conditions of —67 ± 1 mV (n = 37). The maximum diastolic potential of spontaneously beating aggregates in 0.1, 0.3, and 1.0 μM doxorubicin was —63 ± 3 (n = 8), —57 ± 2 (n = 25), and —51 ± 2 mV (n = 33), respectively, and was significantly depolarized in comparison with control values at the two highest concentrations.
Electrophysiological Effects of Doxorubicin in the Heart

Fig. 2. Doxorubicin-induced automaticity and dysrhythmia. Initially quiescent cell aggregates from 7-day-old chick embryo hearts were incubated in tissue culture dishes with doxorubicin for 48 h. For experiments, a tissue culture dish containing treated aggregates was transferred to the thermostatically controlled stage (37°C) of an inverted microscope. The twitch contractions of spontaneously beating aggregates were detected photoelectrically, as described in "Methods." A and B, penwriter records of contractions from two aggregates treated with doxorubicin at a concentration of 0.3 μmol (A) or 1.0 μmol (B). Note that dysrhythmic beating occurred in the aggregate exposed to 1 μmol doxorubicin. A' and B', histograms of cycle lengths corresponding to the records in A and B. Note high variability of cycle lengths in the dysrhythmic case. Time calibration in A applied to A-B.

Fig. 3. Incidence of dysrhythmic beating in heart cell aggregates exposed to doxorubicin. Quiescent aggregates were exposed to doxorubicin in culture for 48 h. Spontaneous beating that resulted from doxorubicin treatment was detected photoelectrically in randomly selected aggregates, and the distribution of cycle lengths over a series of beats was analyzed in each case. The variance of the cycle length distribution is plotted for 92 different aggregates at three doxorubicin concentrations (0.1, 0.3, and 1 μmol, as indicated). Dysrhythmic beating (indicated by a variance >1 x 10^6 ms²) occurred in about 50% of the aggregates examined at the highest doxorubicin concentration. See text for further details.

These changes in maximum diastolic potential thus corresponded to a depolarization of 4, 10, and 16 mV at 0.1, 0.3, and 1.0 μM doxorubicin. The takeoff potential, i.e., the membrane potential at which the action potential is initiated, also was progressively depolarized as a function of the doxorubicin concentration (Table 1). The slope of diastolic (Phase 4) depolarization in spontaneously beating aggregates was increased in a concentration-dependent fashion; the change in this parameter was statistically significant at 1.0 μmol in comparison with 0.1 μM doxorubicin. Finally, at 1.0 μM doxorubicin, there was a small but statistically significant decrease in action potential duration. The overshoot potential was not significantly altered by incubations with doxorubicin.

Intracellular Microelectrode Recordings of Dysrhythmia in Doxorubicin-treated Heart Cell Aggregates. Several examples of dysrhythmia in doxorubicin-treated aggregates are presented in Fig. 5. Fig. 5A shows an intracellular recording from an aggregate which generated action potentials at an uneven rate. The cycle length histogram, which is also given, shows several peaks reflecting this arrhythmic beating. The records from a different aggregate in Fig. 5B provide an example of a sudden deceleration in the rate of beating by the mechanism of the "dropped beat," i.e., a failure of Phase 4 depolarization to achieve the action potential threshold for one or more beats.

The associated histogram shows that most of the 136 cycle lengths were in the 400 to 800 ms range. The exceptions were 4 unusually long interval lengths, indicating roughly 1.2 (2 cases), or 4 dropped beats. Fig. 5C depicts a recording from a third aggregate in which a sudden acceleration in beating occurred, apparently by the generation of an extrasystole. In this instance, the extrasystole appears to have arisen from a depolarizing afterpotential (Fig. 5C1, arrow), which did not always reach the action potential threshold (Fig. 5C2, arrow). In some aggregates, a temporary period of arrest occurred (for 20 to 60 s) during which time the membrane potential was stable. The resting membrane potential of such arrested cells provides an indication of the depolarizing action of doxorubicin (0.3 to 1.0 μM).
Table 1 Effect of doxorubicin on the action potential in aggregates of chick ventricular cells

Aggregates of chick ventricular heart cells were incubated in culture without drug (control) or exposed to doxorubicin for 24 to 30 h. Coverslips containing cultured aggregates were transferred to an experimental chamber where they were superfused with bubbled (95% O2, 5% CO2) culture medium at 37°C. Aggregates were impaled with conventional intracellular microelectrodes, and resting and action potentials were recorded. Control aggregates were paced, with suprathreshold rectangular current pulses, at a frequency of 1 Hz.

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>MDP* (mV)</th>
<th>Overshoot (mV)</th>
<th>APD50 (ms)</th>
<th>APD90 (ms)</th>
<th>APD10 (ms)</th>
<th>Phase 4 slope (mV/s)</th>
<th>Takeoff potentiala (mV)</th>
<th>Cycle length (ms)</th>
<th>No. of cells impaled/no. of cultures</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>-67 ± 1t</td>
<td>+21 ± 2</td>
<td>220 ± 12</td>
<td>174 ± 9</td>
<td>64 ± 3</td>
<td>-66 ± 1</td>
<td>(1.0)</td>
<td>37/10</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin (0.1)</td>
<td>-63 ± 3</td>
<td>+19 ± 5</td>
<td>243 ± 18</td>
<td>202 ± 19</td>
<td>60 ± 9</td>
<td>-49 ± 3</td>
<td>0.7 ± 0.1</td>
<td>8/1</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin (0.3)</td>
<td>-57 ± 2d</td>
<td>+23 ± 2</td>
<td>191 ± 13</td>
<td>147 ± 13</td>
<td>48 ± 5</td>
<td>-47 ± 2</td>
<td>1.0 ± 0.1</td>
<td>29/5</td>
<td></td>
</tr>
<tr>
<td>Doxorubicin (1.0)</td>
<td>-51 ± 2d</td>
<td>+17 ± 2</td>
<td>175 ± 15</td>
<td>137 ± 14d</td>
<td>37 ± 3d</td>
<td>-40 ± 2d</td>
<td>0.9 ± 0.1</td>
<td>33/5</td>
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* MDP, maximum diastolic potential; APD50, action potential duration at 90% repolarization; APD90 and APD10 defined similarly.

† Mean ± SE.

‡ P < 0.05 versus control.

§ P ≤ 0.05 versus 0.1 μM doxorubicin.

Fig. 5. Intracellular microelectrode recordings from cultured cardiac aggregates exhibiting dysrhythmic beating. The aggregates in A to C were exposed to 1.0 μM doxorubicin in culture for 24 h. A1 and A2, continuous recording from an aggregate showing fluctuations in action potential frequency. Time calibration in A2 applies to A1 and A2. In A3, the associated histogram demonstrates a broad distribution of cycle lengths in this aggregate (316 beats analyzed; variance of distribution was 6.0 x 105 ms2). B1, two sweeps illustrating a "dropped beat" (note long pause). In B2, the cycle length histogram for a sequence of action potentials suggests that predominantly rhythmic beating occurred in this aggregate except during occasional quiescent periods (arrow). Also illustrated (B1) are several features of the action potential measured in this study (Table 1). First arrow in upper trace points to the maximum diastolic potential (MDP). The second arrow points to the takeoff potential at which the action potential is initiated. A sharp transition from Phase 4 (diastolic depolarization) to Phase 0 (action potential upstroke) occurs at the takeoff potential. This transition point was estimated from the intersection of imaginary lines (note dashed lines) projected from Phase 4 and Phase 0. (The dashed lines were slightly displaced from Phase 4 and Phase 0 for illustrative purposes.) Action potential plateau and repolarization (Phases 2 and 3) are also indicated. C, intracellular recording from an aggregate that exhibited aberrant automaticity in the form of an extrasystole. Depolarizing afterpotentials are seen in these traces that attained the action potential threshold in C1 (arrow), but not in C2 (arrow).

µmol). As summarized in Table 2, the quiescent resting membrane potentials of treated aggregates (−63 to −30 mV depending on doxorubicin concentration) fell in a range considerably more positive than that of the control resting potentials (−61 to −75 mV).

DISCUSSION

Aggregates of cultured ventricular cardiomyocytes retain electrophysiological characteristics similar to intact cardiac muscle, including an absence of automaticity. Doxorubicin
induced spontaneous firing accompanied by twitch contractions in cultured aggregates at concentrations lower than those known to produce cytotoxic damage. The effects of doxorubicin were determined in vitro at concentrations (0.1 to 1 μM) which correlate well with in vivo levels achievable during the first 24 to 48 h following an i.v. bolus or continuous infusion of a standard dose of doxorubicin (26, 27). Thus, in this experimental preparation, clinically relevant concentrations of doxorubicin had a direct effect on the cardiac cells.

The data in Table 1 indicate a depolarizing action of doxorubicin in that the maximum diastolic potential was progressively shifted toward positive membrane potentials with increasing drug concentration. As confirmatory evidence, the resting potential measured during a pause in spontaneous beating was markedly depolarized in treated aggregates when compared with the quiescent resting membrane potential of controls (Ta-67 ± 1 mV that ranged from -61 to -75 mV.

The action of doxorubicin on automaticity may represent a basis of the concentration and duration of doxorubicin applic-

The occurrence of dysrhythmia in humans during doxorubicin therapy is recognized. In two prospective studies using continuous Holter monitoring, arrhythmias attributable to doxorubicin occurred in 24 to 30% of the patients within 24 h of administration (7, 28). EKG abnormalities are observed in humans (6) and experimental dogs (5) during the course of doxorubicin treatment (e.g., premature ventricular beats, non-sustained ventricular tachyarrhythmias).

It is possible that doxorubicin, by virtue of its depolarizing action, could cause abnormal automaticity in the intact heart through conversion of latent pacemaker cells into ectopic pacemakers. In this regard, preliminary experiments in dog Purkinje fibers demonstrated that doxorubicin (50 μmol) causes a gradual membrane depolarization accompanied by markedly enhanced automaticity during a 6-h continuous exposure (29). Rather similar effects on transmembrane potential and automaticity were produced in cultured aggregates of chick heart cells with considerably lower drug concentrations that were applied for a 24- to 48-h period (present results).

The molecular mechanism for the depolarizing action of doxorubicin is unknown. Doxorubicin probably does not directly affect ion channels, since LeMarec et al. (30) demonstrated in cardiac Purkinje fibers the absence of any electrophysiological action of the acute application of 50 μM doxorubicin. A possible indirect mechanism that could give rise to membrane depolarization is through the formation of doxorubicin-catalyzed free radicals (e.g., superoxide, hydrogen peroxide, or hydroxyl radicals) (9, 31), which are known to react with membrane lipids. Doxorubicin could, as a result, cause a general membrane permeability change and/or indirectly affect the properties of specific ion channels that are embedded in the cell membrane. These membrane actions of doxorubicin could, in turn, give rise to depolarization.

Besides membrane depolarization, doxorubicin (at relatively high concentrations) also causes excessive tissue calcium levels, which may contribute to the cardiac toxicity (i.e., loss of function and cell death) observed in this and many other studies. The underlying mechanism by which doxorubicin disturbs tissue Ca2+ levels may be through interference in various Ca2+ transport processes. In this regard, doxorubicin is known to inhibit certain Ca2+ translocation mechanisms in vesicular preparations of cardiac membranes. These include the Na/Ca exchanger in cardiac sarcolemma (32) and Ca-ATPase activity in sarcoplasmic reticulum (33). A physiological manifestation of the Na/Ca exchanger, which is electrogenic in heart muscle, is the delayed afterdepolarization occurring in cardiac Purkinje fibers in conditions of excessive Ca2+ loading; Binah et al. (34) demonstrated an immediate inhibition of ouabain-induced delayed afterdepolarizations by 50 μM doxorubicin, suggesting that this agent indeed inhibits Na/Ca exchange in intact heart muscle. Azuma et al. (35) reported indirect evidence that doxorubicin, within minutes, causes a transient increase in the slow Ca2+ inward current of intact, perfused chick embryo hearts.

Chick cardiac aggregates, because of their small size and favorable geometry, have been used as a model system to study the ionic currents underlying pacemaker behavior (see Refs. 19 and 21). Since the present investigation establishes that chick cardiac aggregates exhibit doxorubicin-induced automaticity, dysrhythmia, and depolarization at clinically significant drug concentrations, it is now possible to examine the ionic basis of these effects with voltage clamp methods.

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