0014-4754/94/080712-05$1.50 + 0.20

Collagen gels populated with rat neonatal heart cells can be used for optical recording of rhythmic contractions which also show ECG-like potentials

J. E. M. Souren *, R. C. Peters [a] and R. Van Wijk

Department of Molecular Cell Biology, and [a] Department of Comparative Physiology, Utrecht University, Padualaan 8, NL-3584 CH Utrecht (The Netherlands)
Received 11 November 1993; accepted 3 May 1994

Abstract. Neonatal heart cells populated collagen gels show rhythmic contractions which can be recorded optically and electrically. Optical recordings revealed two populations of rhythmically contracting gels: 1) highly coherent contracting collagen gels with normally distributed contraction interval times and contraction amplitudes, and 2) irregularly contracting gels with a multi-modal distribution of contraction interval times and amplitudes. The irregularly contracting gels were shown to be 'semi regular', which means that a short contraction interval was preferentially followed by another short interval. The volume of the collagen gel during the contraction decreased, and our calculations indicate that the myocytes expel 3–10 times their own volume from the gel. Changes in electrical potential were observed depending on the location of the electrodes. These electrical, ECG-like changes in potential were maximal when one electrode was placed in the centre and the other at the edge of the gel. The results of this study indicate that myocyte-populated collagen gels are a very promising system for studies of electrophysiology and coherent contractions.
Key words. Collagen; neonatal heart cells; rat; contraction; electric potentials.

Neonatal rat heart cells retain the ability to beat in vitro[1,2]. It is now well documented that, a few days after isolation, these cells commence beating and then form a coherent synchronous system. Several attempts have been made to develop systems with various geometrical structures such as contracting strands[3–5] and beating aggregates[6]. However, the contractions in these systems are only visible on the microscopical level. Recently, a synchronously contracting system in the macroscopic range was demonstrated using a floating collagen matrix[7]. With this system the coherent contractions of the cell mat as a whole become visible to the eye and can be measured more easily.

In this study we have used two methods for the recording of the contractions. The first measures the change in optical density caused by displacements of the gel. This was used to evaluate the contraction of floating collagen gels over a longer period of time. Second, we showed that collagen gel contractions could be recorded by measuring the extracellular electric field of a pulsating culture and that pulsations coincide with ECG-like changes in the electrical field.

Materials and methods

Preparation of collagen gel cultures. Rat tail collagen stock solution was prepared as described earlier[7]. The collagen stock solution was mixed with 0.032 N NaOH and twice-concentrated Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 20% Fetal Calf Serum (FCS) on ice. In this way gels were prepared with a final concentration of 1× DMEM supplemented with 10% FCS. The mixture (2 ml) was poured into 8 cm^2 Greiner bacteriological dishes in a 37 °C conditioned room. The gels form within 1 min and have an average thickness of 2.5 mm. Neonatal heart cells were isolated from 1- to 2-day-old rats as described earlier[7] and the isolated myocytes were placed on top of the gel 1 h after it set. The dishes were then placed in a humidified incubator at 37 °C with 5% CO_2: 95% air. After 2–3 days coherent contracting collagen gels developed.

Optical recording of rhythmic contractions. Rhythmic contractions were determined by eye using an inverted phase-contrast microscope or by recording the changes in optical density. A densitometer (Kipp & Zonen, Delf, Holland; model densitometer: DD2) was used to measure the change in optical density caused by displacement of the gel edge, using white light and a slit of 2.0 mm in length and 0.2 mm width (fig. 1). The output signal of the densitometer was amplified and recorded or sampled by a computer system using a PCL-711S pc lab card (Advantech Co. Ltd.) with a sampling frequency of > 100 Hz. Drift of the collagen gel in the petri dish as well as electrical noise in the measuring system are the cause of noise in the absorption measurement. The noise level was determined by analysing populated collagen gels which showed no contractions, and data from contracting gels were only used when the difference between minimum and maximum optical density was at least 3 times the maximum noise level measured. The (maximum) signal to (maximum) noise ratio of a typical measurement was > 20. We defined

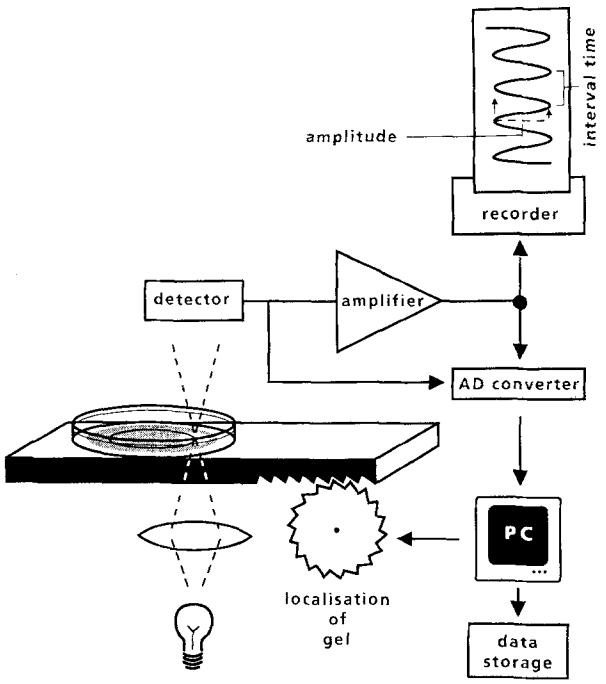

Figure 1. Diagram of the device for optical recording of the beating of neonatal rat heart cell-populated collagen gels.

the lowest absorbance level as reflecting the minimal gel size (the maximal contracted state), and the highest absorbance level as the maximal size (the maximal relaxed state). The maxima and minima of the sampled optical density data were determined and the time interval between two maxima was used as the interval time between two contractions; the difference between the minimum and the subsequent maximum signal of a contraction was used as a measure of the amplitude of that contraction. The change in absorbance is an arbitrary measure of the amplitude. Absorbance is dependent on the local absorbance, collagen density and thickness of the gel. Therefore only the amplitude data obtained from the same collagen gel have been compared. The average optical density was used as a control for detecting a possible displacement of the whole gel. When the average optical density changed more than 5%, data collection was stopped and the collagen gel was put in the starting position before data collection was resumed. Data obtained from the recorder and computer sampling were compared and the data were checked for correct processing.

Electrical recording of rhythmic contractions. For measuring electrical potential differences the culture medium was earthed with a silver wire electrode which was placed between the myocyte gel and the glass rim of the petri dish. For recording of the potentials two glass capillary electrodes were brought gently into contact with or placed just above the top layer of the myocyte-population collagen gel, and connected to a differential preamplifier (PAR 113B). The electrode capillaries were filled with a culture medium-0.5% agar gel in order to avoid unwanted diffusion potentials. The distance between the electrodes was about 1 cm. The agar-culture medium was in direct contact with the silver/silver chloride electrode pellets. The recorded signals were displayed on a Tektronix 5103N oscilloscope with a 5D10 wave form digitizer, and plotted on a Kipp BD90 X-Y recorder. The bandwidth of the preamplifier was adjusted to d.c. < frequency < 30 Hz. The gain was 10^3.

Statistics. For the calculation of the mean, standard deviation, kurtosis and skewness of the distributions and for calculating correlations we used SPSS (statistical package for social sciences).

Results and discussion

When 3×10^6 myocytes were seeded on top of a collagen gel (0.36 mg collagen/ml) in a 8 cm^2 petri dish, they formed a monolayer aggregate by attachment to the collagen gel. Early, scattered, asynchronous cell beatings were increasingly followed by fields of synchronously beating heart cells. When the cells on the gel reached a high degree of synchronization, a rhythmically contracting collagen gel was obtained[7]. The rhythmic contraction of the collagen gel was clearly visible to the naked eye. We asked the question, what is the change in volume of the gel in relation to the average volume of the myocytes? By video and cinematographic recordings we determined the change in surface area during contraction. The measured displacement of the gel at the gel edge was in the range of 30–100 μm. The change occurred all around the gel as a regular 0.3%–1.0% change of the circumference of the gel that resulted in a 0.6%–2.0% decrease of the volume of the gel. In order to correlate the change in gel volume with the total cell volume we estimated the average cell volume of a myocyte at approximately 5.2×10^{-7} μl. The total cell volume of the monolayer, consisting of 3.0×10^6 myocytes, is thus 1.6 μl. Unfortunately, volume changes of individual myocytes cannot be measured in this system because the displacement of the gel is too large to study individual cells. The change in volume of the gel during a contraction has been estimated as between 4.7–15.6 μl. We thus conclude that the myocytes expel about 3–10 times their own total volume from the gel during a contraction.

The cinematographic recording did not give any quantitative data on the regularity of the contraction. To do this, we recorded the contraction of the gel optically by using the displacement of the sharp rise in optical density at the gel boundary (fig. 1). Our automatic system was designed to measure the interval time and the amplitude of the contractions over a long period. With this system we have recorded contractions of a number of gels over long periods (several hours on successive days), and found that different gels showed extensive differences in

stability and frequency of the contractions. The measured interval time of the contractions of the gels was either very stable and regular (30–40% of the contracting gels), with a short average duration (0.3–0.5 s), or was not regular with a broad range of interval times and an average interval time higher than that of the regularly contracting gels. To illustrate this variation we will discuss the data obtained from two gels that we consider as typical representatives of the two classes. The analysis of the difference between the two types of contraction demonstrates the irregular behaviour.

Figure 2A shows an example of the distribution of the interval time of a regularly contracting collagen gel. The average value of the contraction interval time is 0.38 s (SE 0.00048) with a standard deviation of 0.05 s. The distribution has a kurtosis of −0.045 (SE 0.048) and a skewness of −0.084 (SE 0.024), and the interval time ranges from 0.25 to 0.50 s. Comparison of the measured distribution with the best-fitted normal distribution leads to the conclusion that the contraction interval time is almost normally distributed. The distribution of the amplitude of the optical density changes in this gel is given in figure 2B. The average value for the amplitude was standardized at 100 and then had a standard deviation of 3.96. The distribution has a kurtosis of 10.2 (SE 0.055) and a skewness of 0.001 (SE 0.027). This leads to the question of whether variation in the contraction interval time is related to variation in the

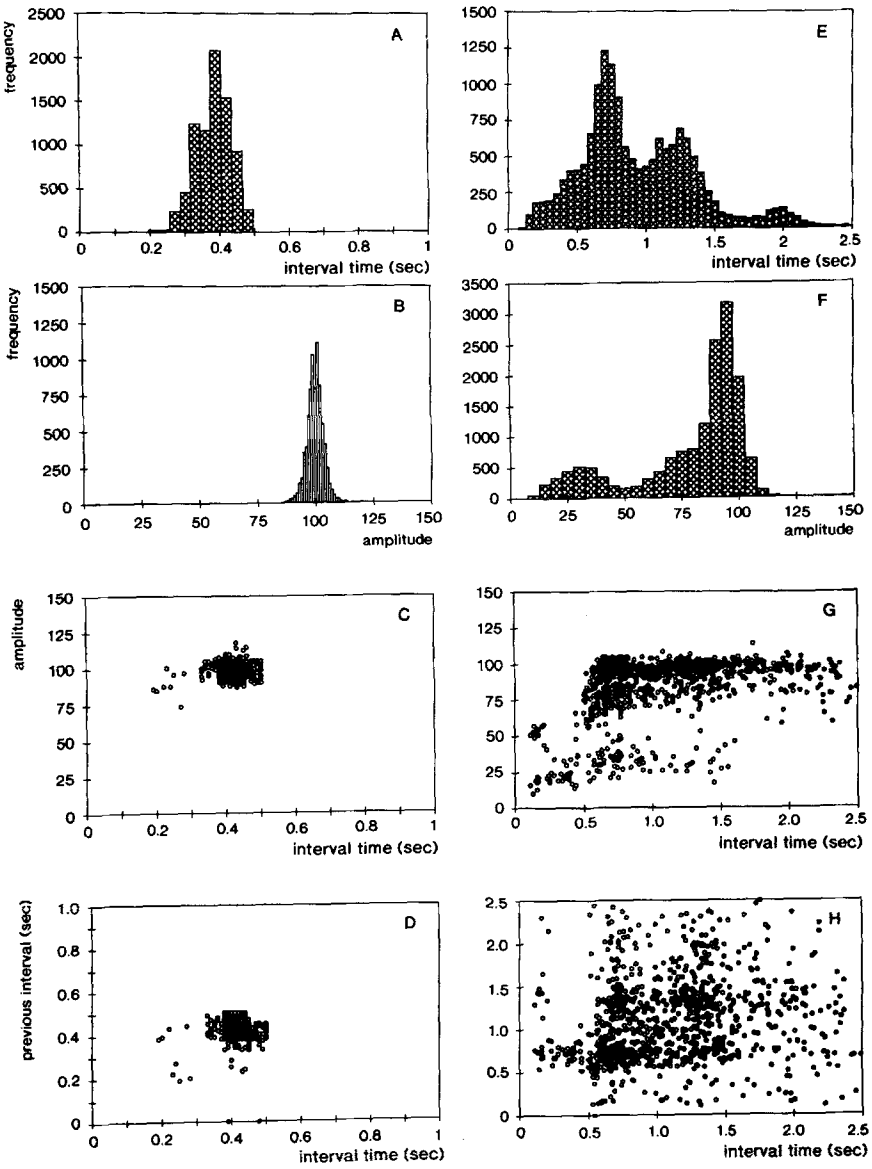

Figure 2. The frequency distribution of interval times and amplitudes of contractions of a regularly (A–D) and an irregularly (E–H) contracting myocyte-populated collagen gel. The frequency distribution of the interval times (A, E) and amplitudes (B, F) is given for a period of 1 h, as is the correlation between these interval times and amplitudes (C, G) and between two subsequent interval times (D, H) during a part of this period (n = 1000 contractions).

amplitude. Figure 2C shows that the contraction interval time is not correlated to the amplitude of the contraction (correlation coefficient = 0.05). Summarizing, we can say that in the regularly contracting gel the distribution of the interval times as well as the distribution of the amplitudes are both almost normal and that no correlation exists between the two parameters.

Figure 2 (E–H) shows an example of the distribution of the contraction interval time and the amplitude of an irregularly contracting collagen gel (fig. 2E and 2F respectively). The distribution of the interval time is multi-modal, with maxima at the interval times of 0.7, 1.3 and 1.9 s. The occurrence of these regular time steps suggest that the longer interval times of 1.3 and 1.9 s occur by omission of 1 and 2 contractions respectively. The distribution of the amplitude is bimodal with maxima at 30 and 95 (fig. 2F). The plot of the interval time against the contraction amplitude for the irregularly contracting gel is shown in figure 2G. From this figure it can be seen that short interval times of 0.7 s and less (i.e. a high frequency) correlate with low and high contraction amplitudes (average ± 30 and 95), while contractions with longer (1.3 and 1.9 s) interval times have only high contraction amplitudes (average ± 95). Data of only 1000 contraction cycles have been plotted in this figure because use of all the data points ($> 10{,}000$) is confusing. However we did test whether the selected data are representative of the whole data set. The correlation coefficient of the total data set is 0.44 ($p < 0.01$), a value higher than found for the regularly contracting gel.

We performed a second analysis with the aim of studying the relation between the interval time of one contraction to the interval time of the next to see whether the occurrence of different interval times was random. As expected a clustering of data points can be seen at different regions of the maxima in the distribution at 0.7, 1.3 and 1.9 s (fig. 2H), in contrast to the regularly contracting gel where only one cluster can be seen (fig. 2D). We divided the interval time of the contractions in 2 groups: 1) contractions with an interval time equal or less than 1.0 s and 2) contractions with interval times of more than 1.0 s. The probability within 1000 data points that an interval time would fall within group 1 was 49%. However if a contraction had a short interval, i.e. was a group 1) interval, then the probability that the next contraction is a group 1) interval was higher (68%) than expected from a random distribution. The same is true for contractions in the 2) group (51%, 68.9%).

For the irregularly contracting gels we conclude that the contraction interval time and the amplitude are multimodally distributed and that subsequent interval times of contractions are not randomly distributed. A contraction with a short interval time is preferentially followed by a contraction with a short interval time. This means that in the irregularly contracting gel there is a tendency towards regular beating. The irregularly contracting gel, which is better termed a 'semi-regularly' contracting gel, might be considered as a gel that does not reach the degree of coherence as a regularly contracting gel.

In order to see whether the degree of coherence, i.e. the self-maintenance of contractions, is characterized by a specific contraction pattern we compared the patterns of regularly and non- (or semi-)regularly contracting gels as obtained by recorder.

In the irregularly contracting gels the time in the relaxed state is longer than in the contracted state and the pattern shows peaks followed by relaxation periods. In contrast, regularly contracting gels show a contraction pattern which follows a sinusoid curve with almost equal times spent in the contracted and in the relaxed state, suggesting a possible resonance of the contraction with the relaxation of the gel. The occurrence of this resonance in only some of the gels is not clear but may be related to the differences in contraction interval times.

In a mycoyte-populated collagen gel which showed spontaneous synchronized rhythmic contractions, we probed for changes in the extracellular field accompanying the contractions. Measurements were performed at room temperature in a low electrical noise environment. The contraction rate at room temperature (20 °C) was approximately 40% of the values obtained at 37 °C as described earlier[7].

The electrical noise in the experimental setup is shown in figure 3A. Changes of the potential, fully synchronous with the contractions of the myocyte-populated collagen gels, were observed when the electrodes made contact with the collagen gel as shown in figure 3B. The size of the measured potentials was 5 to 20 μV. In order to be sure that the measured potentials are real changes in the electrical field caused by the myocytes and not artifacts due to liquid junction potentials at the electrode tips, the electrodes were also placed at positions above the gel. The signal could still be recorded and consisted of ECG-like potential jumps (fig. 3D).

The signal obtained was dependent on the location of the electrodes. The signal was maximal when one of the electrodes was placed in the centre and the other at the edge of the gel (fig. 3D) and the signal was smaller when the two electrodes were at an equal distance from the centre of the gel (fig. 3C).

This suggests a larger time interval between the contractions of the myocytes at the centre and the edge of the gel than of myocytes at an equal distance from the centre of the gel. Further experiments are needed to study the possibility that this is due to a contraction wave of the myocytes which is directed from the centre to the edge of the gel or vice versa.

In summary, the data presented so far demonstrate that automatic recording enables us to study contraction

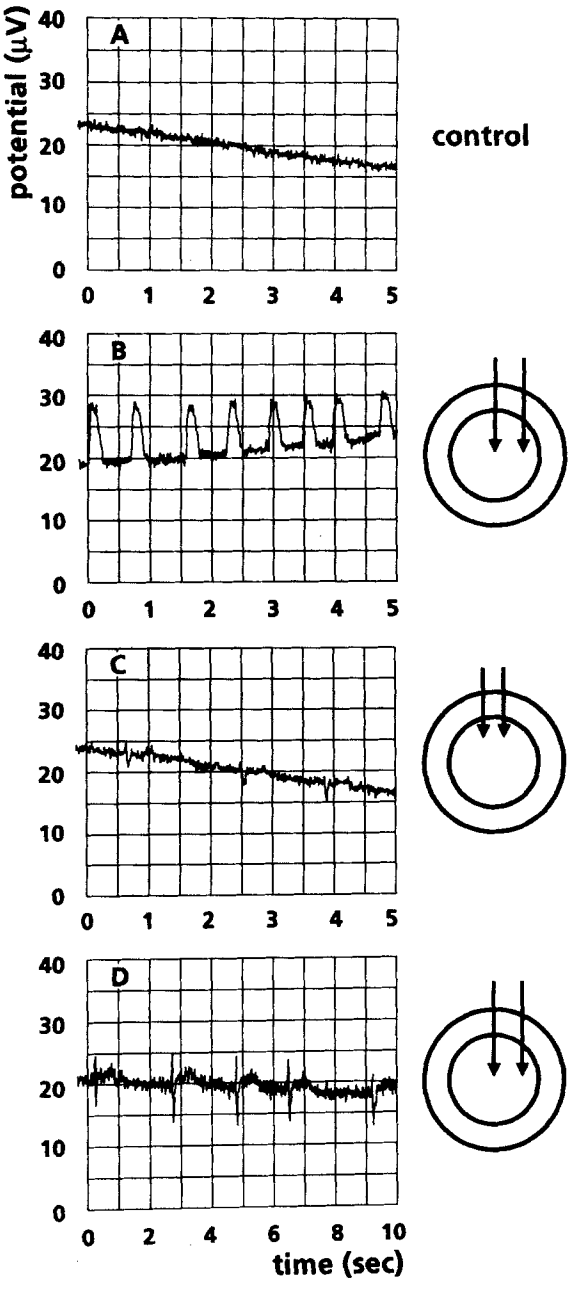

Figure 3. Measurement of the electric field of a myocyte-populated collagen gel. The electrodes were placed either in a Petri dish with culture medium alone (A), or on a myocyte-populated collagen gel (B), or were placed above the gel (C + D). The electrodes were positioned with either one electrode in the centre and one at the edge of the gel (B + D), or both at an equal distance from the centre of the gel (C). The bandwidth of the recording was d.c. < frequency < 30 Hz; gain 10^3.

parameters for long periods of time. In collagen matrixes populated by myocytes 30–40% of the gels produce highly coherent contraction patterns, as shown by sinusoidal curves with a normal distribution. The electric signal reaches a high enough signal to noise ratio for electrical recording to be practical and further developments of the measuring system will complete the automatic monitoring of contractions by electric signals and gel contraction at various temperatures.

The above-mentioned methods allow us to study the effect of various compounds on the electrophysiology of myocytes, which may be extrapolated to the whole organ. The neonatal populated collagen gel is a model system in which the beating of the heart cells is not complicated by diffusional limitations of metabolites since it is a monolayer of heart cells on top of a collagen gel consisting of more than 95% culture medium. The large displacement of the heart cells at the edge of the gel limits microelectrode measurements but this model system is well suited for macroelectrode recordings.

The system could also be a tool to study the effect of various stresses, especially heat shock and hypoxia, on the electrophysiology and biochemistry of these cells.

The results presented on the generated electrical pulses of myocytes in our gel suggest that application of externally applied pulses should lead to contractions of the gel by induced myocytes. Preliminary experiments revealed this to be the case, but further research is necessary.

Acknowledgments. The authors thank C. Schneydenberg for isolation of the heart cells, M. C. C. van Schoot for technical assistance and C. A. van der Mast for critically reading the manuscript.

* To whom correspondence should be addressed.

1 Harary, I., and Farley, B., Expl Cell Res. *29* (1963) 451.
2 Mark, G. E., and Strasser, F. F., Expl Cell Res. *44* (1966) 217.
3 Purdy, J., Lieberman, M., Roggeveen, A. E., and Kirk, R. G., J. cell. Biol. *55* (1972) 563.
4 Lefurgey, A., Hawkey, L. A., Ingram, P., and Lieberman, M., J. struct. Biol. *106* (1991) 42.
5 Rotevatn, S., Murphy, E., Levy, L. A., Raju, B., Lieberman, M., and London, R. E., Am. J. Physiol. *257* (1989) C141.
6 Sachs, H. G., De Haan, R. L., Devl Biol. *30* (1973) 233.
7 Souren, J. E. M., Schneidenberg, C., Verkleij, A. J., and Van Wijk, R., In Vitro cell. devl Biol. *28* (1992) 199.