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EARLY EVENTS IN DEVELOPMENT OF ELECTRICAL ACTIVITY AND CONTRACTION IN EMBYRONIC RAT HEART ASSESSED BY OPTICAL RECORDING

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SUMMARY

1. Spontaneous action potential and contraction in the early embryonic heart of the rat have been monitored optically using a voltage-sensitive merocyanine–rhodanine dye together with a multiple-element photodiode matrix array, and the onset of rhythmical action-potential activity in the early phases of rat cardiogenesis was conclusively determined for the first time.

2. Spontaneous rhythmical action potentials were first generated in the central part of the embryonic heart at the middle period of the 3-somite stage of development, at $9\frac{1}{2}$ days after copulation. Subsequently, contractions coupled with the action potential also appeared at the end of the 3-somite stage.

3. Usually, at the 3-somite stage, spontaneous action signals were synchronized among the different areas in the heart. From this result, it is evident that the paired right and left cardiac primordia are fused completely at the time of initiation of spontaneous electrical activity.

4. In the 3-somite embryonic heart, excitatory waves were conducted radially over the heart, at a uniform rate (0·4–0·8 mm/s), from the pace-making area. However, the regional priority of pace-making activity is not rigid but is flexible.

INTRODUCTION

Complete understanding of the ontogenesis and early development of cardiac functions in the mammal has been hampered by our inability to monitor the electrical activity in early embryonic hearts; the intracellular measurement of electrical events in the early embryonic heart cannot be achieved in very early embryos because the heart cells are too small and frail to be impaled with micro-electrodes. Absorption signals of voltage-sensitive dyes have provided a new and powerful tool for monitoring changes in membrane potential in a wide variety of preparations, and it has been discovered that it is possible to make optical recordings from cells which are

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inaccessible to micro-electrodes (Cohen & Salzberg, 1978; Freedman & Laris, 1981; Salzberg, 1983). An additional advantage of the optical method for recording action potentials is that multiple regions of a preparation can be monitored simultaneously and provide real-time spatially-resolved mapping of activity (Salzberg, Grinvald, Cohen, Davila & Ross, 1977; Grinvald, Cohen, Lesher & Boyle, 1981; Salzberg, Obaid, Senseman & Gainer, 1983; Grinvald, Anglister, Freeman, Hildesheim & Manker, 1984).

Recently, in this laboratory, optical monitoring of action potentials from early embryonic chick hearts has been achieved using voltage-sensitive dyes, and we have already demonstrated the development of spontaneous electrical excitability in early embryonic prebeating chick heart (Fujii, Hirota & Kamino, 1980, 1981*a*, 1981*b*, 1981*c*; Kamino, Hirota & Fujii, 1981; Hirota, Sakai, Fujii & Kamino, 1983*a*). While these results suggested that optical methods might be used to study the early embryonic heart of mammals, the comparative physiology of cardiac function during the early phases of cardiogenesis required investigation.

We have used a voltage-sensitive merocyanine–rhodanine dye together with a 100-element photodiode array to monitor spontaneous action potentials optically from early embryonic rat hearts, and we report here evidence for the ontogenesis of the spontaneous electrical excitability and the initiation of contraction in the embryonic heart, at an early stage of development.

METHODS

Preparation

The experiments were carried out on the embryonic hearts of rats (Sprague–Dawley, Charles River Japan Inc.) at the 2- to 4-somite stages of development, at 9½ days after fertilization. A female rat in pre-oestrus was placed in a cage with a male. If a vaginal plug was present the next morning, it was assumed that copulation had taken place during the early morning hours. The female rats were anaesthetized with ether 9½ days after copulation and the uterus was surgically removed. Subsequently, we opened the uterus wall and decidua, and an embryonic vesicle was then isolated. The isolated embryonic vesicle was kept in an air-equilibrated bathing solution of the following composition (in mM): NaCl, 149; KCl, 5·4; $CaCl_2$, 1·8; $MgCl_2$, 0·5; and Tris HCl buffer (pH 7·4), 10. The embryonic vesicles were opened ventrally by dissecting the yolk sac and amnion in bathing solution, under a dissecting microscope, and the embryo was then exposed, and attached to the silicone bottom (KE 106LTV, Shinetsu Chemical Co., Tokyo, Japan) of a simple chamber by pinning with tungsten wires.

Staining

After the splanchnopleure was carefully peeled away, the isolated embryo was incubated in a bathing solution containing 0·2 mg/ml (0·4 mM) of the merocyanine–rhodanine dye (NK 2761) for 10–20 min. The preparation was then washed with several changes of normal bathing solution. The dye was purchased from Nippon Kankoh Shikiso Kenkyusho (Okayama, Japan).

Optical measurement

The optical method for monitoring of action potential that we have used in the present experiments was essentially similar to that of Salzberg, Obaid, Senseman & Gainer (1983), with slight modification (Hirota, Kamino, Komuro, Sakai & Yada, 1985). The preparation chamber was mounted on the stage of an Olympus Vanox microscope (Type, AHB–L–1, Olympus Optical Co., Tokyo, Japan) mounted on a vibration isolation table. Bright-field illumination was provided by a JC24V–300W halogen–tungsten lamp (Kondo Sylvania Ltd., Tokyo, Japan) driven by a stable d.c. power supply (Model PAD 35–20L, 0–35 V 20 A. Kikusui Electronics Corp., Kawasaki, Japan).

Incident light was collimated, passed through a heat filter (32·5B–76, Olympus Optical Co.), rendered quasi-monochromatic with a 700 ± 11 nm (for measuring of action potential related change) or 610 ± 9 nm (for contraction related change) interference filter (Type 1F-S, Vacuum Optics Company of Japan, Tokyo, Japan), and focused onto the epi-myocardium cell layer of the

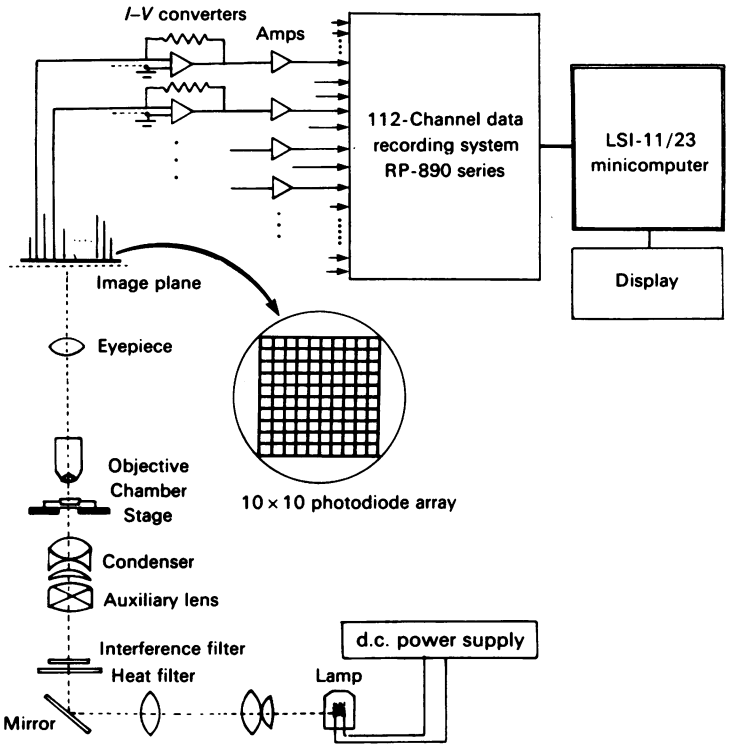

Fig. 1. Schematic diagram of the apparatus used to measure the light intensity transmitted by stained embryonic heart. The microscope objective and eyepiece form a real magnified image of the preparation at the microscope image plane. A 10×10 element array of photodiodes, positioned at the image plane, records the changes in light intensities that are related to embryonic heart activity. The optical signals are amplified and fed into the computer memory *via* a multi-channel data recording system.

heart by means of an aplanatic/achromatic condenser. A long working distance objective (S plan or S plan Apo) and a photographic eyepiece formed a magnified real image of the embryonic heart at the image plane. Magnifications were usually $\times 40$. The transmitted light intensity at the image plane of the objective and photographic eyepiece was detected using 100 elements of a 10×10 square array of silicon photodiodes (MD–100-4PV, Centronic, Ltd., Croydon). Each detector received light from a 0·0014 mm^2 area of the preparation. The image of the preparation was positioned on the array, and a drawing was prepared of the heart superimposed on the photodiode matrix. The outputs of the detectors in the diode array were fed to amplifiers via current-to-voltage (I–V) converters. The amplified outputs from 100-elements of the diode array were first recorded simultaneously on a 112-channel data recording system (RP–890 series with seven input/output processors RP–893, NF Electronic Instruments, Yokohama, Japan), which have been completed in the present stage, and then were fed into a computer (LSI–11/23 system, Digital Equipment Co., Tewksbury, MA, U.S.A.). A schematic diagram of the optical apparatus used is shown in Fig. 1. In addition, the signals were sometimes displayed on two Tektronix 5113 Dual Beam

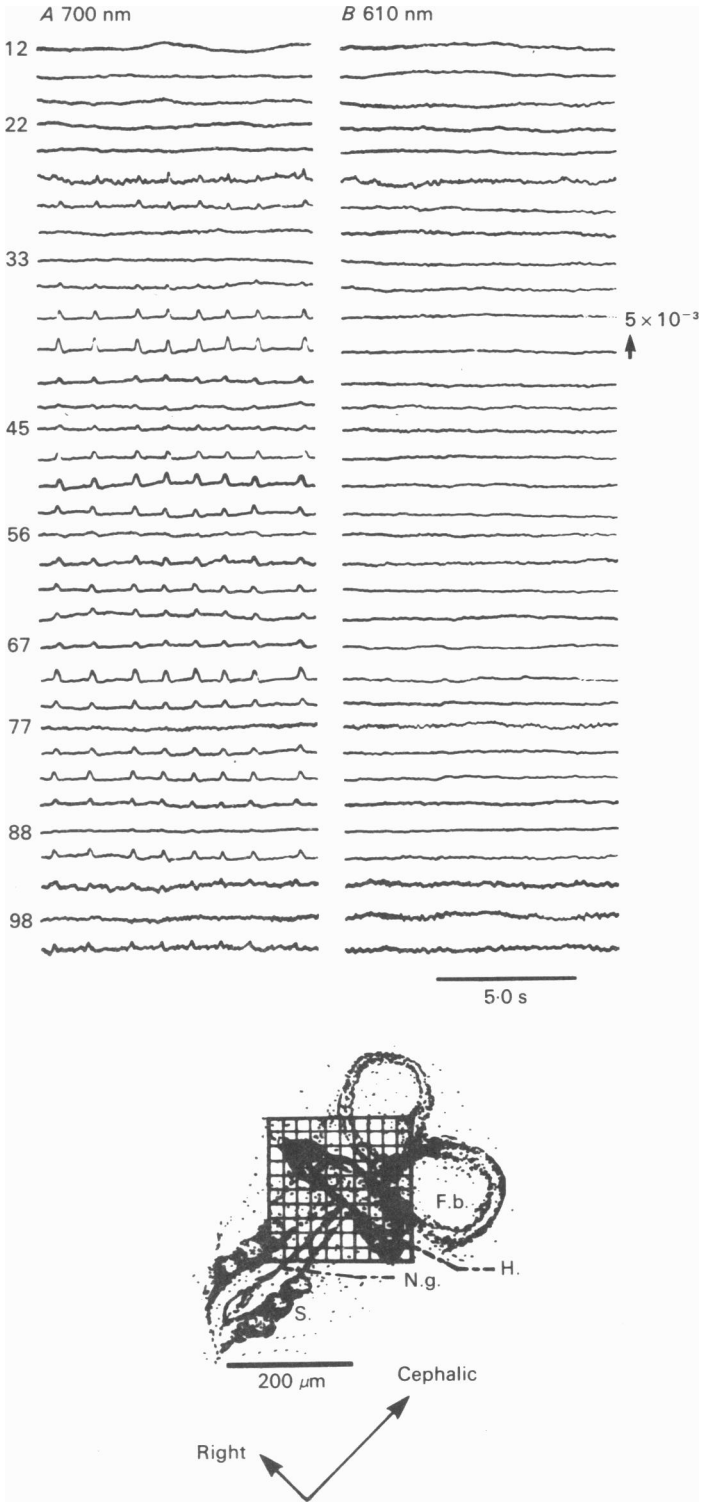

Fig. 2. For legend see opposite.

Oscilloscopes with two 5A18N amplifiers (Beaverton, OR, U.S.A.). Procedures for thermo-regulation were as described previously (Hirota, Fujii, Sakai & Kamino, 1983*b*). The temperature of the bathing solution in the chamber was controlled by means of a thermo-regulator fixed to the stage of the microscope used for the optical measurements. This thermo-regulator was built according to our specifications by the Nippon Blower Company, Tokyo, Japan.

RESULTS

Beginning of spontaneous action potentials

The embryos removed from the uterus in the afternoon of the tenth day were used for study. Generally, the primitive heart appears in the rat embryo at the 3-somite stage of development. The heart as a whole is flat and spread out in the form of a crescent in the pre- and pericephalic region of the embryo (Goss, 1952). Furthermore, in the 3-somite embryonic hearts, the epimyocardium cell layer is composed of a mono-layer (Goss, 1952).

Fig. 2 shows trains of spontaneous optical signals recorded simultaneously from thirty-four adjacent areas in a 3-somite embryonic rat heart stained with a voltage-sensitive merocyanine–rhodanine dye (NK 2761: Kamino *et al.* 1981). Voltage-clamp experiments in squid giant axons provided good evidence that absorption signals of merocyanine–rhodanine dyes were linearly related to membrane potential change (Ross, Salzberg, Cohen, Grinvald, Davila, Waggoner & Wang, 1977; Gupta, Salzberg, Grinvald, Cohen, Kamino, Lesher, Boyle, Waggoner & Wang, 1981), and that the optical response follows the voltage change with a time constant of less than 2·0 μs (B. M. Salzberg, A. L. Obaid and F. Bezanilla, personal communication). Of these merocyanine–rhodanine dyes, NK 2761 provided the largest signal-to-noise ratio in the early embryonic chick hearts, and neither the photo-dynamic damage nor the pharmacological effect of this dye were serious (Kamino *et al.* 1981; Fujii *et al.* 1981*c*). The embryonic heart was imaged onto a 100-element photodiode matrix array. A drawing of the heart shown in Fig. 2 was prepared from a photograph.

Fig. 2. Simultaneous optical recordings of spontaneous action potentials from thirty-four contiguous regions of a 3-somite embryonic rat heart stained with a merocyanine–rhodanine dye (NK 2761). The measurement was made with a 100-element photodiode matrix array, when the trains of spontaneous optical signals were recorded with a 700 ± 11 nm interference filter, the signal size was largest (*A*). The direction of the arrow to the right of the optical traces indicates a decrease in transmission and the length of the arrow represents the stated value of the change in intensity divided by the d.c. background intensity. The fractional change in transmitted intensity is related to the fractional absorption change (Kamino *et al.* 1981). NK 2761 is a suitable molecular indicator for monitoring action potential activity, especially for early embryonic hearts; as can be seen in this recording, the signal size is large (signal-to-noise ratio $> 30:1$) and the dye bleaching time is relatively long. When a 610 ± 9nm interference filter was used, the activity related optical signals were absent, shown in *B*. The lower inset illustrates the location of the image of the embryo on the photodiode matrix array (see also the legend of Fig. 4). This drawing is a ventral view: f.b., forebrain; h., heart; s., somites; n.g., neural groove. The arrows under the drawing indicate the right (Right) and cephalic directions of the view. This embryo had three pairs of somites. The numbers of the elements correspond to the illustration in Fig. 4*B*.

The optical signals shown in Fig. 2 depended on the wave-length of the incident light: the largest signals appeared at a wave-length of 700 nm with the merocyanine–rhodanine dye, and, as shown in Fig. 2*B*, these signals were eliminated by illumination at 610 nm, the isosbestic point of the action spectrum of the absorption signal of the merocyanine–rhodanine dyes (Gupta *et al.* 1981; Fujii *et al.* 1981*c*). On

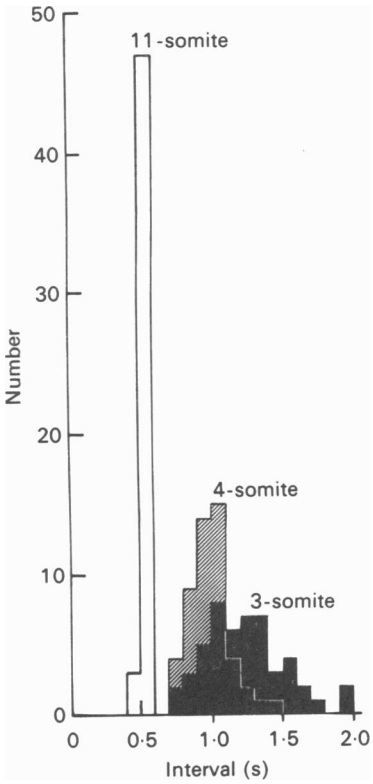

Fig. 3. Time interval histograms of spontaneous optical signals recorded from the embryonic rat hearts at the 3-, 4- and 11-somite stages of development: filled columns, 3-somite; shaded columns, 4-somite; and open columns, 11-somite. These histograms were obtained from fifty intervals measured at 34·2–35·8 °C. The ordinate is the number of intervals in a given bin. The bin width is 0·1 s. In the preparation at the 11-somite stage, the peak-to-peak time intervals of the optical signals resulting from contraction were measured. In this Figure, although the histograms in the 3- and 4-somite preparations were partly superimposed, it is clear that the time interval histogram shifts dramatically from right to left as development proceeds from the 3-somite to the 11-somite stage, and that the broad histogram gradually narrows as development proceeds.

the basis of the spectral characteristics of the optical signals shown in Fig. 2, it is clear that the signals effectively represent spontaneous action potentials occurring in the 3-somite embryonic heart. Control experiments using much older (11 day) embryonic hearts also demonstrated that the optical signals represent voltage changes (see Appendix). The empirical criteria required to distinguish between optical

signals resulting from the action potential and those corresponding to contraction were described previously (Fujii *et al.* 1980; Hirota *et al.* 1985). We have not been able to obtain signals from 2-somite embryonic hearts stained with the dye. It appears, therefore, that in the embryonic heart of the rat spontaneous action potential activity is first generated at the 3-somite stage of development, prior to beginning of contraction. In addition, the optical action signals were insensitive to tetrodotoxin, and were obtained in a Na^+-free solution, but did depend on the external Ca^{2+} concentration. From these results, we concluded that the spontaneous action potential is a Ca^{2+}-dependent action potential similar to that found in the 7–9-somite embryonic chick heart (Sakai, Fujii, Hirota & Kamino, 1983*a*). Furthermore, simultaneous recording of spontaneous optical action signals from many different areas, shown in Fig. 2, provides clear evidence for action potential synchrony in the 3-somite embryonic rat heart.

Rhythm generation. In the 3-somite embryonic rat heart, recurrence of spontaneous action potentials was apparently rhythmic, and the rate was relatively slow, although sometimes irregular and spasmodic potentials were observed. To study in more detail the stabilization of a spontaneous rhythm during the early phases of cardiogenesis, we compared the time intervals of between action signals during various stages of development.

Fig. 3 shows time interval histograms of the spontaneous optical signals obtained from 3-, 4- and 11-somite embryonic hearts. This Figure shows the striking developmental changes in the cardiac rhythm generation. In the 3-somite embryonic heart, the mean time interval was 1·24 s, and the interval shifted to 1·00 s in the 4-somite embryonic heart and to 0·54 s in the 11-somite embryonic heart. With the decrease in the mean time interval, the standard deviation of the time intervals also decreased, from 0·28 s in the 3-somite embryonic heart to 0·15 s in the 4-somite embryonic heart and to 0·03 s in the 11-somite embryonic heart. This Figure clearly shows that the rate of occurrence of spontaneous action potentials is relatively slow and that the rhythm is somewhat poor in the 3-somite embryonic heart; that the heart rate increased with development, and that the rhythm is completely organized by the 11-somite stage, suggesting that development of the cardiac rhythm is related to the increase in heart rate. The mean values of heart rates were $50{\cdot}9 \pm 13$ (S.D.)/min (five preparations, at 34·7–36·2 °C) for the early 3-somite embryonic precontractile hearts and $56{\cdot}5 \pm 14{\cdot}6$ (S.D.)/min for the later 3-somite embryonic contractile hearts (five preparations, at 35·0–38·3 °C).

Spread of the action potential activity

Fig. 4 illustrates two examples of spontaneous electrical excitation. In *A*, we recorded simultaneously from twenty-seven separate loci in a 3-somite embryonic rat heart, using a 100-element photodiode matrix array. The grid superimposed on the drawing of the heart indicates the size and position of the photodiode array elements, which correspond to the positions of absorption signals measured simultaneously. In this recording, very small signals were detected by elements 44, 45, 55, 56, and 66 only from a limited area of the central part of the heart. We think that this recording was made at the very beginning of spontaneous action potential activity.

Fig. 4*B* shows the recording from thirty-four areas of a slightly developed 3-somite

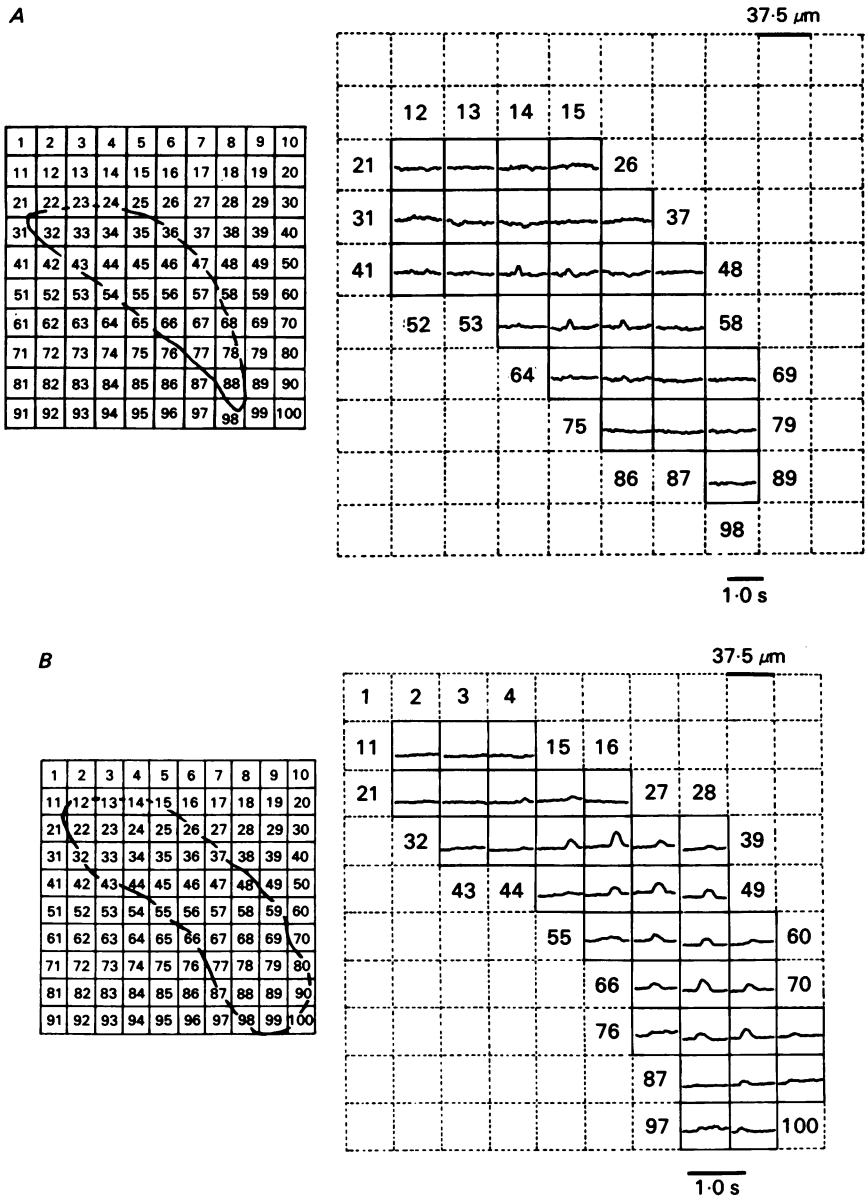

Fig. 4. Two examples of real-time mappings of spontaneous electrical activity in two 3-somite embryonic hearts constructed by multiple-site optical recording of transmembrane voltage (m.s.o.r.t.v.) using a 100-element photodiode matrix array. In this Figure and Fig. 7 the traces are arranged so that their relative positions in the Figure correspond to the relative positions of the areas of the heart imaged onto the detectors. The insets on the left indicate the location of the images of each heart (indicated by a closed curve) in the array elements. The signals were obtained with a 700 ± 11 nm interference filter. The d.c. background intensities detected by each element were almost equal in the unstained heart, and the resting intensities detected by each element were also equal after staining. Thus, the signal size is proportional to the fractional change in the voltage-related absorption change. In *A*, the fractional changes of the optical action signals were smaller than 2×10^{-4}, and they were located in a limited area (loci 44, 45,

embryonic heart. In this preparation, there is a marked difference, in size, among the signals recorded from the thirty-four areas. The larger signals were recorded in loci 35, 36, 37, 46, 47, 48, 57, 58, 68, 69, 79 of the heart, and the signals were small or absent at other positions. In these experiments, the optical signal detected by one element of the photodiode array reflects a sum of action potential changes originating from

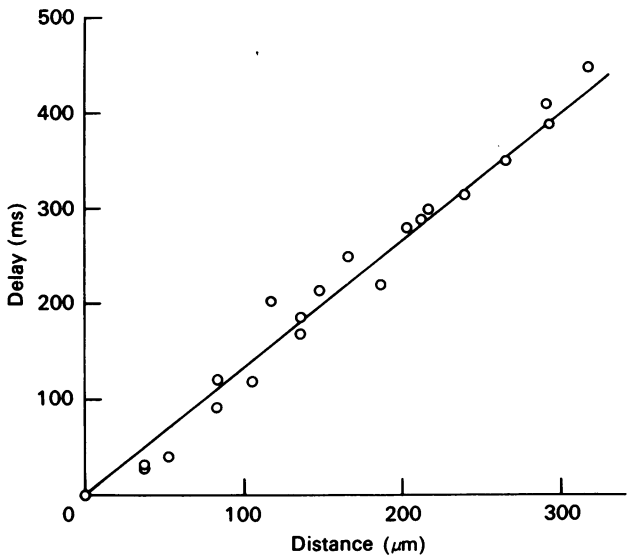

Fig. 5. The delay in the foot of the optical action signal as a function of distance from the pace-making area in which the action potential first occurred. Data were obtained from the heart used in the experiment shown in Fig. 4*B*. In this heart, the first occurrence of the action potential was detected by element 89 located in the left pre-atrial region. The ordinate is the delay (in ms) between the initiation of action signals at position 89 and each other position; the abscissa is the distance (μm) of the measured position from position 89. The conduction velocity of the excitatory wave was estimated to be 0·74 mm/s.

a population of the cardiac cells. Therefore, the signal size is probably proportional to the number of active cells in the region of the field imaged onto one element of the photodiode array. On this assumption, it seems probable that the regional differences in size of these signals reflect a regional variation in the active (excitable) cell populations in the embryonic heart. Accordingly, the real-time maps illustrated in Fig. 4 suggest that there is a maximum in the number of active cells in the central part of the cono-ventricular region of the 3-somite embryonic rat heart. However, we cannot rule out the possibility that the regional differences in the optical signal

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55 and 56). Therefore, we suppose that the preparation shown in *A* was monitored at the time of initiation of spontaneous electrical excitation. In *B*, the signal size was largest in the centre of the heart and declined toward the edge. The detectors positioned over the edge either had very small signals (elements 34, 38, 45, 59, 80 and 99) or no changes could be detected (elements 12, 13, 14, 22, 23, 26 and 33). The signals were not present with illumination at 610 nm.

size correspond to those of the amplitude of the action potentials generated in the single cells.

In addition, there were progressive delays between the feet of the optical action signals in the recording shown in Fig. 4. These delays reflect the conduction time of the excitatory wave. For example, in the multiple site optical recording of spontaneous action potentials shown in Fig. 4*B*, it is evident that spontaneous action potentials first occur at position 89 in the left pre-atrial region, corresponding to the pace-making area, and the excitation then propagates over the cono-ventricular region of the heart.

In Fig. 5 we have plotted the delay in the optical action signals observed in the experiments shown in Fig. 4*B*, as a function of the radial straight-line distance from the centre of position 89. The delays were measured as the time-to-foot of the signal. The delays in the signals were linearly related to the distance from position 89. This result indicates that the excitation propagates radially and uniformly over the cono-ventricular area from the pace-making area at the left pre-atrium, in the 3-somite embryonic rat heart at the time of initiation of action potential activity. In this preparation, the conduction velocity was estimated to be about 0·7 mm/s. A similar pattern of propagation was observed in 7–9-somite embryonic pre-contractile chick hearts (Hirota *et al.* 1983*a*). However, the conduction velocity in the 3-somite embryonic rat heart is somewhat slower than that found in the 7–9-somite embryonic chick heart.

Regional priority of pace-making activity. Excitations which originate in the pace-making area spread over the entire region of the embryonic heart. We have already found, in the 8-somite embryonic precontractile chick heart, 'switching phenomena'; the site exhibiting pace-making priority was first situated in the right pre-atrium, and then switched over to the left pre-atrium, or vice versa (Sakai, Hirota, Fujii & Kamino, 1983*b*). Similar phenomena also were often observed in the embryonic rat heart.

Fig. 6 illustrates an example of translocation of the pace-making area in a 3-somite embryonic rat heart. This preparation exhibited considerable variability in the regional priority of pace-making activity; the pace-making area was first situated at the right pre-atrium, thereafter it shifted to the left pre-atrium. The direction of the propagation of excitation was also flexible in the early embryonic heart; if the pace-making area was situated on the right pre-atrium, the excitation propagated to the left side from the right; opposite propagation resulted from location of the pace-making area on the left pre-atrium. Thus, it is most likely that the pace-making area is not yet fixed in embryonic hearts at the 3-somite stage of development.

Initiation of spontaneous contraction

The contraction that follows the action potential is also detectable, as an optical signal in the early embryonic heart. At the time of initiation of contraction in embryonic chick hearts stained with a voltage-sensitive dye, the optical signal consists of two components: the first component (first signal) corresponds to the dye-related absorption change accompanying the action potential, and the second component (second signal) is mechanical in origin (Fujii *et al.* 1980, 1981*a*; Hill & Courtney, 1982): particularly in embryonic heart at the developmental stage at which contraction is initiated, we conclude that the second signal corresponds to a light-scattering change

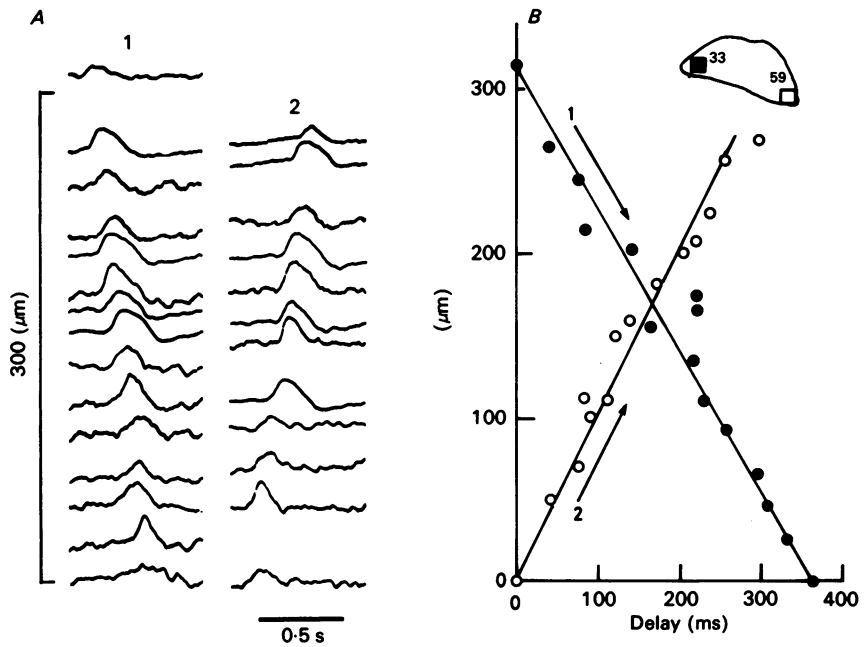

Fig. 6. Demonstration of the translocation of the pace-making area. *A*, simultaneous optical recording of spontaneous action potentials from multiple sites in a 3-somite embryonic rat heart. During the course of the measurement, the conduction pattern was transformed from type 1 (the left-hand recording) into type 2 (the right-hand recording). In the situation of type 1, the excitation, which first occurred on the right pre-atrium (position 33 in the drawing illustrated in the upper right-hand corner), was conducted progressively in toward the left pre-atrium; in the situation of type 2, the excitation first occurred on the left pre-atrium (position 59 in the drawing), and then propagated toward the right pre-atrium. In this experiment, after the optical signals were recorded on a data recording system, they were displayed on two Tektronix 5113 Dual Beam Storage Oscilloscopes with two 5A18N amplifiers (Beaverton, Oregon, U.S.A.). The oscilloscope gains were adjusted for each signal in order to make it easier to identify the foot of the signals. *B*, graphic representation of the results shown in *A*. The delay of the optical signals is plotted as a function of the distance from the pace-making area (position 33 for type 1, indicated by a downward arrow; position 59 for type 2, indicated by an upward arrow). The ordinate is distance (in μm) of the measured position from the pace-making area. The abscissa is the delay (in ms) of the beginning of each optical signal relative to the pace-making area. Position 33 (for type 1) or position 59 (for type 2) is regarded as the zero-point reference for the timing of occurrence of the action potential. The conduction velocity is exhibited before and after the change in propagation direction; it was estimated to be 0·95 mm/s travelling to the left and 1·0 mm/s to the right, respectively.

due to small contractions of the myofibrils (Hirota *et al.* 1985). The first signal varies with the wave-length of incident light, while the second signal is independent of wave-length and is detectable with white light or at the isosbestic point of the action potential related first signal (e.g. 610–620 nm with the merocyanine–rhodanine dyes; Hirota *et al.* 1985). The optical signal in a stained heart is often biphasic, the direction of the second signal is opposite to that of the first signal. Thus, even when the contractions are too small to be detected visually using a microscope, they may be identified by the second signal.

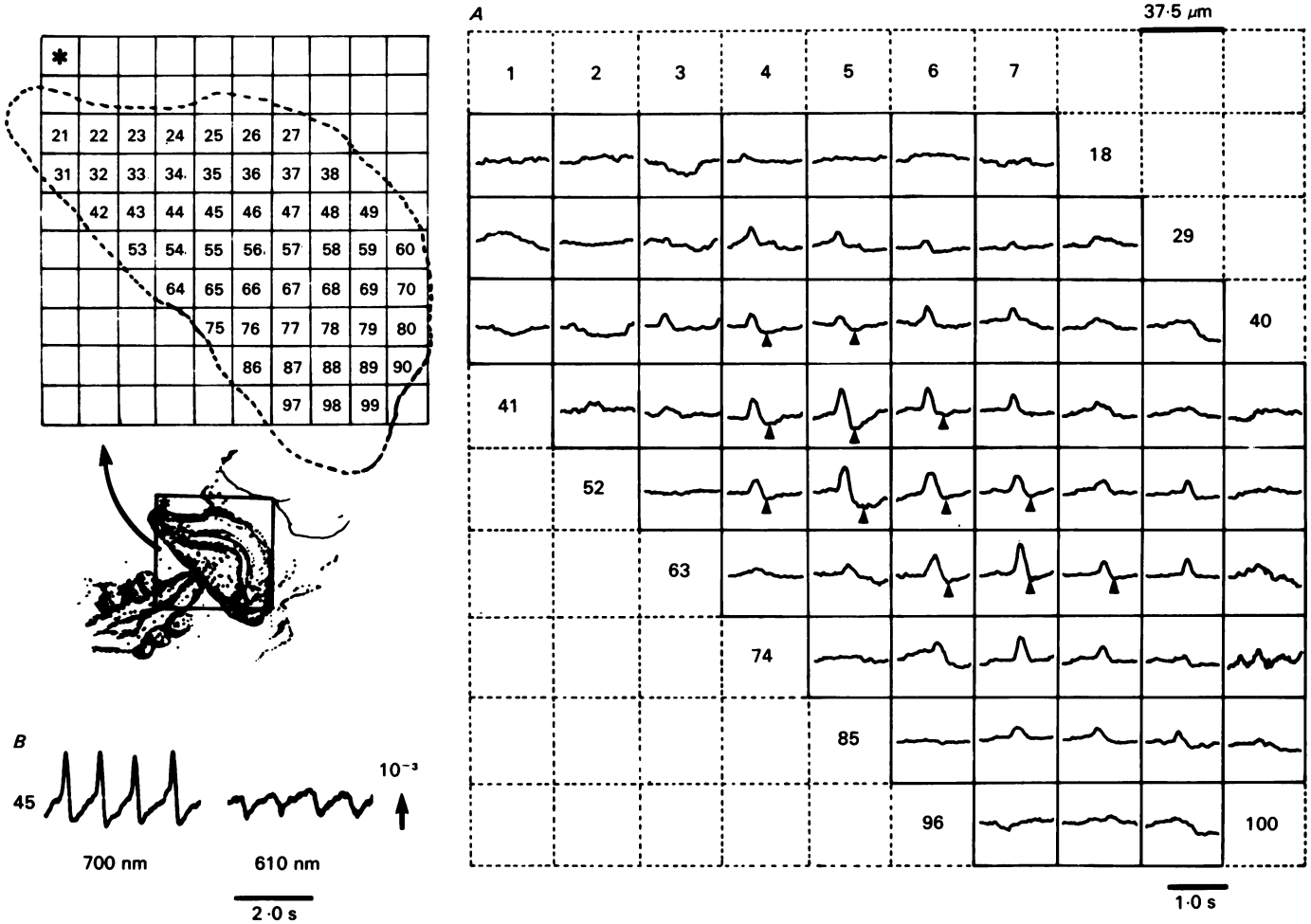

Fig. 7. For legend see opposite.

Evidence for the onset of contraction coupled with the spontaneous action potential in a 3-somite embryonic rat heart is presented in Fig. 7. The heart was stained with NK 2761, and optical signals were recorded simultaneously from sixty-two contiguous areas of the heart using a 100-element photodiode array. The heart appeared to be at a slightly later developmental stage than that of the heart shown in Figs. 2 and 4.

The signals, shown in Fig. 7*A* were obtained at 700 nm. In this recording, in addition to the monophasic signals related to the action potential, biphasic signals were detected by elements, 34, 35, 44, 45, 46, 54, 55, 56, 57, 66, 67 and 68 positioned over the image of the ventricular regions. Of the biphasic signals, the signals from elements 45 and 55 exhibited the largest second signal, and, although the first components of the signals from elements 44, 46, 56, 66 and 67 were relatively large, the second components were small. At 610 nm only the second components were detected. As an example, Fig. 7*B* shows representative signals detected by element 45, the left trace was recorded at 700 nm, and the right trace was at 610 nm. Therefore, it is most likely that the second components in Fig. 6*A* reflect the contractions following spontaneous action potentials. We found that the first contraction was generated in the central portion of the ventricular region at the 3-somite stage of development, in the rat embryo. Of the forty 3-somite embryonic hearts examined in the present work, spontaneous action potentials without contraction were detected in 20 % of the preparations, contractions coupled with action potentials appeared in 15 % of the preparations, and in the others there was no signal.

Similar experiments were carried out using various preparations at the 3–4-somite stages and the results are summarized in Fig. 8. Toward the end of the 3-somite stage

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Fig. 7. Real-time mapping of the spontaneous action potential and mechanical activation, constructed by simultaneous recording of spontaneous optical signals from sixty-two separate areas of a 3-somite embryonic rat heart at approximately the time of initiation of the contraction. *A*, the preparation was stained with NK 2761, and the measurement was made using a 700 ± 11 nm interference filter together with a 100-element photodiode matrix array, at 36·2–36·8 °C. The location of the photodiode matrix array relative to the preparation is illustrated on the left: the region of the array on which the heart was imaged is surrounded by a closed curve (in the upper illustration). Note that biphasic signals consisting of first and second components (indicated by the arrowheads) are seen in the central part of the heart. In particular, the appearance of the second signals was concentrated in the central part of the ventricular region, and the contractile area was estimated to be about 0·0168 mm^2. Elements 14, 24, 25, 26, 27, 28 (respectively), 33, 37, 47, 58, 59, 69, 76 (perhaps), 77, 78, 79, 87, 88 and 89 detected only the action potential related first signals; in this heart the active area was estimated to be about 0·0448 mm^2. Therefore, the contractile area constituted about 27·5 % of the active area. *B*, enlargement of the optical signals detected by element 45. The first components which were detected at 700 nm were absent at 610 nm and only the second components remained. Generally, the appearance of such a second component reflects the generation of a small contraction, which was not detected visually. The second component does not vary with the wavelength of the incident light and is detectable from unstained hearts. The light intensity detected from the edge of the image of the heart often fluctuated, so that changes seen in positions 13, 16, 17, 21, 23, 31, 32, 39, 49, 50, 60, 70, 80, 90, 97, 98 and 99 are regarded as artifacts. The resting light intensities received by the individual detectors, within the image of the heart, were almost equal.

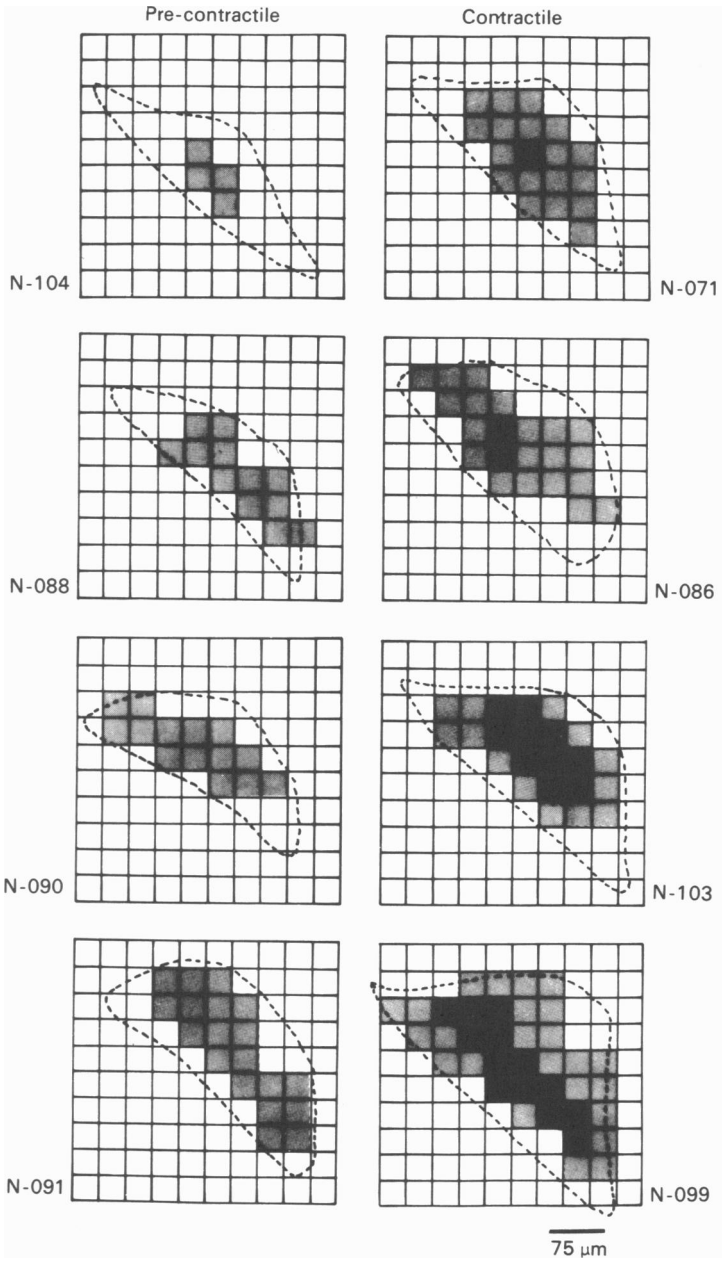

Fig. 8. Developmental changes in areas where action potential related optical signals and/or contraction related optical signals were detected, in the 3–4-somite rat embryonic hearts. Preparations, N-104, N-088, N-090, N-091, N-071, N-086 and N-103 were at 3-somite stage; and preparation N-099 was at 4-somite stage. The shadowed areas show the loci where the action potential related signals appeared at 700 nm; and filled area is the loci where the contraction related signals were detected at 610 nm. The areas surrounded by a dashed line correspond to the ventral area of the hearts. Measurements were made at 36·1–36·4 °C for N-104; 33·4–33·9 °C for N-088; 33·9–34·1 °C for N-090; 32·7–33·2 °C for N-091; 35·9 °C for N-071; 34·0–34·5 °C for N-086; 34·6–35·0 °C for N-103; and 37·6–37·8 °C for N-099.

the contraction was usually generated first in the limited area in the central region of the heart. The contractile area is enlarged as development proceeds, and eventually by the 4-somite stage, the area covers the whole region of the heart. This pattern of the development of contraction differs from that found in the chick embryo (Hirota *et al.* 1985).

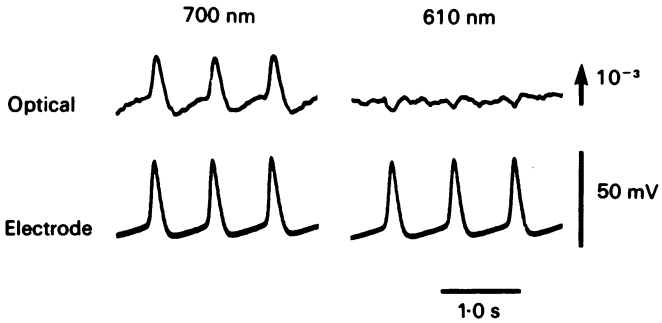

Fig. 9. An optical change accompanying an action potential in an 11-day-old embryonic rat heart stained with a merocyanine–rhodanine dye (NK 2761). The transmission change was recorded simultaneously with an electrode measurement of the action potential in one of the cono-ventricular cells within the light beam. The optical changes were detected with a 700 ± 11 or 610 ± 9 nm interference filter; the action potentials were measured with an intracellular micro-electrode filled with 3 M-KCl and of the resistance of about 30 MΩ at about 36·1 °C. For this experiment, in order to reduce the contraction, the measurements were made in a NaCl-hypertonic solution together with high Ca^{2+} concentration. The direction of the vertical arrow to the right of the optical traces indicates the direction of a decrease in transmitted intensity from the heart, and the length of the arrow represents the stated value of the change in intensity divided by the d.c.-background intensity.

DISCUSSION

Using an optical method for monitoring of action potentials, we have determined for the first time the moment of initiation of spontaneous electrical excitation and contraction during the early phases of mammalian cardiogenesis.

In the early embryonic chick heart, spontaneous rhythmical action potentials are first generated at the 7-somite stage, and the first contraction coupled with electrical excitation begins reliably at the middle period of the 9-somite stage. This sequence of events in the development of cardiac function in the early phases of rat cardiogenesis, as shown in Figs. 2, 4 and 7, is evidenced already at the 3-somite stage. Therefore, from the standpoint of the development of cardiac function in the rat embryo, the 3-somite stage is critical.

On the basis of the result obtained by multi-site recording of spontaneous action potentials shown in Fig. 2, it is strongly suggested that embryonic heart cells are electrically coupled over the entire region in the embryonic primitive rat heart at the 3-somite stage, and it is most likely that in rat embryos fusion of the paired right and left rudiments (primordia) has been completed by the middle period of 3-somite stage, at the time of initiation of spontaneous electrical excitation.

Since the report of Goss in 1938 (Goss, 1938), it has been commonly accepted that

the embryonic rat heart exhibits its first contraction on the left side, at about $9\frac{1}{2}$ days of development, before fusion of the paired left and right rudiments is complete (Goss, 1952; DeHaan, 1959, DeHaan, 1980). However, we have never obtained evidence to support this idea. Indeed, the real-time mapping of the contraction shown in Fig. 8 argues additionally that the paired cardiac primordia are fused completely at the time of initiation of spontaneous contraction. Thus, our present findings strongly suggest that conventional ideas concerning the onset of contraction of the embryonic rat heart need to be corrected.

TABLE 1. Comparison of early developmental events of cardiac functions of rat and chick

	Rat (Sprague–Dawley)	Chick (White Leghorn)
Electrical		
Stage of beginning of spontaneous rhythmic action potential:	3-somite stage (the middle period)	7-somite stage (the middle period)
Regionally synchronized?	Yes	Yes†
Heart rate	50·9 ± 13 (S.D.)/min ($n = 5$)*	46·6 ± 8·2 (S.D.)/min ($n = 23$)‡
Ionic property	Ca^{2+} dependent	Ca^{2+} dependent**
Effect of TTX	Insensitive	Insensitive**
Conduction pattern	Radially and at a uniform rate	Radially and at a uniform rate§
Conduction velocity	0·55 ± 0·15 (S.D.) mm/s ($n = 7$)*	1·5 ± 0·53 (S.D.) mm/s ($n = 31$)‡
Contractile		
Stage of beginning of contraction	3-somite stage (the later period)	9-somite stage (the middle period)¶
Area of the first contraction	Central part of the cono-ventricle	Right margin of the cono-ventricle‖

Notes: *, measured in the 3-somite prebeating hearts at 34·7–36·2 °C; †, Fujii *et al.* 1981*c*; ‡, Hirota *et al.* 1983*b*, measured in the 7–9 somite prebeating hearts at 36·8–37·4 °C; **Sakai *et al.* 1983; §, Hirota *et al.* 1983*a*; ¶, Fujii *et al.* 1980; ‖, Hirota *et al.* 1985.

We have indicated earlier (Fujii *et al.* 1981*a*) one element of a photodiode array detects signals from many active cells at once in a multicellular tissue (Senseman & Salzberg, 1980). We may thus assume that the observed size of the optical signals is a function of the active membrane area and of the optical signal generated by a unit area of active membrane undergoing an action potential. Here, the active membrane area in the field of optical recording by one element depends on the number of active cells. This idea admits the possibility that functional differentiation into active cells from native ones progresses most prominently in the central area of the cono-ventricular region during early phases of rat cardiogenesis. On the other hand, the merocyanine–rhodanine dye-related absorption change is also related linearly to the membrane potential change (Ross *et al.* 1977). Therefore, an additional possibility is that electrical excitability is largest in the central area in the cono-ventricular region of the rat heart at the 3-somite stage.

The radial propagation of excitation at a uniform rate seems to be common to the early embryonic hearts of the rat and chick. Thus, we suggest tentatively that the preferential conduction pathway(s) is not yet differentiated in the earliest phases of

cardiogenesis in vertebrates. The propagation of action potentials in the early embryonic heart would be favoured by a low-resistance pathway for current flow between the cell interiors (Berger, 1972; DeMello, 1977). One possible mechanism is that gap-junctions are sites of intercellular communication (Heppner & Plonsey, 1970; DeHaan, 1972; Komuro, Hirota, Yada, Sakai, Fujii & Kamino, 1985). Although little information is available on the structural development of gap-junctions in the embryonic and developing heart, gap-junctions have been observed in the 11-somite chick heart (Manasek, 1968; McNutt, 1970) and in the 10-day-old embryonic mouse heart (Gros, Mocquard, Schrevel & Challice, 1981). In addition, the propagation pattern shown in Fig. 5 suggests that gap-junctions are uniformly formed over the entire region of the heart.

The 'switching phenomenon' of pace-making area demonstrated in Fig. 6 shows clearly that the regional priority of pace-making activity is not rigid, as we already have suggested in a previous paper (Sakai *et al.* 1983*b*). In experiments using double hearts produced experimentally or congenitally in chick embryos, we have found that intrinsic spontaneous rhythmic activity exists independently in the right- and left-hearts, and that there are differences in the frequency of the spontaneous action potentials (Fujii, Sakai, Hirota & Kamino, 1983; Hirota, Fujii, Sakai & Kamino, 1984; Yada, Sakai, Komuro, Hirota & Kamino, 1985). Therefore, it is reasonable to postulate that the regional gradient of rhythmicity determines the regional pace-making priority in early embryonic hearts, and that the fluctuation of the regional gradient of rhythmicity results in the 'translocation' of the pace-making area (Kamino *et al.* 1981).

The first contraction of the chick embryonic heart muscle, at the 9-somite stage, is completely limited to the cono-ventricular part and almost entirely confined to its right margin (Patten & Kramer, 1933; Hirota, Sakai, Fujii & Kamino, 1981; Hirota *et al.* 1985). In contrast, the contractile system and/or excitation–contraction coupling system in the rat embryo is first completed in the central part in the cono-ventricular region at the middle period of 3-somite stage, after the paired right and left primordia are fused.

The present work described some of the comparative physiology of the early embryonic rat heart; the embryonic rat and chick hearts share many similarities and some differences in electrical and contractile properties, as shown in Table 1. We believe that our findings provide new insights into the early development of cardiac function in mammals, and feel that these similarities and differences would be very useful for comparative developmental physiology of cardiac function.

APPENDIX

The optical signal related to an electrode measurement of the action potential

Fig. 9 shows a simultaneous electrical measurement of a spontaneous action potential and the optical changes in an 11-day-old embryonic rat heart stained with the merocyanine–rhodanine dye. It is clear that the large optical signal at 700 nm has a time course similar to that of the action potential measured by a micro-electrode, and is absent at 610 nm. Thus, the large optical signal accurately reflects the membrane potential change associated with the action potential.

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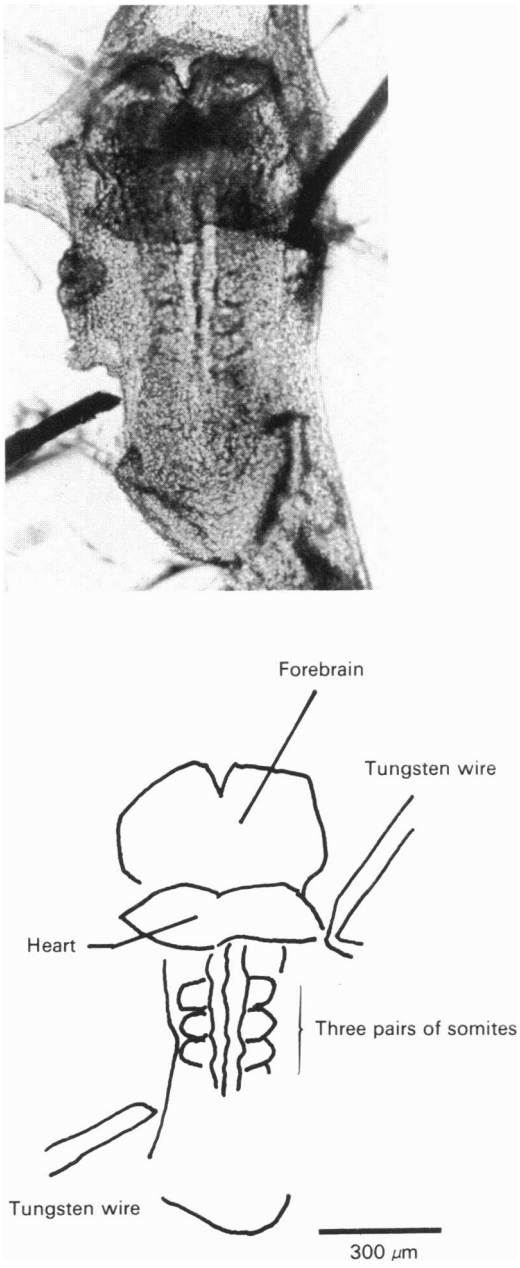
Forebrain
Tungsten wire
Heart
Three pairs of somites
Tungsten wire
300 μm

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EXPLANATION OF PLATE

Photomontage of the ventral view of a rat embryo with three pairs of somites. This photograph was taken in a chamber for optical measurement, after completion of the optical recording.