Proc. Natl. Acad. Sci. USA
Vol. 77, No. 1, pp. 679–683, January 1980
Physiological Sciences

Correlation between relaxation and automaticity in embryonic heart cell aggregates

(cardiac relaxation/potassium permeability/pacemaker physiology/intracellular calcium)

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Communicated by Harry Grundfest, October 12, 1979

ABSTRACT **Diastolic depolarization in cardiac muscle is due to a decline in potassium permeability that has been ascribed to removal of intracellular free calcium. A continued decline in tension during the pacemaker potential might therefore occur. In this study, contractile responses of chicken embryonic heart cell aggregates are recorded with a photodiode. Photodiode output is well correlated with the position of the aggregate's edge. Movements of different edges are synchronous, and their amplitude and duration vary appropriately during experimental maneuvers that alter the magnitude and duration of contractile force. Edge movement during relaxation has two phases, a rapid phase lasting about 100 msec and a slow phase that may last over 10 sec. The slow phase is not due to viscoelasticity because its time course does not depend on the magnitude or duration of the initial deformation. The rate of relaxation is correlated with the rate of depolarization during the pacemaker potential. Reduction in automaticity during cooling, spontaneous variation, and overdrive pacing are associated with impairment of the slow component of relaxation. Electrophysiological evidence suggests that the diastolic potassium permeability of the aggregates is controlled by intracellular calcium. A possible explanation for the correlation between the slope of the pacemaker potential and the slow component of relaxation is that both phenomena reflect a common physiological process—i.e., the removal of free calcium from the cytoplasm.**

A promising approach to the study of cardiac physiology is the use of isolated cells or cell clusters obtained by enzymatic dispersion of intact tissue. Spheroidal aggregates of chicken embryonic myocardial cells are sufficiently isopotential for voltage clamp (1, 2) and can be used to investigate changes in electrical properties during development. Although the small size of these aggregates does not permit direct measurement of tension, deformation of the aggregate during contraction can be recorded by optical monitoring of edge movement (3–5). The edge movement presumably reflects the magnitude and time course of the contractile response, but the relation of edge position to instantaneous force has not been established experimentally.

In the present study, edge movements of beating embryonic heart-cell aggregates are recorded during injection of current pulses that modify the amplitude, duration, and frequency of action potentials. These observations confirm that the edge movements reflect the strength and duration of contractile force. The edge movements differ from recordings obtained in native fibers with force transducers because they reveal a slow phase of relaxation that can continue for hundreds of msec. The rate of relaxation during the slow phase varies during experimentally induced changes in automaticity and is correlated with the rate of diastolic depolarization. Although the process of relaxation is not well characterized in these cells, several observations suggest that its time course reflects the removal of intracellular free calcium. The correlation between automaticity and relaxation may be due to mediation of the pacemaker current by a calcium-activated conductance, as has been reported in other cardiac cells (6), nerve cells (7, 8), and skate electroreceptors (9).

METHODS

Trypsin-dispersed ventricular cells were prepared from 9- to 12-day-old chicken embryos by a modification of DeHaan's procedure (10, 11). Cells grown on a hydrophobic surface form spheroidal aggregates 50–150 μm in diameter. Aggregates obtained in the present study retained immature electrophysiological properties (12–14) of rapid beating (120–200 min^{-1}), slow action potential upstroke (30–40 V/sec), low maximum diastolic membrane potential (−60 to −75 mV), and tetrodotoxin insensitivity. After 4–12 days in culture, the aggregates were plated onto glass slides, immersed in physiological saline on the stage of an inverted microscope, and penetrated with microelectrodes. The composition of the saline was 137 mM NaCl/2.7 mM KCl/0.4 mM NaH_2PO_4/1.8 mM $CaCl_2$/1.0 mM $MgCl_2$/5.5 mM dextrose/6.0 mM Hepes at pH 7.4 and 37 ± 0.2°C. Microelectrodes were filled with 2 M KCl and had a resistance of 20–40 MΩ. Constant current stimuli were applied by use of a second microelectrode or a bridge circuit, and edge movement was recorded with a photodiode placed in the image plane of an eyepiece lens. Illumination was provided by a 100-W quartz halogen lamp. Recordings of edge movement were calibrated by direct measurement on a TV monitor screen or by use of an eyepiece reticule.

RESULTS

Characteristics of Edge Movement. Simultaneous recordings of membrane potential and edge movement are shown in Fig. 1*A*. The photodiode was focused on a moving edge, and the amount of light detected was proportional to the instantaneous edge position. Correlation of the photodiode output with direct measurements of edge position gave a highly linear relationship (Fig. 1*B*). The edge movements reflect changes in the shape of the aggregate that result from activation of the contractile filaments. When the positions of several edges of the same aggregate were directly measured, the instantaneous displacement of each edge was well correlated with that of every other edge ($r > 0.95$). A high degree of mechanical synchrony was expected because aggregates are isopotential during all phases of the action potential (1). The amplitude of the edge movements was increased by positive inotropic interventions such as ouabain treatment and strong depolarization (Fig. 2*B*), which indicated that edge displacement was an index of con-

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^Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetate.

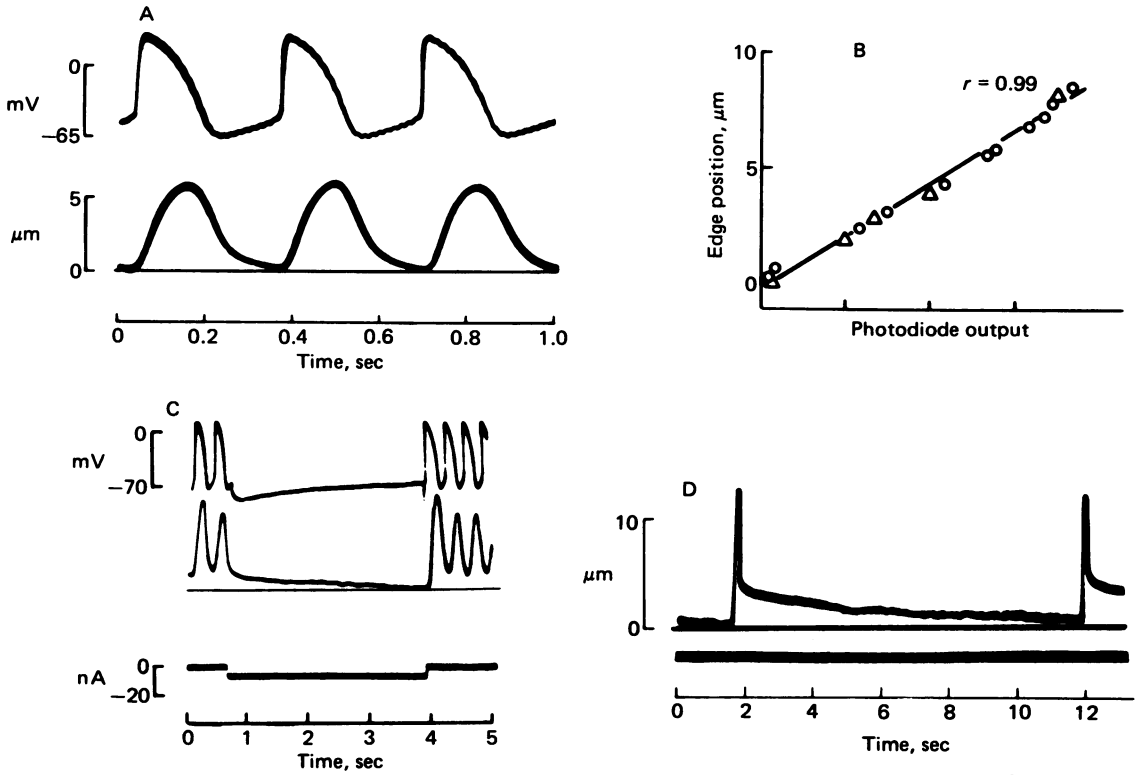

FIG. 1. Electrical activity and edge movements in beating aggregates prepared from trypsin-dispersed chicken embryonic ventricular cells. (*A*) (*Upper*) Intracellular potential; (*Lower*) photodiode output. (*B*) Photodiode output from another aggregate is correlated with direct measurements of edge position by simultaneous high-speed (4 msec) photography of a TV monitor and an oscilloscope screen. O, Contraction; Δ, relaxation. (*C*) Edge movement (*Middle*) during injection of an inhibitory constant-current stimulus (*Bottom*) through the microelectrode. Relaxation continues for 3 sec, and the recording of membrane potential obtained with a bridge circuit (*Top*) shows progressive diastolic depolarization. The rhythm in *C* was reset by a hyperpolarizing current pulse prior to the beginning of the sweep. Contractions are stronger after hyperpolarizing pulses that produce sufficient prolongation of the interbeat interval. (*D*) Edge movement is shown during injection of a sustained 20-nA inhibitory current. Beat frequency is slowed to 0.1 Hz, but edge movement remains continuous.

tractile force. When the contractile force decayed, the aggregate relaxed to its presystolic shape. The return of a displaced edge was hastened if the action potential plateau was prematurely terminated by a hyperpolarizing current pulse (Fig. 2*A*). Premature repolarization would abbreviate the movement of calcium into the cytoplasm, leading to earlier calcium removal. Edge movement during relaxation is therefore governed, at least in part, by the decline in intracellular free calcium.

Evidence for a Slowly Decaying Contractile Force. Edge movement never ceased during the pacemaker potential. Continuous movement was apparent both from the optical recording (Fig. 1*A*) and from direct observation of the edge. When the rate of beating was slowed by hyperpolarizing current, the edge relaxed beyond its normal presystolic position (Fig. 1*C Bottom*) and continued moving, even when the cycle length was increased to 10 sec (Fig. 1*D*). This movement was accompanied by progressive diastolic depolarization (Fig. 1*C Top*). Slow recoil between beats could reflect viscoelastic properties of the aggregate, rather than persistance of contractile force. Two observations demonstrated that this was not the case. (*i*) The time course of the slow edge movements did not depend on the mechanical history of the aggregate. In Fig. 2*B*, beat 2 was augmented by depolarizing current, and the next beat, beat 3, was also stronger. Subsequent beat frequency was unchanged, however, and the slow movements between beats were superimposable on those recorded in the absence of stimulation (Fig. 2*C*). If the edge movements were due to viscoelasticity, then the time required for recoil would vary with the magnitude and duration of the initial deformation. Absence of a significant viscoelastic effect means that the shape of the relaxing aggregate is determined by persisting contractile force. (*ii*) Existence of a weak, slowly decaying force could be confirmed by growing the cells as a web attached to a stiff nylon bristle. When the elastic properties of the bristle were studied by loading it with small weights, displacement was found to be a linear function of force. Attachment of cells caused the bristle to become bent, and each contraction produced an additional phasic deflection that was smaller than the movement of unrestrained cell aggregates. When the bristle's movement was recorded by the photodiode, continuous relaxation between beats was confirmed. The change in force detected by the bristle during the last half of each cycle was 1% of the peak force, which was much smaller than the relative movement of unrestrained aggregates during the same period.

Relationship of Edge Movement to Automaticity. Although the slow component of relaxation did not reflect the mechanical history of the aggregate, its time course changed substantially during variations in automaticity. Fig. 3*A* is from an aggregate that exhibited spontaneous alternations in cycle length. Edge movements during contraction were identical for the two beats, but the slow phase of relaxation was substantially faster during the short cycle (beat 1) than during the long cycle (beat 2). Changes in the slow phase of relaxation were also observed when automaticity was modified by overdrive pacing with intracellular current pulses. When an aggregate was driven faster than its intrinsic rate, the pacemaker potential became less steep, leading to a prolonged pause when stimulation ceased (ref. 15 and Fig. 4*A*). The optical recording in Fig. 4*A* shows that relaxation was also impaired after overdrive so that recoil of the edge from a given position took several times longer than

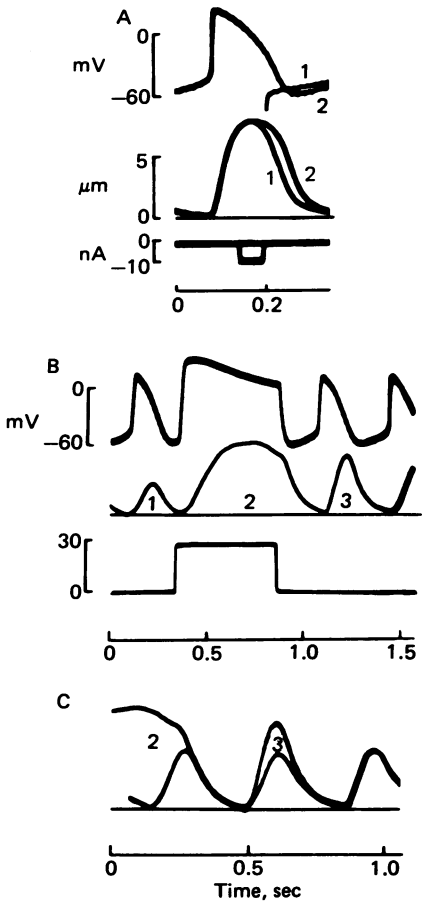

FIG. 2. Edge movement during changes in the action potential. (*A–C*) (*Top*) Membrane potential; (*Middle*) edge movement; (*Bottom*) current. (*A*) Premature termination of the action potential by a hyperpolarizing pulse (trace 1) causes earlier return of the displaced edge. Membrane potential is not recorded during the stimulus because of electrode polarization. (*B*) The action potential during beat 2 is prolonged by a strong depolarizing current. Edge excursion is much greater, and relaxation is delayed until the stimulus terminates. The following beat (beat 3) is also potentiated. (*C*) Edge movements in *B* are superimposed on those obtained during an earlier sweep in which no stimulus occurs. Beat frequency is the same in both sweeps, and the edge movements during the pacemaker potential are superimposable.

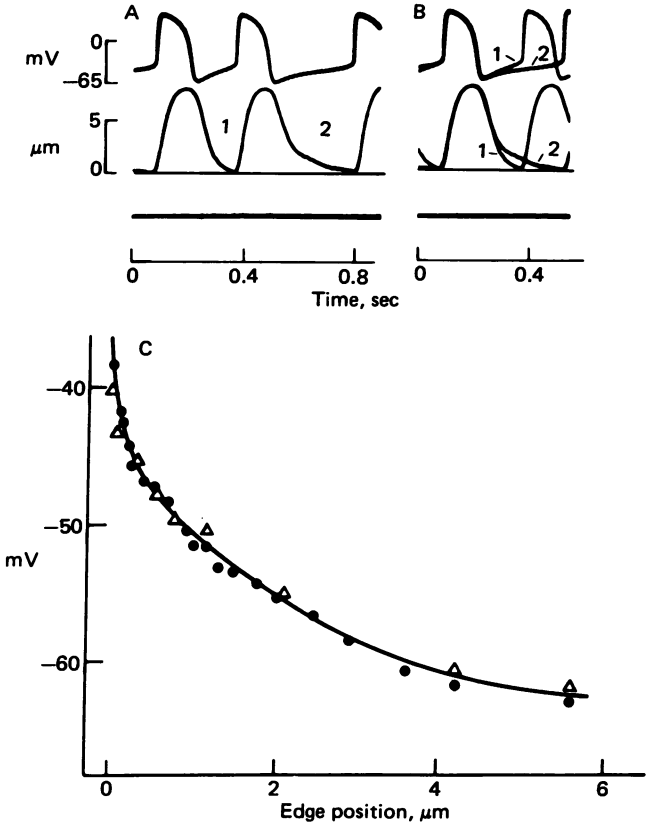

FIG. 3. Edge movements (*Middle*) during two consecutive beats of different cycle length are shown in *A* and superimposed in *B*. Movements during contraction are identical, but the return of the displaced edge is faster during the short cycle (trace 1) than during the long cycle (trace 2). Edge position at the onset of excitation is identical. (*C*) Edge position plotted as a function of membrane potential. Data points are taken at regular intervals throughout the pacemaker potentials. ●, Beat 1; △, beat 2. The fixed relationship between edge position and membrane potential indicates that potassium permeability and the shape of the aggregate during the pacemaker potential are both determined by a common factor. Similar results are obtained during cooling and overdrive suppression.

usual (see Fig. 4*B*). The opposite occurred when the rate of beating was slowed by a hyperpolarizing stimulus. In Fig. 4*C*, onset of an inhibitory current produced an abrupt increase in cycle length. As the stimulus continued, the pacemaker potential spontaneously steepened (not shown), which led to a secondary increase in beat frequency. This spontaneous acceleration was accompanied by an increase in the rate of relaxation during the slow phase, as shown by the slope of the fine lines in Fig. 4*C*. Changes similar to those in Fig. 4 were observed in every aggregate in which the rate of beating was altered for several seconds by excitatory or inhibitory stimuli.

In each of the above examples, the change in automaticity did not alter the degree to which the aggregate had relaxed when the membrane reached threshold. Edge position at threshold also remained constant when the duration of the preceding contraction was increased by a prolonged depolarizing stimulus (Fig. 2*B*) or shortened by an inhibitory stimulus (not shown). A more general relationship between edge position and diastolic membrane potential is found when they are considered as functions of each other. In Fig. 3*C*, the edge movements shown in Fig. 3*A* are plotted along the horizontal axis and simultaneous transmembrane potentials are plotted along the vertical axis. Nearly identical curves were obtained. A uniform relationship between edge position and membrane potential was also obtained when automaticity was modified by cooling, premature termination of the action potential, or overdrive pacing. Thus, in each of these experiments, there was an exact correlation between the shape of the aggregate and the diastolic membrane potential. This correlation suggests that mechanical relaxation and diastolic depolarization are quantitatively related to a common physiological parameter.

DISCUSSION

Mechanical Properties of Heart Cell Aggregates. The foregoing observations indicate that chicken embryonic heart cell aggregates have a weak component of contractile force that persists for many seconds after each beat. This persisting force has not been described in native cardiac fibers under normal conditions (e.g., ref. 16). Although it is possible that the slow phase of relaxation is unique to embryonic tissue, it is also possible that the edge movements are especially sensitive to this force. Contraction in spheroidal aggregates differs from that in native fibers because movement is restricted by the internal compliance of the tissue rather than by external loading. In the absence of viscoelasticity, the deformation of the aggregate

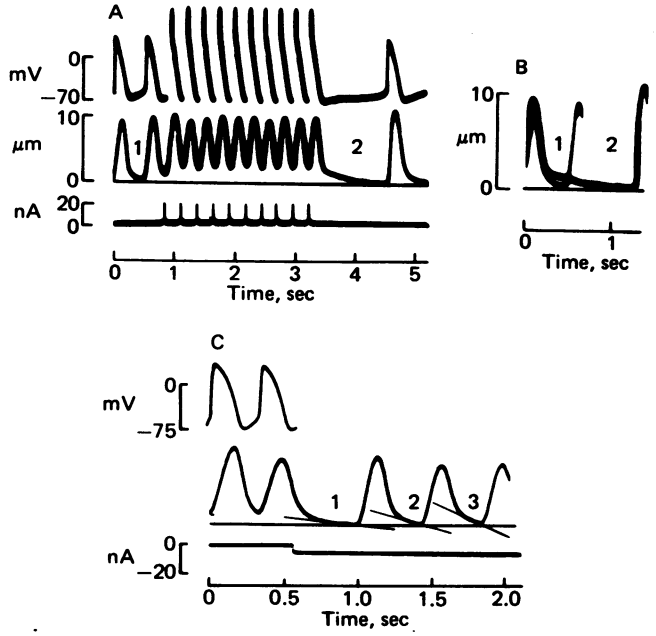

FIG. 4. Edge movement after changes in beat frequency. (*A*) Spontaneously beating aggregate is driven faster than its intrinsic rate by a burst of brief intracellular current pulses (*Bottom*) applied through the recording electrode. After the burst, the slope of the pacemaker potential (*Top*) is reduced (beat 2) and its duration is increased 5-fold. The recording of edge movement (*Middle*) shows that relaxation is impaired, but the position of the edge when the membrane reaches threshold is unchanged from control (beat 1). (*B*) Edge movement after beats 1 and 2 are superimposed. Movement during contraction and during the rapid phase of relaxation is relatively unchanged, but the slow component of relaxation is even slower than usual. (*C*) Edge movement during a maintained inhibitory stimulus. Cycle length increases markedly at simulus onset (*Bottom*), but subsequently decreases as the stimulus continues. This spontaneous acceleration is accompanied by an increase in the rate of relaxation, as shown by the fine lines. Membrane voltage is not recorded during the stimulus because of electrode polarization. However, similar experiments performed with a second microelectrode show that the decrease in cycle length results from an increase in the slope of the pacemaker potential, rather than a change in threshold.

reflects the instantaneous force exerted by the contractile filaments. Because the myoplasm is incompressible and the crossbridges are randomly oriented, the resistance of the aggregate to deformation should increase during contraction. The maximum deformation is typically only a few percent of the aggregate's diameter, which is comparable to the movement that occurs in native fibers during recordings of "isometric tension" (e.g., ref. 17). However, as the aggregate relaxes, the removal of crossbridges should increase its compliance so that the movement produced by small changes in force will be larger. Increased compliance of spheroidal aggregates during relaxation may explain why the slow phase is more conspicuous than a web of cells that is externally restrained by a stiff elastic bristle.

Does Relaxation Reflect the Removal of Intracellular Free Calcium? The slow phase of relaxation in heart-cell aggregates could reflect persistance of free calcium in the cytoplasm or persistance of crossbridges in the absence of calcium. In tissues with a well-developed calcium-sequestering system, such as skeletal muscle and mammalian ventricular myocardium, the decline in force is thought to lag behind calcium removal so that relaxation involves only the detachment of previously activated crossbridges (18, 19). The rate of relaxation in mammalian myocardium depends on loading (18), which presumably affects the number of crossbridges formed, but is relatively independent of action potential duration (20, 21), which regulates calcium entry across the surface membrane. In contrast, the rate of relaxation in tissues with a poorly developed calcium-sequestering system is thought to be limited by calcium removal (18, 19, 22). Relaxation of frog myocardium from peak tension is independent of loading (18), but can be accelerated by premature termination of the action potential (23), which contrasts with the result obtained in mammalian ventricle (20). Furthermore, relaxation of frog myocardium under voltage clamp is markedly slowed by reduction of extracellular sodium (22). This observation has been taken as evidence that relaxation reflects the removal of calcium across the surface membrane in exchange for sodium (22).

Four observations suggest that the relaxation of chicken embryonic heart-cell aggregates reflects the rate of calcium removal. (*i*) Relaxation from peak tension can be accelerated by premature termination of the action potential (Fig. 2*A*). (*ii*) The time course of relaxation is independent of the magnitude of the peak contraction (Fig. 2*C*). Relaxation is therefore load-independent. (*iii*) There is a paucity of sarcoplasmic reticulum relative to surface membrane (24). (*iv*) The slow phase of relaxation in isolated chicken embryonic ventricular cells is prolonged by increasing extracellular calcium or lowering extracellular sodium (25). Miura *et al.* (25) therefore postulate that the slow phase is due to calcium extrusion across the surface membrane.

If relaxation of heart-cell aggregates were governed by calcium removal, then the shape of the aggregate could serve as an index of instantaneous free calcium. The antomical, electrical, and mechanical uniformity of the aggregate suggest that changes in intracellular calcium are similar in each cell. Because the cells are small spheres with a mean radius of 5.4 μm (2), 47% of the myoplasm must lie within 1 μm of the surface membrane and less than 8% can lie more than 3 μm away. Removal of free calcium from the contractile filaments should therefore be fairly synchronous.

The relationship between free calcium and tension may, of course, be altered by changes in myofilament length. Fabiato and Fabiato (17) have shown that a 40% increase in sarcomere length from 2.2 to 3.1 μm increases the calcium sensitivity of disrupted frog ventricular cells by 0.5 logarithm units. However, this observation does not necessarily invalidate the use of deformation as an index of free calcium. The movement of embryonic heart-cell aggregates during the slow phase of relaxation is only about 1% of the aggregate's diameter. Although direct measurement of sarcomeres is not possible, their movement is probably much smaller than in the Fabiato experiment (17). Furthermore, it is not clear that length-dependent changes in calcium sensitivity would produce time dependence of the relationship between deformation and free calcium. Even if the curve of force against calcium shifts appreciably during relaxation, instantaneous deformation at a given level of free calcium could be relatively independent of the rate of calcium removal.

Does Removal of Free Calcium Also Mediate Automaticity? Besides regulating tension, intracellular calcium may control diastolic membrane potential by affecting potassium permeability. Removal of free calcium apparently mediates the decline in potassium permeability during the pacemaker potential in sheep Purkinje fibers (6). The potassium permeability of these fibers is augmented by intracellular injection of calcium and reduced by injection of a calcium-chelating agent ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetate (EGTA) (6, 26). Injection of calcium/EGTA buffers increases potassium permeability of resting fibers only if the free calcium in the buffer exceeds 5×10^{-7} M (26). Because this

level is just above the threshold for production of tension in disrupted cardiac cells (17), slight relaxation during the pacemaker potential might be expected. Diastolic depolarization in embryonic heart-cell aggregates also results from a decline in potassium permeability (27) that may be due to calcium removal. The pacemaker current is activated only when the membrane is sufficiently depolarized to elicit inward calcium current (2, 28). Outward current at the end of a depolarizing pulse is suppressed by D-600, which blocks calcium influx (2). Prevention of calcium influx by brief superfusion with cobalt or EGTA produces sustained depolarization to −25 mV and linearizes the membrane, these effects being reversible (29).

Simultaneous control of mechanical relaxation and potassium permeability by intracellular calcium would explain the observed correlation between edge position and diastolic membrane potential. Changes in automaticity and the slow component of relaxation during cooling, spontaneous variation, and electrical pacing would then be ascribed to changes in the rate of calcium removal. Persistance of free calcium was previously proposed to explain the diminished automaticity of Purkinje fibers after overdrive (30). The impaired relaxation of heart-cell aggregates after overdrive (Fig. 4*A*) is consistent with this model, as is the faster relaxation after a reduction in beat frequency (Fig. 4*C*). Variations in electrogenic pump current, which affect automaticity during prolonged changes in activity (31), are probably not important during the brief changes described here because membrane potential remains constant for several seconds when a beating aggregate is hyperpolarized to E_K by a current pulse. These observations suggest that the slow phase of relaxation in embryonic heart-cell aggregates may provide information about the control of spontaneous beating.

I thank Drs. D. Brutsaert, P. Cranefield, D. Gadsby, H. Grundfest, D. Harrison, D. Miura, R. W. Tsien, and R. Zucker for their helpful comments on this work. R. Assisi and D. Bragg gave expert technical assistance. W.T.C. was supported by the California Heart Association.

1. DeHaan, R. L. & Fozzard, H. (1975) *J. Gen. Physiol.* **65,** 207–222.
2. Nathan, R. D. & DeHaan, R. L. (1979) *J. Gen. Physiol.* **73,** 175–198.
3. Bucher, O. M. (1957) *Exp. Cell Res.* **13,** 109–115.
4. Sinclair, A. J., Miller, H. A. & Harrison, D. C. (1970) *J. Appl. Physiol.* **29,** 747–749.
5. Thompson, E. J., Wilson, S. H., Schuette, W. H., Whitehouse, W. C. & Nirenberg, M. W. (1973) *Am. J. Cardiol.* **32,** 162–166.
6. Isenberg, G. (1977) *Pfluegers Arch.* **37,** 77–85.
7. Meech, R. W. & Standen, N. B. (1975) *J. Physiol. (London)* **249,** 211–239.
8. Eckert, R. & Lux, H. D. (1976) *J. Physiol. (London)* **254,** 129–151.
9. Clusin, W. T., Spray, D. C. & Bennett, M. V. L. (1975) *Nature (London)* **256,** 837–839.
10. DeHaan, R. L. (1967) *Dev. Biol.* **16,** 216–249.
11. Barry, W. H., Pitzen, R., Protas, K. & Harrison, D. C. (1975) *Circ. Res.* **36,** 727–734.
12. Sperelakis, N. & Shigenobu, K. (1972) *J. Gen. Physiol.* **60,** 430–453.
13. McDonald, T. F., Sachs, H. G. & DeHaan, R. L. (1972) *Science* **196,** 1248–1250.
14. McDonald, T. F. & Sachs, H. G. (1973) *Pfluegers Arch.* **354,** 151–164.
15. Pelleg, A., Vogel, S., Belardinelli, L. & Sperelakis, N. (1978) *Circulation* **58,** II-104.
16. Gibbons, W. R. & Fozzard, H. (1975) *J. Gen. Physiol.* **65,** 207–222.
17. Fabiato, A. & Fabiato, F. (1978) *J. Gen. Physiol.* **72,** 667–699.
18. Brutsaert, D. L., DeClerck, N. M., Goethals, M. A. & Housmans, P. R. (1978) *J. Physiol. (London)* **283,** 469–480.
19. Brutsaert, D. L., Claes, V. A. & DeClerck, N. M. (1978) *J. Physiol. (London)* **283,** 481–491.
20. Morad, M. & Trautwein, W. (1968) *Pfluegers Arch.* **299,** 66–82.
21. Morad, M. & Goldman, Y. (1973) *Prog. Biophys. Biophys. Chem.* **6,** 259–313.
22. Roulet, M., Mongo, K. G., Vassort, G. & Ventura-Clapier, R. (1979) *Pfluegers Arch.* **379,** 259–268.
23. Morad, M. & Orkland, R. K. (1971) *J. Physiol. (London)* **219,** 167–189.
24. Shimada, Y. & Fischman, D. A. (1975) in *Developmental and Physiological Correlates of Cardiac Muscle,* eds. Lieberman, M. & Sano, T. (Raven, New York), pp. 81–100.
25. Miura, D., Biedert, S., Smith, T. W. & Barry, W. H. (1979) *Circulation* **60,** II-213 (abstr.).
26. Isenberg, G. (1977) *Pfluegers Arch.* **371,** 51–59.
27. Sperelakis, N. & Shigenobu, K. (1972) *J. Gen. Physiol.* **60,** 430–453.
28. Nathan, R. D. (1979) *Biophys. J.* **25,** 199a.
29. Clusin, W. T. (1979) *Biophys. J.* **25,** 80a.
30. Vassalle, M. (1977) *Circ. Res.* **41,** 269–277.
31. Gadsby, D. C. & Cranefield, P. F. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 1783–1787.