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Reentrant waves in a ring of embryonic chick ventricular cells imaged with a Ca^{2+} sensitive dye

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Abstract

According to the classic model initially formulated by Mines, reentrant cardiac arrhythmias may be associated with waves circulating in a ring geometry. This study was designed to study the dynamics of reentry in a ring geometry of cardiac tissue culture. Reentrant calcium waves in rings of cultured embryonic chick cardiac myocytes were imaged using a macroscope to monitor the fluorescence of intracellular Calcium Green-1 dye. The rings displayed a variety of stable rhythms including pacemaker activity and spontaneous reentry. Waves originating from a localized pacemaker could lead to reentry as a consequence of unidirectional block. In addition, more complex patterns were observed due to the interactions between reentrant and pacemaker rhythms. These rhythms included instances in which pacemakers accelerated the reentrant rhythm, and instances in which the excitation was blocked in the vicinity of pacemakers. During reentrant activity an appropriately timed electrical stimulus could induce resetting of activity or cause complete annihilation of the propagating waves. This experimental preparation reveals many spontaneously occuring complex rhythms. These complex rhythms are hypothesized to reflect interactions between spontaneous pacemakers, wave propagation, refractory period, and overdrive suppression. This preparation may serve as a useful model system to further investigate complex dynamics arising during reentrant rhythms in cardiac tissue.

Keywords: Cardiac arrhythmias; Reentrant tachycardia; Pacemakers; Refractory period; Overdrive suppression; Unidirectional block

1. Introduction

Reentrant rhythms are widely implicated in life-threatening cardiac arrhythmias. Early experiments demonstrated circulating excitation in rings cut from animal hearts (Mines, 1914). More recently, circulating excitation was observed in rings of tissue in canine preparations using multiple electrodes spaced around the ring (Bernstein and Frame, 1990). The concept of reentrant excitation circulating around a barrier as developed in these experiments provides an important conceptual model for understanding the effects of electrical stimulation delivered during reentrant tachycardia (Stevenson et al., 1988; Callans et al., 1993). Dynamics of excitation circulating on a ring have also been analyzed theoretically. The stability of circulation depends on the size of the ring relative to the physiological properties of the tissue (Quan and Rudy, 1990; Courtemanche et al., 1993; Vinet and Roberge, 1994). Theoretical models display resetting and entrainment similar to experimental observations (Glass and Josephson, 1995; Glass

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et al., 2002; Sinha et al., 2002; Comtois and Vinet, 2002).

The current work was motivated by our interest in developing an in vitro experimental system that contains both excitable medium and pacemakers, and is suitable for experimental manipulations. In early work, cardiac tissue that was grown in strands and rings was used to analyze biophysical properties and model arrhythmias (Lieberman et al., 1972, 1981; Shigeto et al., 1982). More recently, the development of optical recording techniques has facilitated the analysis of dynamics of propagation for cardiac cells in tissue culture grown in a variety of geometric patterns (Koidl et al., 1980; Rohr et al., 1991; Rohr, 1995; Bub et al., 1998, 2002; Fast et al., 1998; Nagai et al., 2000; González et al., 2000; Arutunyan et al., 2001, 2002).

In this work, we describe a tissue culture model in which cells are grown as a ring and monitored by a macroscopic imaging system. In recent articles (Nagai et al., 2000), we described reentrant rhythms in a ring geometry and developed mathematical models of the observed dynamics (Nagai et al., 2000; González et al., 2000). In this work we extend our experimental observations to a wide variety of complex rhythms generated by the interaction of spontaneous pacemaker activity and propagating waves.

2. Materials and methods

2.1. Cell culture

Cell cultures were prepared according to previously established procedures (Bub et al., 1998). Briefly, ventricular cells were isolated from White Leghorn chick embryo hearts after 7 days of incubation in ovo. The ventricles were dissociated into single cells and plated at densities of 4.0×10^4 cells/ml in plastic dishes patterned with a ring design (see below). The cells were maintained in the medium at 5% CO_2 at 36 °C for 3–4 days before experiments were undertaken. After several of the experiments, the rings were fixed for microscopic examination using a Masson trichrome stain (Sigma Diagnostics). Fig. 1 shows a microphotograph of a portion of a ring. The optical recording experiments were carried out in preparations during the third and fourth days after culturing.

2.2. Construction of the ring

Dishes were first coated with a thin film of cell adhesive (Cell-Tak, Collaborative Biomedical Products). To create the ring geometry, we constructed a template from a cylindrical piece of Plexiglas. On one end of the template, an annular depression with dimensions of 8 mm outer diameter and 6 mm inner diameter was machined into the Plexiglas. The raised portions of the template were first coated with silicon polymer (3140 Dow-Corning) and then applied to the culture dishes. The regions of culture dishes that contacted the siliconized regions of template did not favor cell adhesion. Cells that contacted siliconized areas tended to form beating aggregates that poorly adhered and were easily aspirated leaving an annular region.

2.3. Fluorescent imaging

Cellular activity was measured by monitoring the fluorescence signal generated by an intracellular calcium sensitive dye, Calcium Green-1 (Molecular Probes) using methods described previously (Bub et al., 1998).

Cell cultures were maintained at 36 ± 1 °C during the recordings. In some experiments, electrical stimulation was delivered by means of a bipolar teflon coated platinum electrode (diameter 120 μm, separation 1 mm). Cells were stimulated with rectangular pulses of 20 or 30 ms duration at twice diastolic threshold current delivered from a Frederick-Haer (Pulsar 4i) stimulator.

The still figures in the manuscript are supplemented by animations on a public web site http://www.cnd.mcgill.ca/bios/glass/hortensia/index.htm.

3. Results

In five preparations there was only evidence of localized spontaneous pacemakers. The activity pattern associated with a single pacemaker is characterized by a pair of bidirectional waves originating from one position on the ring and colliding at the antipodal point. Table 1 gives a summary of the experiments in which there was a stable pacemaker.

Although the above pattern was frequently observed, in most cases it was not maintained stably over

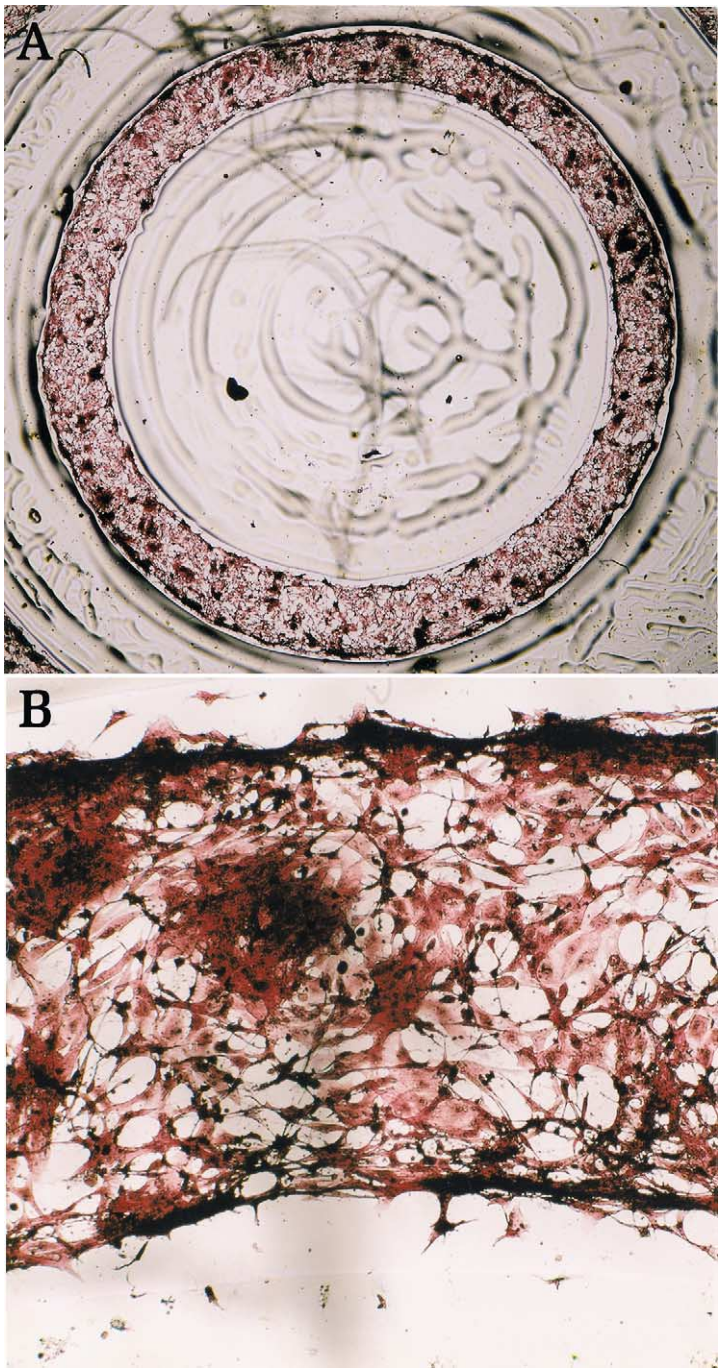

Fig. 1. Microscopic images of a section of the preparation stained with Masson trichrome stain. (A) The entire ring. The inner diameter of the ring is 6 mm and the outer diameter is 8 mm. (B) A section of the ring. The denser regions corresponds to the region of a pacemaker.

Table 1
Pacemaker characteristics

Experiment	Period of pacemaker (s)	Conduction time (s)	Velocity (mm/s)
A	2.1 ± 0.1	0.6 ± 0.1	18.3 ± 3.4
B	2.3 ± 0.1	0.7 ± 0.1	15.7 ± 2.5
C	1.8 ± 0.1	0.7 ± 0.1	15.7 ± 2.5
D	1.7 ± 0.2	0.5 ± 0.1	22.0 ± 4.9
E	2.6 ± 0.1	0.6 ± 0.1	18.3 ± 3.4
Mean ± S.D.	2.1 ± 0.4	0.6 ± 0.1	19.3 ± 3.3

the course of the recording. One way in which the single localized pacemaker activity could be modified was by the spontaneous initiation and/or termination of additional pacemakers. An example is shown in Fig. 2. The fluorescent images in Fig. 2A reveal a pacemaker at 5 o'clock with a initial cycle length of 2.8 s. However, there is a second pacemaker at 11 o'clock (period of about 2.3 s) which arises spontaneously for 2 cycles, one of which is displayed (see the third frame of Fig. 2A and the schematic representation in Fig. 2B). When the second pacemaker is active there is a shifting of the time delay between the activation times at 5 o'clock and 11 o'clock (see the fourth and fifth complexes in Fig. 2C).

In five preparations spontaneous reentrant rhythms were maintained stably for variable lengths of time ranging from five minutes to one hour. The results are summarized in Table 2.

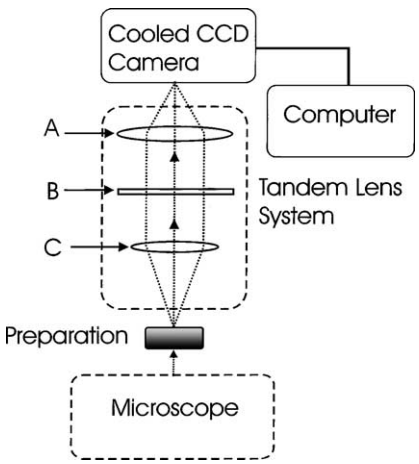

Fig. 2. Schematic diagram of the optical imaging system. (A) Imaging lens; (B) Emission filter; (C) Objective lens; (D) Dichroic mirror; (E) Excitation filter; (F) Heat filter.

In five preparations, reentrant waves started spontaneously. This occured as a consequence of spontaneous pacemaker activity followed by a block of one of the two waves of excitation initiated by the pacemaker.

Fig. 3 illustrates this situation. Fig. 3A shows the pattern corresponding to the third cycle of the trace in Fig. 3C. The propagating waves from the preceding three cycles initiated by the pacemaker at 12 o'clock with a period of approximately 3.3 s were bidirectionally blocked. However, the third cycle displayed a unidirectional block at about 8 o'clock in the fifth frame of Fig. 3A, leading to a wave circulating in a clockwise direction. This wave circulated for four cycles and then blocked at 2 o'clock. This was followed by reinitiation of the pacemaker at 12 o'clock, the last complex in Fig. 3C.

This example illustrates unidirectional block, an essential component of the classic mechanism that leads to reentrant rhythms. A key problem is to understand the anatomical substrate underlying the unidirectional block. In these experiments, the unidirectional block usually occured at a site that was associated with a slower pacemaker (more than 40 times in one preparation and more than 60 times in five preparations). Less frequently, the block occured in a region that

Table 2
Reentry characteristics

Experiment	Period (s)	Velocity (mm/s)
F (cw)	1.1 ± 0.3	19.7 ± 6.3
G (ccw)	0.8 ± 0.1	27.4 ± 3.7
H (ccw)	1.5 ± 0.1	14.6 ± 1.1
I (cw)	0.8 ± 0.1	27.4 ± 3.7
J (cw)	1.0 ± 0.1	22.0 ± 2.4
Mean ± S.D.	1.0 ± 0.2	23.9 ± 3.4

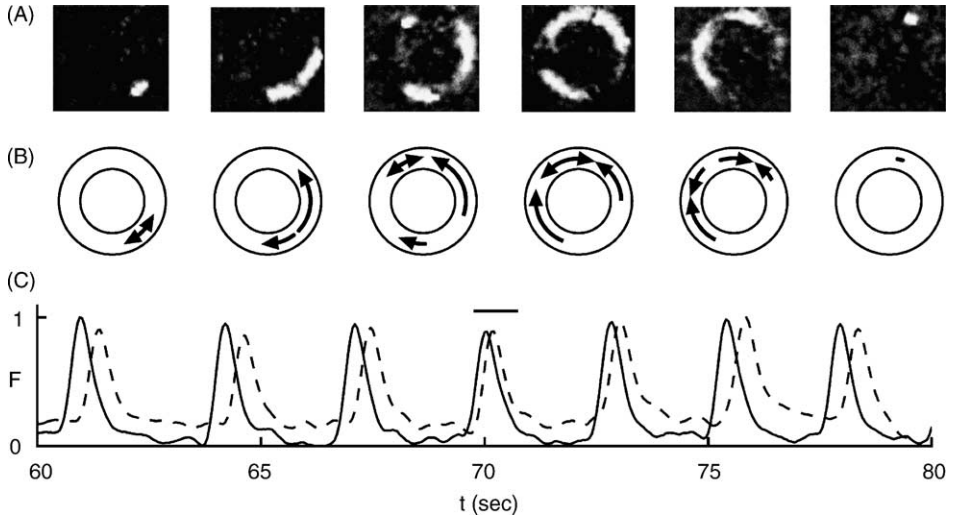

Fig. 3. Fluorescence from a ring showing the propagation from two pacemakers. There is a pacemaker at 5:00 o'clock with a period of 2.8 s and a second pacemaker at 11:00 o'clock with a cycle of 2.3 s that is evident for two cycles. The excitation waves from both pacemakers collide at 1 o'clock and 9 o'clock. The data are represented using three different methods. (A) Fluorescent images recorded by the CCD camera at 200 ms intervals. The lighter region corresponds to the region of higher Ca^{2+} concentration. (B) Schematic diagram of wave propagation corresponding to the images in panel A. (C) Normalized intensity of fluorescence. In this and the following figures, the normalized intensity of fluorescence F recorded from 11 o'clock is indicated by a dashed curve and 5 o'clock is indicated by a solid curve. The bar over the trace in panel C indicates the time interval for which fluorescence is displayed in panel A. The same format and conventions are used in the following figures. In the fourth and fifth complexes the lag between the excitation maxima at 5 o'clock and 11 o'clock is reduced due to the presence of the second pacemaker at 11 o'clock.

appeared to be of lower density (six times in two preparations).

In three cases, following initiation of reentry, the period increased from the initial value which was from 40 to 70% of the final value. The period increased to its final value over a time scale of 5–15 revolutions to a value which was maintained for the duration of the observations. The combinations of multiple pacemakers and spontaneous initiation and termination of reentrant waves led to complex rhythms.

Fig. 4 shows an example of complex rhythms from the same preparation. Following a period of quiescence there is a wave initiated by the pacemaker at 12 o'clock (first row of fluorescent images). Following a quiescent period of 1.75 s (not displayed), there is a second wave initiated by the pacemaker at 12 o'clock (second and third rows of fluorescent images). In addition, a second pacemaker at 5 o'clock spontaneously fires (third panel, second row of fluorescent images). The counterclockwise circulating waves from the pacemaker at 5 o'clock collides with the clockwise wave from the pacemaker at 12 o'clock and both annihilate. However, the clockwise wave from the pacemaker at 5 o'clock continues circulating, presumably because the original counterclockwise wave from the pacemaker at 12 o'clock was unidirectionally blocked at 8 o'clock (second row, fourth panel) as previously shown in Fig. 4. The newly initiated counterclockwise reentrant wave is itself blocked before a complete circulation at 2 o'clock (third row, fifth panel). Following another quiescent period of 1.75 s (not shown), there is again an initiation of a reentrant wave by the pacemaker at 12 o'clock (fourth row of fluorescent images). This time however, there is initiation of a reentrant wave.

Although the main purpose of this work was to establish whether the tissue cultured cells could support circulating waves, and to investigate the spontaneous rhythms set up by the interaction of reentry and pacemakers, it is important to verify whether the response of the preparation to electrical stimulation is similar to responses observed in experimental (Bernstein and Frame, 1990), clinical (Callans et al., 1993) and theoretical (Glass and Josephson, 1995; Glass et al.,

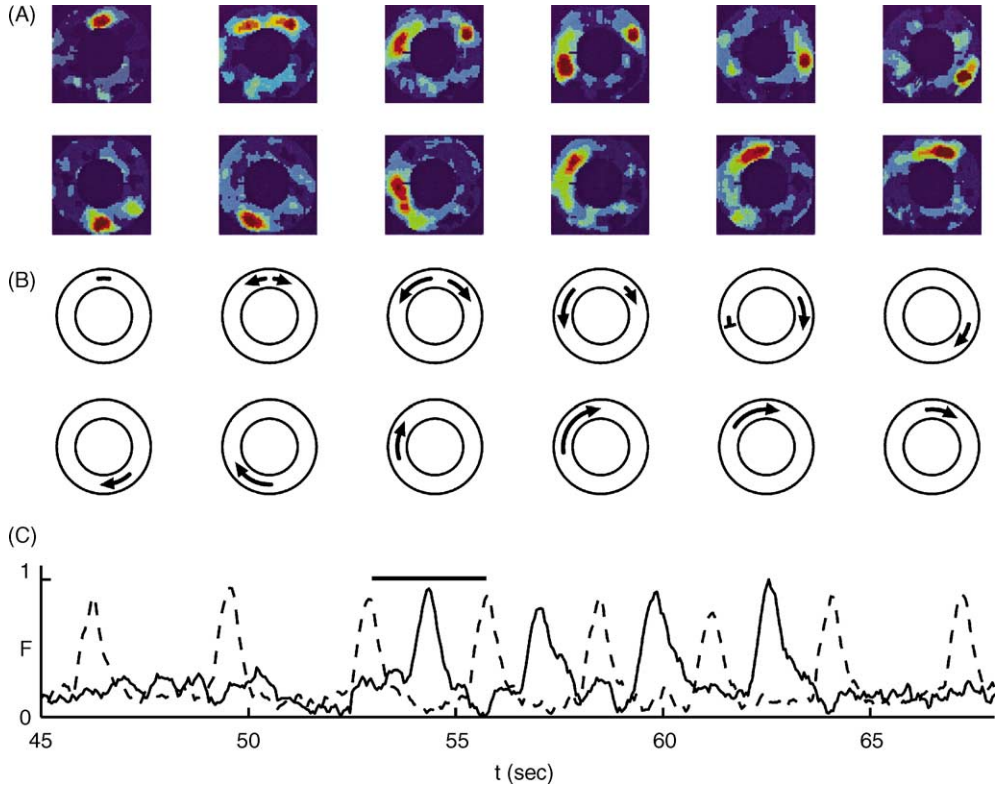

Fig. 4. Initiation of reentrant excitation. There is a pacemaker at 12 o'clock and unidirectional block at 8 o'clock. Following unidirectional block, there is a single wave circulating in a counter clockwise direction with a velocity of 8.4 mm/s and a period of 2.8 s. (A) Fluorescent images recorded at 250 ms intervals; (B) schematic diagram; (C) normalized intensity of fluorescence.

2002; Sinha et al., 2002; Comtois and Vinet, 2002) studies.

Electrical stimulation in this preparation has a variety of effects, depending on the precise timing and location of the stimulus and the number of pulses delivered (González et al., 2000). If the stimulus is applied a sufficiently long time after a wave traveling in a clockwise direction traverses a region, two new traveling waves will be induced. The antidromic wave collides with the original wave and both annihilate, while the newly initiated orthodromic wave continues circulating. The net effect is that there is once again a single clockwise wave which is reset.

A different effect is observed from electrical stimulation delivered with a short latency (approximately in the range of 80 and 120 ms) following an activation (Fig. 5). Electrical stimulation applied with a short latency following the passage of a wave circulating in a counterclockwise direction (Fig. 5A second row, second panel) initiated only a single wave, traveling clockwise. This wave collides with the original counterclockwise traveling wave to lead to annihilation of the reentrant rhythm. The initiation of a single wave verifies theoretical predictions of a "vulnerable phase" of cardiac tissue (Starmer et al., 1993). The annihilation of the reentrant wave following collision of the induced wave with the original reentrant wave also follows theoretical predictions (Glass and Josephson, 1995; Sinha et al., 2002; Comtois and Vinet, 2002).

In one ring the tissue remained indefinitely quiescent following the annihilation. In the other two preparations, one of which is illustrated in Fig. 5, the original pacemaker pattern reinitiated after a silent period of 1–5 s. Following the annihilation, waves were generated by a pacemaker oscillation Fig. 6.

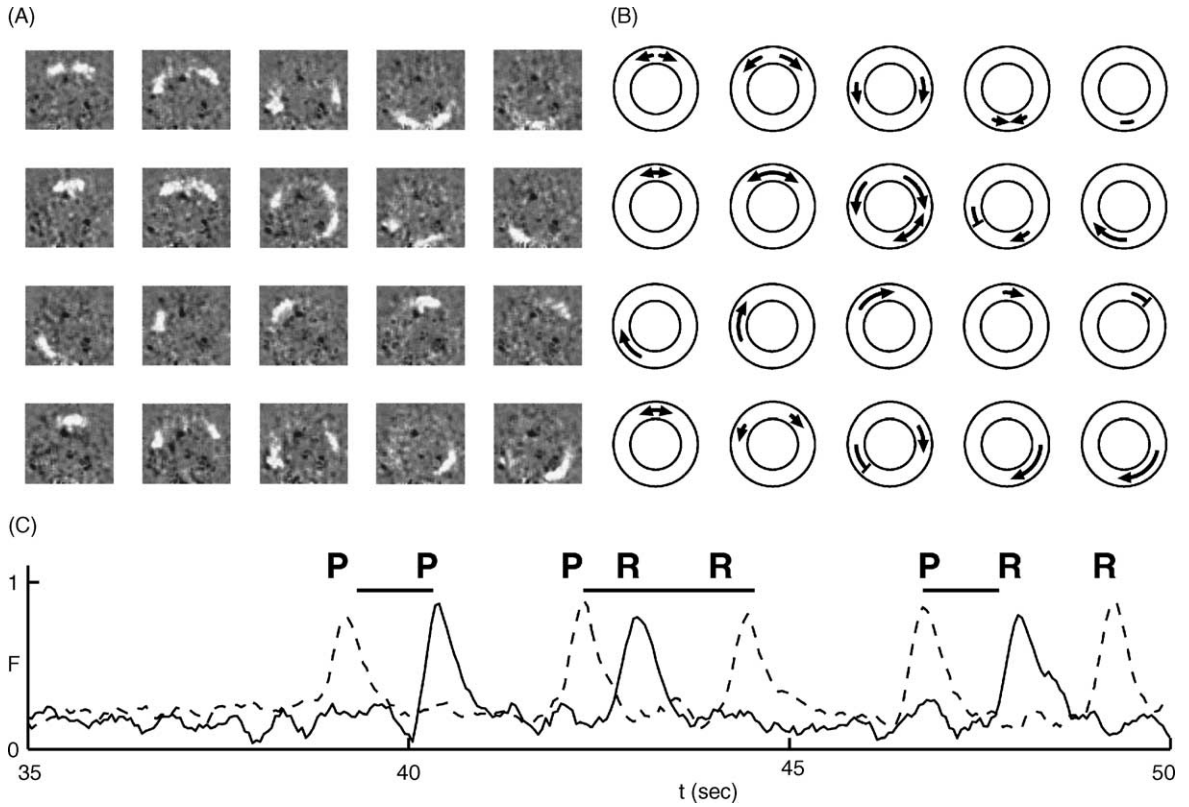

Fig. 5. Complex rhythms in a preparation with pacemakers and reentry: (A) fluorescent images recorded at 250 ms interval. There are two quiescent intervals of 1.75 s that are not recorded separating the recordings in the first and second rows, and the third and fourth rows; (B) schematic diagram; (C) normalized intensity of fluorescence. P denotes a complex from a pacemaker, and R denotes a complex from a reentrant wave. See the text for details.

4. Discussion

We have demonstrated a variety of different rhythms either arising spontaneously or induced by electrical stimulation in cultured chick cardiac ventricular myocytes grown in a ring morphology. The experiments have implications both for experimental investigations of reentrant arrhythmias.

In this work, cardiac cells grown in a specific geometry were used to generate an anatomical substrate that is believed important in the genesis of reentrant arrhythmia. Since the tissue culture can potentially be manipulated to a variety of different geometries, extension of these methods can provide experimental methods for investigation of the influence of geometry on dynamics. This is an alternative to a previously described method in which photosensitive reagent was used to coat dishes and construct geometries by illumination projected through masks which define the desired geometry (Rohr et al., 1991).

Previous work has imaged propagation in tissue culture at high spatial and temporal resolution by using voltage sensitive dyes (Rohr et al., 1991; Rohr, 1995; Fast et al., 1998). Voltage sensitive dyes require high light intensities and the duration of the experimental observations is often of the order of tens of seconds. Calcium sensitive dyes are often used as a indicator of cell activation in experiments where long recording times and high spatial resolution are a priority (Bub et al., 1998, 2002; Arutunyan et al., 2001, 2002). Although the calcium sensitive dyes do not directly reflect the electrical excitation of the cells, there is no reason to expect that the main observations here would be changed if we had directly recorded

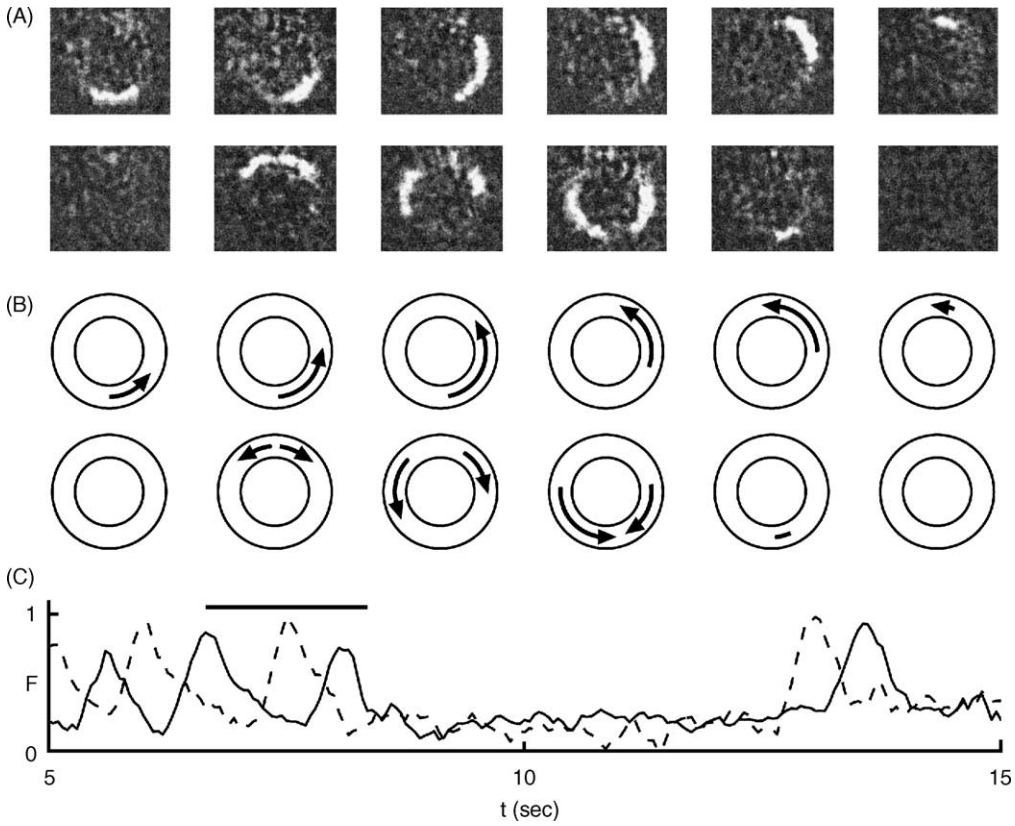

Fig. 6. Annihilation of a reentrant rhythm. The stimulus at 12 o'clock (second row, first panel of A) leads to the initiation of a single wave propagating in a clockwise orientation. This collides with the original wave travelling in a counterclockwise direction leading to annihilation of the reentrant rhythm. Following a pause, pacemaker oscillations reappear. (A) Fluorescent images recorded at 150 ms intervals; (B) schematic diagram; (C) normalized intensity of fluorescence.

membrane voltage. Measurements in neonatal myocyte cultures stained with both voltage and calcium sensitive dyes demonstrated that the calcium transient follows the action potential upstroke with a delay of 5.3 ± 1 ms (Fast and Ideker, 2000).

The velocity of propagation in the cultured cells is in the range of 20–30 mm/s which is significantly slower than the reported conduction velocities in intact heart. Reported propagation speed in rat tissue culture preparations ranges from 4.5 to 18 cm/s (Freud, 1972), and from 9 to 103 cm/s (Rohr et al., 1991). To resolve the dynamics of the comparatively slower conduction velocities observed here, the 50 ms frame rate is appropriate. The slower conduction velocities here are most likely due to a combination of less developed intercellular coupling than in normal tissue, lower cell density, and slower upstroke velocity.

These experiments demonstrate a range of different rhythms including waves spreading from a single pacemaker and annihilating at the antipodal point; simple reentry; resetting; and annihilation of reentry by a single stimulus; as well as complex rhythms in which there was a repeated initiation and termination of reentrant rhythms. We believe that these complex rhythms are a consequence of the interaction between pacemakers and reentry in the geometrical configuration of a ring of tissue.

To understand the hypothesized mechanism, we first describe an experiment in which an aggregate spontaneously beating embryonic chick heart cells is stimulated at a fixed delay following an action potential (Kunysz et al., 1997). If the delay was sufficiently long, but shorter than the intrinsic period of the pacemaker, the stimulation would give rise to an

immediate firing of the pacemaker. This in turn would trigger another stimulus and another firing of the pacemaker. However, as the process proceeded there was a buildup of overdrive suppression of the pacemaker (Vassalle, 1977), and eventually the stimulus would fail to elicit an action potential. This would lead to a long delay until the next spontaneous firing of the action potential. However, once there was a spontaneous action potential, the stimulus delivered after a fixed delay could once again elicit an action potential and the process could repeat, leading to bursting rhythms (e.g. see Fig. 2 in (Kunysz et al., 1997)).

In the current experiments localized pacemakers serve to initiate bidirectional waves. However, due to local heterogeneities, there is often unidirectional block. Once unidirectional block occurs there is a reentrant rhythm. This effectively leads to excitation of the pacemaker at a fixed delay following the initial excitation, similar to what was artificially generated in (Kunysz et al., 1997). Thus, from a theoretical perspective, even a single pacemaker in a ring with heterogeneities could generate repetitive rhythms. However, in our experiments the situation is more complex since in several preparations there is clear evidence for multiple pacemaker regions simultaneously in the ring. We hypothesize that the interactions of these multiple pacemakers via the ring of tissue combined with physiological mechanisms that lead to decreased excitability following rapid activity lead to complex rhythms observed in these experiments (Nagai et al., 2000). Ectopic activity originating from a localized source occurs in paroxysmal bursts in cell culture models of ischemic border zones (Arutunyan et al., 2001, 2002).

The above description gives insight into mechanisms that lead to complex rhythms observed in physiological settings. Although concepts such as refractory period, reentry, pacemakers, and overdrive suppression may seem simple when taken in isolation, when these are all combined in experimental systems, they lead to a wide diversity of rhythms that evolve in unexpected ways.

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