A comparative study of collagenase- and trypsin-dissociated embryonic heart cells: reaggregation, electrophysiology, and pharmacology

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The electrophysiological and pharmacological properties of aggregates prepared from cells of 7-day-old chick embryo heart ventricles depend on the enzyme used for cell dissociation. The mean beat rate of aggregates formed from trypsin-dissociated cells was about 53 beats/min whereas aggregates formed from collagenase-dissociated cells had a mean beat rate of more than twice this value. Spontaneous activity of most aggregates formed from trypsin-dissociated cells was inhibited by elevating external potassium or by adding tetrodotoxin to the medium. A similar response to potassium was seen in all aggregates formed from collagenase-dissociated cells. However, approximately half of the aggregates formed from collagenase-dissociated cells were tetrodotoxin insensitive. Intracellular microelectrode recordings demonstrated that aggregates formed from collagenase-dissociated cells typically had reduced action potential maximal upstroke velocities and depolarized threshold potentials in comparison to those recorded from aggregates formed from trypsin-dissociated cells. In the presence of tetrodotoxin the maximal upstroke velocity of aggregates formed from either collagenase- or trypsin-dissociated cells decreased markedly. In the case of the collagenase-treated cells, the spontaneous activity which persisted in the presence of tetrodotoxin was abolished by the slow channel blocker D-600. Computer simulation of membrane depolarization supports the view that aggregates formed from collagenase-treated cells have a reduced fast inward sodium current and a significant leakage current. Aggregates prepared from trypsin-dissociated cells display properties which more closely resemble those of intact 7-day embryonic ventricular tissue. We therefore conclude that, contrary to previous reports, collagenase is not the enzyme necessarily best suited for cell dissociation in all tissue culture studies.

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Les propriétés électrophysiologiques et pharmacologiques d'agrégats provenant de cellules de ventricules cardiaques d'embryons de poulets âgés de 7 jours dépendent de l'enzyme utilisée pour la dissociation cellulaire. La fréquence cardiaque moyenne des agrégats provenant de cellules dissociées avec de la trypsine était d'environ 53 battements/min alors que celle des agrégats provenant de cellules dissociées avec de la collagénase était au moins deux fois plus élevée. On a pu inhiber l'activité spontanée de tous les agrégats formés par les cellules dissociées avec de la trypsine en élevant le potassium externe ou en ajoutant de la tétrodotoxine au milieu de dissociation. On a pu observer une réponse au potassium similaire chez tous les agrégats provenant des cellules dissociées avec de la collagénase. Toutefois, près de la moitié de ces derniers étaient insensibles à la tétrodotoxine. Des enregistrements intracellulaires avec des microélectrodes ont démontré que, comparés aux agrégats provenant de cellules dissociées avec de la trypsine, les agrégats provenant de cellules dissociées avec de la collagénase présentaient généralement de plus faibles pointes de vitesse de montée du potentiel d'action et des potentiels de seuil dépolarisés. En présence de tétrodotoxine, la pointe de vitesse des agrégats provenant de l'une ou l'autre catégorie de cellules diminua nettement. Toutefois l'activité spontanée des cellules traitées à la collagénase, qui s'était maintenue en présence de la tétrodotoxine, fut éliminée par l'inhibiteur de voie lente, D-600. Une stimulation sur ordinateur de la dépolarisation membranaire supporte l'hypothèse qui veut que les agrégats formés par des cellules traitées à la collagénase aient un courant sodique entrant plus faible et un courant de fuite important. Les agrégats préparés avec des cellules dissociées avec de la trypsine présentent des propriétés qui ressemblent beaucoup plus à celles du tissu ventriculaire intact des embryons âgés de 7 jours. Nous concluons donc, contrairement à ce qui a déjà été dit, que la collagénase n'est pas nécessairement la meilleure enzyme à utiliser pour la dissociation cellulaire lors d'expériences avec la culture cellulaire.

[Traduit par le journal]

Introduction

Tissue culture preparations have been widely used to study membrane properties of developing cardiac muscle. These preparations are usually made from enzyme-dissociated cells.

Trypsin, a nonspecific protease, is the enzyme most commonly used to dissociate cells from the hearts of embryonic and neonatal chick (Cavanaugh 1955; DeHaan and Gottlieb 1968; Fischman and Moscona 1971; Hyde et al. 1969; Lieberman 1967; Lompre et al. 1979; McDonald et al. 1972; Robinson and Legato 1980; Shrier and Clay 1980; Sperelakis and Lehmkuhl 1964; Wollenberger 1964), embryonic mouse (Fischman and Moscona 1971), and neonatal rat (Harary and Farley 1963; Jongsma 1972; Jongsma et al. 1975; Kasten 1971; Legato 1972; Mark and Strasser 1966; Schanne et al. 1977). Crude collagenase has also been used to dissociate embryonic (Cavanaugh et al.

1963; Smith and Berndt 1964) and adult cardiac cells (Berry et al. 1970; Glick et al. 1974; Kono 1969; Powell and Twist 1976; Dani et al. 1977; Clark et al. 1978; Rajs et al. 1978; Isenberg and Klockner 1980; Bustamante et al. 1982; Hume and Giles 1981). Cavanaugh et al. (1963) observed that crude collagenase yielded more complete dissociation of embryonic chick heart and was less damaging than trypsin, as judged by several criteria including cell appearance and viability. Masson-Pévet et al. (1976) also concluded that collagenase was superior to trypsin for cell dissociation, based on an ultrastructural study of newborn rat heart dissociated by the two enzymes.

The purpose of this study was to determine whether there were any electrophysiological or pharmacological differences between the cell membranes of cultured cells dissociated by these two agents. We compared the properties of heart cell aggregates prepared from 7-day-old chick embryo heart ventricles dissociated with trypsin or collagenase. The aggregate morphology, beat rate, electrophysiology, and pharmacology were found to depend on the dissociating enzyme used. In particular, aggregates prepared from collagenase-dissociated cells displayed a reduced upstroke velocity of the action potential and a decreased tetrodotoxin sensitivity. Under the conditions used in this study, we conclude that aggregates prepared from trypsin-dissociated cells display electrophysiological and pharmacological properties more typical of intact 7-day ventricular tissue than those prepared from collagenase-dissociated cells. Computer simulations based on an ionic model from voltage-clamp measurements (Ebihara and Johnson 1980; Clay and Shrier 1981*a*) suggest that the experimental results can be explained by a reduced fast inward sodium current and an increased leakage current in collagenase-dissociated cells.

Methods

Preparation

Fertilized eggs from White Leghorn hens were incubated at 37°C and 85% humidity. Seven-day-old embryos were removed and decapitated, and their hearts were aseptically excised. The apical portions of the heart ventricles were isolated, fragmented, and dissociated into single cells using enzymatic dissociation combined with mechanical agitation (DeHaan 1970). One-half the tissue was dissociated in trypsin, and the other half in collagenase. An inoculum of 5×10^5 to 7×10^5 cells was added to 3 mL of culture medium 818A contained in a 25-mL Erlenmeyer flask. The flask was gassed with a mixture of 5% CO_2 – 10% O_2 – 85% N_2, sealed with a silicone rubber stopper, and placed on a gyratory table (70 revolutions/min) for 48–72 h at 37°C to allow spherical aggregates to form (Sachs and DeHaan 1973; DeHaan and DeFelice 1978).

Dissociation media

(*a*) Trypsin-dissociation medium DM8 (Clapham et al. 1980) consisted of 5×10^{-5} g/mL crystalline trypsin (Worthington Biochemical; 245 U/mg) and 5×10^{-6} g/mL deoxyribonuclease (Worthington; 9.1×10^4 U/mg) in calcium- and magnesium-free phosphate buffered solution at pH 7.3. (*b*) Collagenase-dissociation medium consisted of 5×10^{-5} g/mL collagenase (Sigma; Type I, 146 U/mg) and 5×10^{-6} g/mL deoxyribonuclease (Worthington) in calcium- and magnesium-free phosphate buffered solution at pH 7.3.

Culture media

Medium 818A (Sachs and DeHaan 1973) contained 20% medium 199 (Grand Island Biological Company (GIBCO)), 2% heat-inactivated horse serum (K.C. Biological), 4% fetal calf serum (GIBCO), and 0.5% gentamycin (Schering) in potassium-free Earle's balanced salt solution (millimolar): NaCl, 116.0; $MgSO_4$, 0.8; NaH_2PO_4, 0.9; $CaCl_2$, 1.8; $NaHCO_3$, 26.3; glucose, 5.5. The potassium concentration of the final medium was adjusted to 1.3 m*M*.

Drugs

Tetrodotoxin (TTX) (Sigma) was dissolved in distilled water (1 mg/mL) and diluted to final concentrations of 1×10^{-8} g/mL to 1×10^{-5} g/mL. D-600, a generous gift of Knoll AG (Ludwigshafen am Rhein, Germany), was dissolved in distilled water and diluted to a final concentration of 2×10^{-7} g/mL.

Electrophysiology

The contents of each flask were transferred to a plastic tissue culture dish (Falcon No. 3001) on the warmed stage of a dissecting microscope. The aggregates adhered firmly to the bottom of the dish in an hour or less. The temperature was maintained at 37 ± 0.5°C by a temperature controller (continuously monitored). The bath (volume 0.5 mL) was continuously perfused at a rate of 1 mL/min with medium 818P (medium 818A without serum) equilibrated with a gas mixture of 5% CO_2 – 10% O_2 – 85% N_2. Drugs were added to the perfusion medium.

Rhythmically beating aggregates were viewed at a magnification of ×50 and their sizes were measured with an ocular reticle whose smallest division represented 18 μm. Measurements could be made to about half a division.

Aggregate diameter was taken to be the mean of the major and minor diameters in the horizontal plane. Aggregate volume was calculated assuming the aggregate to be a prolate spheroid. Contractions were visually monitored to obtain beat rates.

Electrical activity was recorded intracellularly using micropipettes filled with 3 *M* KCl. The electrode impedance ranged from 30–70 MΩ. The signals were recorded using an amplifier with negative capacity compensation and the extracellular medium was coupled to a current-to-voltage converter that maintained the bath at virtual ground via an agar bridge and an Ag:AgCl wire. The membrane voltage was amplified (×50) and stored on magnetic tape (HP 3964) for off-line analysis with a digital oscilloscope (Nicolet).

The action potential duration was measured as the time from maximal upstroke velocity ($\dot{V}_{max}$) to 50% action potential repolarization (APD_{50}) and from $\dot{V}_{max}$ to maximum diastolic potential (APD_{100}). Threshold potential was estimated from the break between diastolic depolarization and the

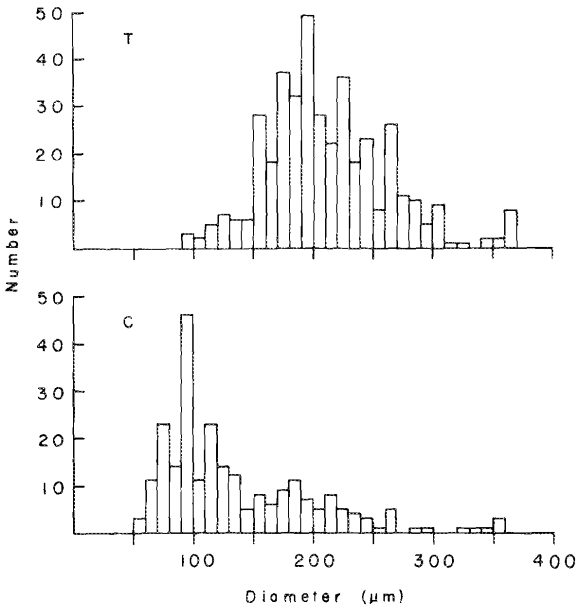

FIG. 1. The size distribution of T aggregates (n = 403) and C aggregates (n = 243).

more rapid upstroke phase. Measurements of $\dot{V}_{max}$ were made with an electronic differentiator which was linear from 5 V/s to 1000 V/s.

In this study n always refers to the number of aggregates sampled and does not represent multiple measurements from a single preparation.

Computer simulations

The computations were carried out on an HP 1000 Series-F computer (Hewlett–Packard) located in the Department of Anaesthesia Research at McGill University.

Results

Aggregation

Aggregates formed during 48–72 h of gyration culture from trypsin-dissociated ventricular cells (T aggregates) were compared with those formed from collagenase-dissociated cells (C aggregates). Figure 1 shows the distribution of diameters of T aggregates from 15 cultures and C aggregates from 12 cultures (of which 8 were paired cultures). Most (72%) T aggregates ranged in diameter between 150 and 250 μm whereas most (70%) C aggregates ranged between 50 and 150 μm: only 7% of T aggregates were smaller than 150 μm while only 16% of C aggregates were greater than 200 μm. The mean diameter (± SD) of T aggregates (210.3 ± 54.3 μm, n = 403) was significantly different ($P < 0.01$) from that of C aggregates (134.1 ± 63.8 μm, n = 243).

Spontaneous beat rate

When T and C aggregates were placed in tissue culture dishes they remained spheroidal for several hours and beat spontaneously with a regular rhythm. It was shown by Sachs and DeHaan (1973) that aggregate beat rate is inversely related to aggregate volume. There is evidence of a similar volume–beat rate trend for T aggregates (Fig. 2). Moreover, there is good correspondence between the beat rates found here in the 10^6–10^7 μm^3 volume range and those reported previously (Sachs and DeHaan 1973). In contrast, beat rates of C aggregates tend to be independent of aggregate volume. For example, the mean beat rate (± SD) of C aggregates with volumes in the range between 10^5 and 10^6 μm^3 (115.1 ± 25.2 beats/min; n = 70) is not

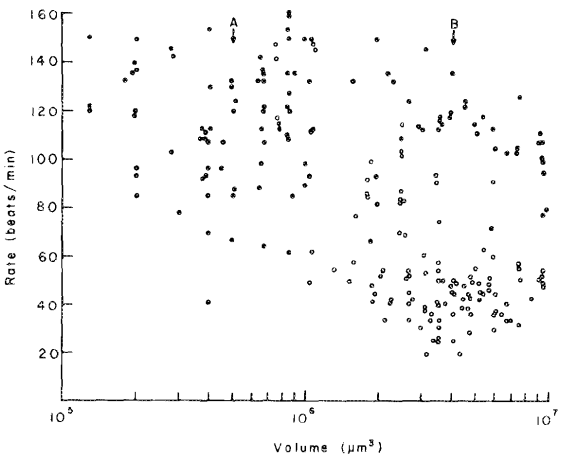

FIG. 2. Rate–volume plot of T aggregates (○, n = 107) and C aggregates (●, n = 114). Arrows at A and B are drawn at volumes calculated using 100 and 200 μm diameters, respectively. Beat rates were obtained by visually monitoring contractions.

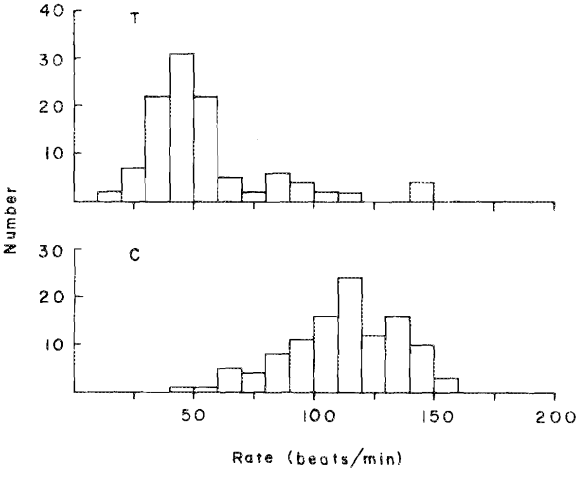

FIG. 3. The distribution of beat rates of T aggregates (n = 107) and C aggregates (n = 114).

TABLE 1. Effect of external potassium concentration on spontaneous activity

	Beat rate (Beats/min)		
	1.3 m*M* $[K^+]_o$	2.5 m*M* $[K^+]_o$	% decrease
T aggregates (n = 10)	52.3±2.9	23.8±6.8	54
C aggregates (n = 8)	91.6±15.6	51.2±12.8	44

NOTE: Beat rates given are means ± SD.

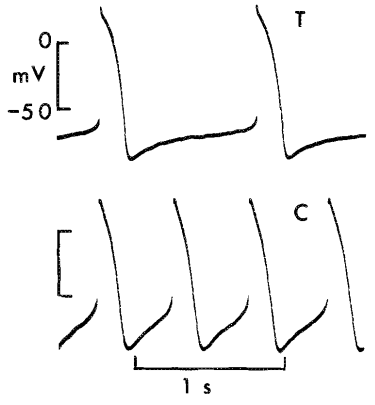

FIG. 4. Typical intracellular recordings of spontaneous activity in T aggregates and C aggregates.

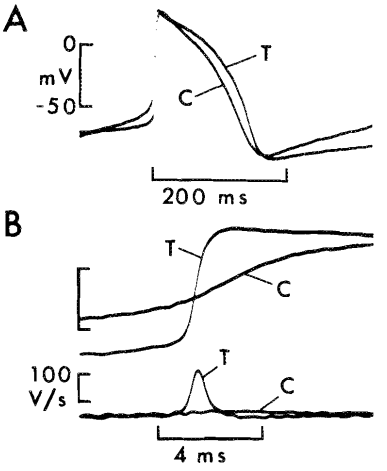

FIG. 5. (A) Superimposed action potentials recorded from a T aggregate and a C aggregate. (B) Upper pair of traces: superimposed upstroke phases displayed at a faster sweep speed. Vertical calibration bar is 50 mV. Lower pair of traces: differentiated upstroke phases.

significantly different from that of C aggregates in the volume range 10^6 to 10^7 μm^3 (113.1 ± 19.3 beats/min; n = 44). However, in the volume range 10^6 to 10^7 μm^3, the mean beat rate of C aggregates was approximately double that of T aggregates (53.3 ± 32.3 beats/min; n = 104). Figure 3 shows the distribution of spontaneous beat rates for T and C aggregates.

It has been previously demonstrated that increasing the external potassium concentration $[K^+]_o$ will slow or abolish spontaneous activity in isolated embryonic heart cells (DeHaan 1970) and in T aggregates (DeHaan and DeFelice 1978). Table 1 shows the decrease in spontaneous beat rate of both T aggregates and C aggregates (120–150 μm diameter) that occurred when $[K^+]_o$ was increased from 1.3 to 2.5 m*M*. In both cases, a further elevation of $[K^+]_o$ to 3.5 m*M* resulted in irregular beat rates characteristically lower than 1 beat/min. At $[K^+]_o$ of 4.5 m*M* all spontaneous activity in both groups was arrested.

It has been previously reported that more than 80% of T aggregates stopped beating in 10^{-5} g/mL TTX (McDonald et al. 1972). These results were replicated in five experiments (n = 75) in which TTX at doses between 10^{-7} and 5×10^{-7} g/mL stopped the beating of 70% of the T aggregates. Any residual spontaneous beating was generally irregular and sporadic bursts of activity were occasionally observed. In contrast, the response of C aggregates to TTX was less uniform. In six experiments (n = 51) spontaneous activity persisted in the presence of 10^{-7} to 5×10^{-7} g/mL TTX: in one of these experiments spontaneous activity persisted when the TTX concentration was increased to 10^{-5} g/mL. In eight other experiments (n = 56) TTX at doses between 10^{-7} and 5×10^{-7} g/mL stopped the beating of 75% of the C aggregates. Residual activity was sporadic.

Electrophysiology

Spontaneous electrical activity recorded from T and C aggregates is shown in Figs. 4 and 5. The pooled results for the action potential parameters measured from 12 experiments (n = 14) using T aggregates and from 11 experiments (n = 25) using C aggregates are presented in Table 2. Recordings were made after impalements had stabilized, as reflected by a constant interbeat interval and stable action potential parame-

TABLE 2. Electrophysiological parameters

Condition	Action potential amplitude (mV)	Overshoot potential (mV)	Maximum diastolic potential (mV)	Action potential duration		$\dot{V}_{max}$ (V/s)	Interbeat interval (ms)
				APD_{50} (ms)	ADP_{100} (ms)		
T aggregates ($n = 14$)	118.8±5.0	28.0±4.1	−90.8±6.3	122.1±19.8	170.5±27.6	120.9±35.4	1032.3±703.6
C aggregates ($n = 25$)	113.2±9.4	24.5±4.8	−88.7±7.1	109.0±16.3	164.6±30.9	24.3±13.4*	424.8±105.7*
C aggregates and TTX ($n = 6$)	113.0±8.5	23.2±5.5	−89.9±5.9	128.3±34.5	181.2±35.2	5.9±1.4	606.8±345.4

NOTE: Values given are means ± SD; parameters from C aggregates which are significantly different from T aggregates ($P < 0.01$, Student's t-test) are denoted by *.

ters. Action potential of T and C aggregates which had significantly different interbeat intervals and maximum upstroke velocities did not have significantly different action potential durations.

The addition of TTX (10^{-7} to 5×10^{-7} g/mL) led to a progressive reduction of $\dot{V}_{max}$ in T aggregates and prolongation of the interbeat interval (Fig. 6). In addition, there was a depolarization in the level of the threshold potential. After several minutes the pacemaker depolarization failed to reach threshold and spontaneous action potential generation was blocked. The mean resting potential (± SD) of these aggregates was found to be −57.3 ± 9.7 mV ($n = 12$).

A similar response to TTX was found in some C aggregates (Fig. 6). Following cessation of activity the mean resting potential of these preparations was −54.9 ± 7.2 mV ($n = 8$). However, in six other C aggregates spontaneous activity was sustained in the presence of TTX, with reduced upstroke velocities and depolarized threshold potential levels (Fig. 7 and Table 2).

The addition of the slow channel blocker D-600 (Kohlhardt et al. 1972) at a concentration of 2×10^{-7} g/mL to a C aggregate beating in the presence of TTX blocked spontaneous activity. The ability of D-600 to block spontaneous activity can also be observed in C aggregates and T aggregates in the absence of TTX: in two of three T aggregates and four of four C aggregates D-600 (2×10^{-7} g/mL) blocked spontaneous activity. A comparison of electrical activity of the T aggregates before and after D-600 shows that D-600 reduced the overshoot, shortened the action potential duration, and reduced the interbeat interval, but had no effect on $\dot{V}_{max}$ (Fig. 8). Figure 8 also shows that the response of C aggregates to D-600 was similar but there was a decrease of $\dot{V}_{max}$.

Simulation of pacemaker and upstroke phases

Simulations of the pacemaker process and the upstroke phase of the action potential of T and C aggregates were carried out by numerical integration of a system of differential equations obtained from voltage-clamp measurements of background (I_{bg}) and time-dependent pacemaker (I_{K_2}) currents in 7-day trypsin-dissociated ventricular aggregates (Clay and Shrier 1981*a*). Measurements of the TTX sensitive, fast inward sodium current (I_{Na}) made in trypsin-dissociated aggregates were also incorporated into the model (Ebihara et al. 1980; Ebihara and Johnson 1980). The parameters of the model are scaled for a 200-μm diameter aggregate which has approximately 2.15×10^{-2} cm^2 of membrane surface area (Clay and Shrier 1981*a*). All currents are given in nanoamperes, voltages in millivolts, conductances in millisiemens, rate constants in inverse seconds, and times in seconds. The

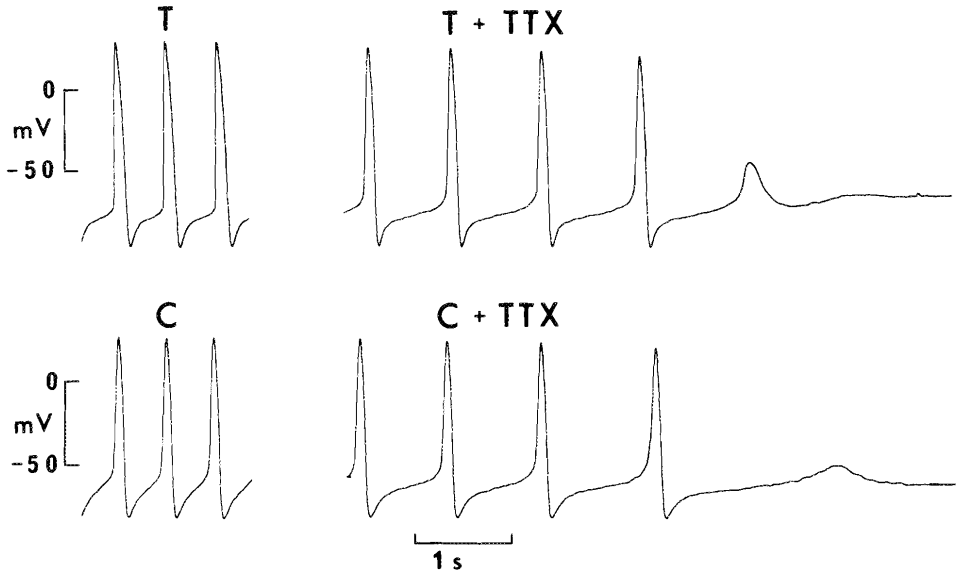

FIG. 6. Left hand panel: spontaneous activity in T aggregates (upper trace) and C aggregates (lower trace). Right hand panel: blockade of spontaneous activity by addition of TTX (10^{-7} g/mL).

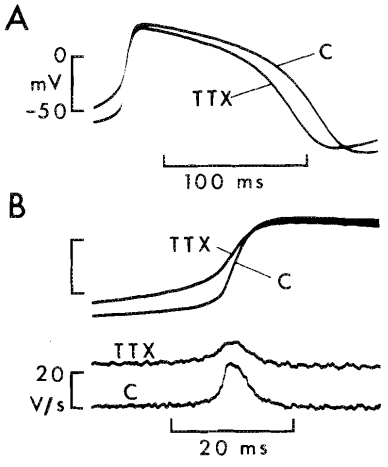

FIG. 7. (A) Superimposed action potentials recorded from a continuous impalement of a single cell within a C aggregate before (C) and after (TTX), 15-min exposure to 10^{-7} g/mL TTX. (B) Upper pair of traces: superimposed action potential upstroke phases displayed at a faster sweep speed. Vertical calibration bar is 50 mV. Lower pair of traces: differentiated upstroke phases.

transmembrane voltage is denoted by V.

I_{bg} is given by the sum of two time-independent currents:

$$[1] \quad I_{bg} = I_{K_1} + I_{Nab}.$$

I_{K_1} is given by

$$[2] \quad I_{K_1} = 220(y^4)(p - q)/(1 + y + y^2 + y^3 + y^4) + 1.1(V + 50)/(1 - \exp(-(V + 50)/25))$$

where

$$[3] \quad p = (1 + \exp(-(V + 90)/25))^{-1}; q = 1 - p; y = q/p.$$

I_{Nab} is given by

$$[4] \quad I_{Nab} = 0.23(V - 40).$$

The I_{K_2} component is given by

$$[5] \quad I_{K_2} = 550 s x^2 (w - z)/(1 + x + x^2)$$

with

$$[6] \quad w = (1 + \exp(-(V + 112)/25))^{-1}; z = 1 - w; x = z/w$$

and

$$[7] \quad ds/dt = -(\alpha_s + \beta_s)s + \alpha_s$$

with rate constants

$$[8] \quad \alpha_s = 0.8(V + 55)/(1 - \exp(-0.2(V + 55)))$$
$$\beta_s = 0.059 \exp(-0.105(V + 55)).$$

The TTX sensitive, fast inward sodium current (I_{Na}) is given by

$$[9] \quad I_{Na} = \bar{g}_{Na} m^3 h (V - 40)$$

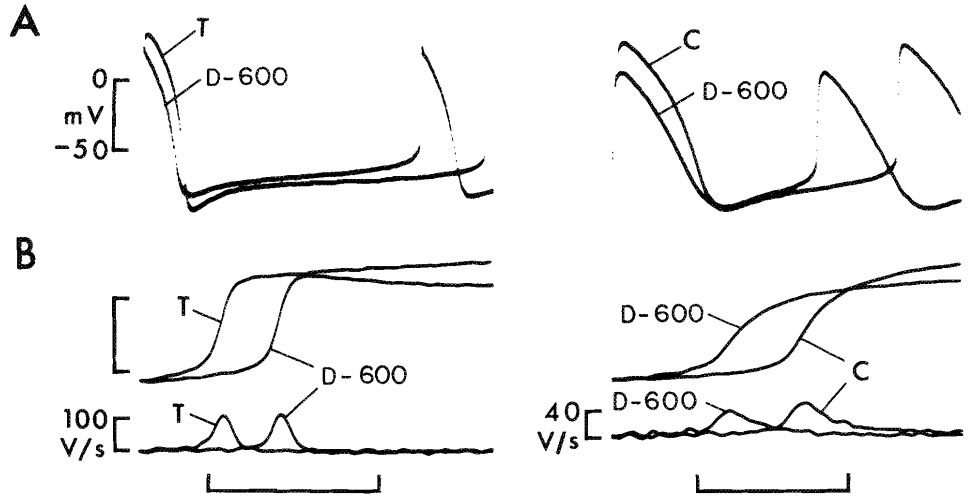

FIG. 8. Comparison of the effects of D-600 (2×10^{-7} g/mL) on action potentials recorded from T aggregates and C aggregates. Left hand panel: (A) Superimposed action potentials recorded from continuous impalement of a cell within a T aggregate before (T) and after (D-600) 20-min exposure to D-600. (B) The upper pair of traces show superimposed upstroke phases from these recordings, while the lower pair of traces show the upstroke velocities. Scale: 50 mV vertical, 0.5 s horizontal (A), 5 ms horizontal (B). Right hand panel: (A) Superimposed action potentials recorded from continuous impalement of a cell within a C aggregate before (C) and after (D-600) 12-min exposure to D-600. (B) The upper pair of traces show superimposed upstroke phases from these recordings, while the lower pair of traces show the upstroke velocities. Scale: 250 ms horizontal (A), 5 ms horizontal (B).

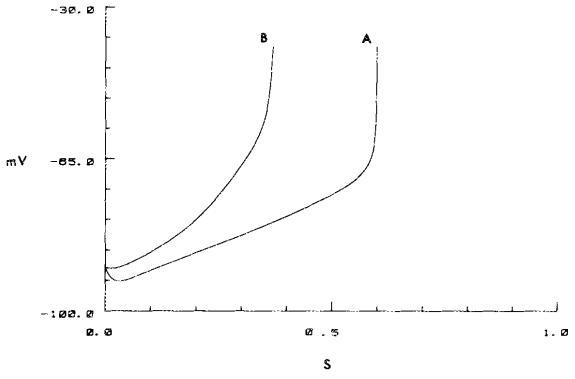

FIG. 9. Simulation of membrane potential during pacemaker and upstroke phases of the action potential. (A) Membrane potential when $\bar{g}_{Na} = 323$ mS and $I_{app} = 0$ nA. (B) Membrane potential when $\bar{g}_{Na} = 60$ mS and $I_{app} = 5$ nA.

with

$$[10] \quad dm/dt = (\alpha_m + \beta_m)m + \alpha_m$$
$$dh/dt = (\alpha_h + \beta_h)h + \alpha_h$$

where

$$[11] \quad \alpha_m = 100(V + 45)/(1 - \exp(-0.1(V + 45)))$$
$$\beta_m = 4 \times 10^3 \exp(-(V + 65)/18)$$
$$\alpha_h = 135 \exp(-(V + 80)/6.8)$$
$$\beta_h = 356 \exp(0.079V) + 3.1 \times 10^8 \exp(0.35V).$$

The net ionic membrane current is given by

$$[12] \quad I_{ionic} = I_{Na} + I_{K_2} + I_{bg}.$$

The expression for I_{Na} in Eq. 9 is the same as that used by Hodgkin and Huxley (1952) to describe I_{Na} in nerve. The expressions for the rate constants α_m and β_m in Eq. 11 have the same functional form as in the Hodgkin-Huxley equations. The rate constants for α_h and β_h were taken from Ebihara and Johnson (1980). The membrane voltage was obtained by numerically integrating

$$[13] \quad dV/dt = (I_{ionic} + I_{app})/C$$

where I_{app} is the externally injected current. The parameter C is the input capacitance of an aggregate, which has been determined previously to be 0.023 μF for aggregates of 200 μm diameter (Clay et al. 1979). The initial condition on membrane voltage was set to be −90 mV, which closely approximates maximum diastolic potential (MDP) for both T and C aggregates. We also set the activation parameter s equal to 1, since s is fully activated at the action potential peak and does not change rapidly during repolarization. We initially set $m = 0$ and $h = 1$, which approximate steady-state values of these parameters at MDP. The time course of

voltage change from these initial conditions was determined by numerically integrating Eq. 13 using the algorithm of Rush and Larsen (1978) with a 50-μs integration time step. Each simulation was terminated at −40 mV. In the case of T aggregates I_{app} was 0 and $\bar{g}_{Na}$ was set at 323 mS, whereas in C aggregates $\bar{g}_{Na}$ was reduced to 60 mS to reflect the reduction of $\dot{V}_{max}$ from 120 V/s in T aggregates to 24 V/s in C aggregates. To simulate the more rapid pacemaker depolarization in C aggregates, I_{app} was set to 5 nA. The results shown in Fig. 9 qualitatively mimic the major differences in the electrophysiological properties of T (line A) and C (line B) aggregates: a more rapid pacemaker depolarization and a slower upstroke phase of C aggregates. Moreover, the simulations also show the depolarization of threshold potential observed in C aggregates (Fig. 5). Finally, we note that neither reduction of $\bar{g}_{Na}$ nor the change of I_{app} alone could account for the observed differences between T and C aggregates.

Discussion

The results of this study indicate that collagenase-treated cells formed smaller aggregates than trypsin-treated cells. The size of aggregates has been shown to depend upon the tissue origin of the cells used, their developmental stage, the mode of cell isolation, and the conditions of cell rotation (Moscona 1965). When the speed of rotation is too great, the shearing forces exceed the mutual adhesiveness of the cells and aggregation is prevented. At slower rotation rates, aggregates form from cells which, upon contact, attach firmly enough to resist the shearing forces. Under the same conditions smaller aggregates will be formed from less cohesive cells (Moscona 1965). The fact that C aggregates were smaller in size than T aggregates indicates a possible decrease in cell−cell adhesivity in C aggregates. However, Cavanaugh et al. (1963) found that collagenase-dissociated cells adhered more rapidly to glass culture dishes and tended to flatten faster. The adhesion of cells to glass may involve a process different from that involved in cell−cell adhesion. It has been postulated that cell recognition and selective cell adhesion are mediated by interaction of specific cell surface components (Moscona 1960). Furthermore, isolation techniques (e.g., enzyme type and concentration) and culture conditions can influence adhesiveness.

In this study, C aggregates on average beat more rapidly than T aggregates. A comparison of the spontaneous activity of C and T aggregates shows that there is a marked difference in the rate of pacemaker depolarization (Fig. 4). The rate of pacemaker depolarization reflects a rather fine balance of inward and outward currents. Indeed, it has been observed that a few nanoamperes of steady applied outward current can completely block the generation of spontaneous action potentials in aggregates of cardiac cells, whereas a steady inward current can increase the beat rate (Scott 1979). Similarly, immediately after microelectrode impalement, rapid beat rates are often observed. These are presumably the result of injury-induced nonspecific leakage currents (Shrier and Clay 1982). The simulations presented in this study are consistent with the hypothesis that treatment with crude collagenase leads to membrane disruption giving rise to a nonspecific membrane leakage current (Fig. 9).

Modifications of membrane currents by enzyme treatment may conceivably account for the difference in spontaneous activity between C and T aggregates. The fast inward sodium current has a component which maintains steady levels of activation in the pacemaker voltage range (the window current, I_{Na2}). This window current not only affects action potential parameters (Atwell et al. 1979), but also pacemaker depolarization rate and consequently spontaneous beat rate (McAllister et al. 1975; Shrier and Clay 1982). In computer simulations, a reduction of the fast inward sodium current (which accounts for the reduced $\dot{V}_{max}$ in C aggregates) results in a slowing of the pacemaker depolarization rate. When $\bar{g}_{Na}$ is reduced from the control value of 323 to 60 mS, spontaneous activity ceases. Changes in the time-dependent pacemaker current (I_{K_2}) in 7-day ventricular T aggregates have been shown to be primarily responsible for the cessation of spontaneous activity when $[K^+]_o$ is elevated from 1.3 to 4.5 m*M* (Clay and Shrier 1981*a*). The degree of slowing of beat rates of C aggregates at elevated $[K^+]_o$ was similar to that found in T aggregates. Furthermore, T and C aggregates ceased beating at similar $[K^+]_o$. This suggests that I_{K_2} is present in C aggregates and at least in some respects is similar to I_{K_2} in T aggregates. A possible role of other currents (primarily background currents) cannot be ruled out. Indeed, the cessation of spontaneous activity of ventricular tissue during embryonic development appears to be due to a reduction of the background inward sodium current I_{Nab} (Clay and Shrier 1981*b*). Although we have shown that a nonspecific leakage current suffices to account for the differences in beat rate, possible contributions of I_{K_2} and I_{bg} cannot be ruled out. To evaluate the contribution of I_{K_2} and I_{bg} would require tracer-flux measurements coupled with voltage-clamp experiments.

In C aggregates, upstroke velocities with a mean of 24.3 V/s were observed, suggesting that the fast sodium current became markedly reduced following collagenase treatment and cell dissociation. TTX was effective in reducing $\dot{V}_{max}$ of these aggregates by a factor of four (Table 2), demonstrating the retention of a TTX-receptor interaction. The observed depolarization of threshold potential after TTX was added (Fig. 7) is also

consistent with the hypothesis that TTX blocks the fast sodium current in C aggregates. The ability of C aggregates to maintain spontaneous activity in the presence of TTX could result from residual TTX-insensitive fast sodium channels or alternatively from slow inward Na–Ca channels. The observation that D-600 abolished the spontaneous action potentials in TTX-treated C aggregates and slowed the upstroke phase of C but not T aggregates (Fig. 7) is consistent with the latter alternative. However, it should be noted that D-600 increased beat rate and subsequently blocked spontaneous activity in T and C aggregates not treated with TTX. This may be attributed to known effects of D-600 on outward potassium currents (Kass and Tsien 1975).

Cultured cardiac cells which have a reduced TTX sensitivity or $\dot{V}_{max}$ have been described as dedifferentiated (Sperelakis et al. 1976). C aggregates display these properties; however, other electrophysiological parameters of cells at earlier stages of development are absent. There is no decrease of the MDP, overshoot, or action potential duration in C aggregates to values typical of younger embryonic ventricular cells (McDonald and DeHaan 1972; McDonald and Sachs 1975). Consequently, we conclude that the effect of cell dissociation using crude collagenase does not lead to a complete dedifferentiation of membrane function after 2–3 days of gyration culture.

Schanne et al. (1977) have demonstrated that trypsin-dissociated neonatal rat cell colonies develop TTX-insensitive action potentials and develop slow responses after 4–6 days in culture. This change has been attributed to the trypsinization procedure and not to dedifferentiation. It is conceivable that this type of dissociation-induced transformation could also occur in T aggregates but with a much prolonged time course.

Enzyme-dissociated cells which are cultured as monolayers have been reported to be TTX insensitive and to lack a fast action potential upstroke (Sperelakis and Lehmkuhl 1965). However, it has been recently demonstrated that trypsin-dissociated cells in monolayer culture can retain TTX sensitivity and a rapid upstroke velocity (Lompre et al. 1979; Robinson and Legato 1980). Excessive trypsinization was suggested as a possible explanation for the poor recovery observed in the earlier monolayer culture studies (Robinson and Legato 1980).

Cells from 7-day embryonic chick heart in TTX-insensitive monolayer cultures can recover TTX sensitivity when dissociated and reassembled into aggregates (Sachs et al. 1973). This suggests that recovery depends on cell–cell interactions. The absence of TTX sensitivity in some C aggregates suggests that there may be some disruption in the recovery process normally seen in T aggregates. The beat rates of C aggregates are independent of aggregate size, and the mean beat rate and distribution of beat rates are similar to those determined from single myocytes obtained from 7-day embryonic hearts (Sachs and DeHaan 1973). The similarities between C aggregates and single cells may be due to loss of some cell–cell interactions in C aggregates.

It is of interest to note that TTX sensitivity and rapid action potential upstrokes are fully expressed in aggregates prepared from collagenase-dissociated neonatal rat heart cells (de Bruijne and Jongsma 1980). However, aggregates of 7-day embryonic chick heart cells prepared using collagenase at a concentration ten times higher than that used in this study demonstrated slow upstroke velocities (32 V/s) which were reduced (to 5 V/s) upon addition of 10^{-6} g/mL TTX (Mackenzie and Standen 1982).

A mixture of trypsin and collagenase has been shown to be effective for the isolation of viable single myocardial cells from adult rats (Jacobson 1977). On the other hand, an earlier report (DeHaan 1967) indicated that dissociation of embryonic chick heart cells using combinations of collagenase and trypsin gave less satisfactory results than when trypsin was used alone.

A more recent study by Haworth et al. (1980) showed that exposure of adult cardiac cells to trypsin after collagenase treatment can be beneficial. It was proposed that trypsin may allow the gap-junction channels in the intact cells to close, thereby conferring upon them Ca^{2+} resistance. This approach might also be beneficial for the dissociation of embryonic heart cells. However, the properties of adult and embryonic isolated cardiac cells differ: unlike adult heart cells, embryonic heart cells in culture retain the ability to redevelop electrical coupling, presumably by the formation of functional nexal units (DeHaan and Hirakow 1972; Clapham et al. 1980).

The observation that T aggregates appear more similar electrophysiologically to the intact ventricle than C aggregates is somewhat unexpected since it has been shown that trypsin can enter cardiac cells during isolation and can do some intracellular damage (Kasten 1971). However, it should be noted that highly purified collagenase is almost completely ineffective in dissociating cardiac cells (Masson-Pévet et al. 1976; Kono 1969). The ability of crude collagenase to dissociate heart muscle would appear to reside to a significant extent in the impurities present in the collagenase. In particular, the collagenase used in our study showed a significant level of protease (264 U/mg) and some low level clostripain activity (1.1 U/mg). In contrast, trypsin was present in exceedingly low concentrations (0.04 U/mg) and it is most unlikely that trypsin contamination would play a significant role. We have not investigated the role of the contaminants present in crude collagenase. There is recent evidence that pro-

tease dissociation media can have effects equivalent to that of crude collagenase with regard to dissociation of cardiac muscle (Bustamante et al. 1982). It also appears that prolonged exposure to protease-containing dissociation media may give rise to cell damage (J. O. Bustamante, personal communication), which might account for some of the effects we observed.

The ability to obtain isolated cells for tissue culture seems to depend on the combination of enzymes, culture conditions, and the tissue under study. Based on our results we conclude that crude collagenase may not always be superior to trypsin as a means of dissociating heart cells as previously proposed (Cavanaugh et al. 1963; Masson-Pévet et al. 1976). In fact, aggregates prepared from trypsin-dissociated cardiac ventricular cells from 7-day chick embryos display functional properties more typical of intact 7-day ventricular tissue than those prepared from collagenase-dissociated cells.

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