Functional Pacemaking Area in the Early Embryonic Chick Heart Assessed by Simultaneous Multiple-Site Optical Recording of Spontaneous Action Potentials

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ABSTRACT Pacemaking areas in the early embryonic chick hearts were quantitatively assessed using simultaneous multiple-site optical recordings of spontaneous action potentials. The measuring system with a 10- × 10- or a 12 × 12-element photodiode array had a spatial resolution of 15–30 μm. Spontaneous action potential–related optical signals were recorded simultaneously from multiple contiguous regions in the area in which the pacemaker site was located in seven- to nine-somite embryonic hearts stained with a voltage-sensitive merocyanine-rhodanine dye (NK 2761). In the seven- to early eight-somite embryonic hearts, the location of the pacemaking area is not uniquely determined, and as development proceeds to the nine-somite stage, the pacemaking area becomes confined to the left pre-atrial tissue. Analysis of the simultaneous multiple-site optical recordings showed that the pacemaking area was basically circular in shape in the later eight- to nine-somite embryonic hearts. An elliptical shape also was observed at the seven- to early eight-somite stages of development. The size of the pacemaking area was estimated to be ~1,200–3,000 μm^2. We suggest that the pacemaking area is composed of ~60–150 cells, and that the pacemaking area remains at a relatively constant size throughout the seven- to nine-somite stages. It is thus proposed that a population of pacemaking cells, rather than a single cell, serves as a rhythm generator in the embryonic chick heart.

INTRODUCTION

A complete understanding of the ontogenetic development of physiological functions in the early embryonic heart has been difficult to attain. One reason

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J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/88/04/573/19 $2.00 573

Volume 91 April 1988 573–591

for this difficulty is that the conventional electrophysiological techniques have been applied only with great difficulty to the early embryonic heart cells.

Using optical methods for monitoring electrical activity with voltage-sensitive dyes (Fujii et al., 1980), spontaneous electrical activity in early embryonic precontractile chick hearts has been discovered, and information on the early development of cardiac function in the embryo has been rapidly expanded. Applying optical methods for monitoring of membrane potential activity (Cohen and Salzberg, 1978), we have described the development of electrophysiological events (Hirota et al., 1987), the initiation of excitation-contraction coupling (Kamino et al., 1983; Hirota et al., 1985*a*), and the appearance of the pacemaker potential and rhythm generation (Fujii et al., 1981) in the six- to nine-somite embryonic chick hearts.

In addition, optical measurements using a combination of several single photodiodes or a multiple-element photodiode array have facilitated simultaneous recordings of electrical activity from many regions in one preparation (for a review of the method, see Cohen and Lesher, 1986). Using such methods, we have investigated the conduction patterns of excitation and the relative position of the pacemaker site in early embryonic hearts (Kamino et al., 1981, 1987; Hirota et al., 1983, 1985*b;* Komuro et al., 1985; for a review, see Viragh and Challice, 1983; for a monograph, see Canale et al., 1986).

Throughout those studies, we suspected that the pacemaking area could be assessed quantitatively by combining the use of a multiple-element photodiode array with higher magnification of the image. We report here the quantitative evaluation of the location, shape and size of the pacemaking area in the seven- to nine-somite embryonic chick hearts. A preliminary report of this work appeared in an abstract for the 63rd Annual Meeting of the Physiological Society of Japan (Sakai et al., 1986).

METHODS

Preparations

Fertilized white Leghorn eggs were incubated usually for 30–40 h in a forced-draft incubator (type P-03, Showa Incubator Laboratories, Urawa, Japan) at a temperature of 37°C and 60% humidity, and were turned once each hour. For optical measurements in the present experiments, the seven- to nine-somite embryos were used. The isolated embryos were kept in a bathing solution with the following composition (millimolar): 138 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, and 10 Tris HCl buffer (pH 7.2). The solution was equilibrated with air and allowed to warm to 37°C. Most of the egg yolk and vitelline membrane attached to the embryo and splanchnopleure were carefully removed in the bathing solution, under a dissection microscope.

Potential-sensitive Dye Staining

The isolated embryos were incubated for 10–15 min in a bathing solution containing 0.1–0.2 mg/ml of a merocyanine-rhodanine dye (NK 2761; Kamino et al., 1981) obtained from Nippon Kankoh-Shikiso Kenkyusho Co., Okayama, Japan. The preparation was then washed with several changes of normal bathing solution.

Optical Recording

The apparatus used was as described (Hirota et al., 1985*b*). The preparation chamber was mounted on the stage of an Olympus Vanox microscope (type AHB-L-1). Bright field illumination was provided by a 300-W tungsten halogen lamp (type JC-24V-300W, Kondo Sylvania Ltd., Tokyo, Japan) driven by a stable DC power supply (model PAD 35-20L, 0-35V 20A, Kikusui Electronics Corp., Kawasaki, Japan). Incident light was collimated, passed through a heat filter (32.5B-76, Olympus Optical Co., Tokyo, Japan) and an interference filter (type 1F-W, Vacuum Optics Co. of Japan, Tokyo, Japan) at 702 ± 13 nm for measuring of action potential–related change or 611 ± 10.5 nm for testing the contraction-related artifacts, and focused on the preparation 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 33 and 50. The transmitted light intensity at the image plane of the objective and photographic eyepiece was detected using a 10 × 10 or 12 × 12 square array of silicon photodiodes (MD-100-4PV or MD-144-4PV, Centronic, Ltd., Croydon, England). Each of the 1.4- × 1.4-mm^2 active elements of arrays is separated by 100-μm insulating regions. The image of the area of the heart in the embryo was positioned on the array and a drawing was prepared of the heart superimposed on the photodiode matrix. The output of each detector in the diode array was fed to an amplifier via a current-to-voltage converter. The amplified outputs from 100 or 128 elements of the detector were first recorded simultaneously on a 128-channel recording system (RP-890 series, NF Electronic Instruments, Yokohama, Japan) and then fed into a computer (LSI-11/73 system, Digital Equipment Corp., Marlboro, MA). The 128-channel data recording system is composed of a main processor (RP-891), eight I/O processors (RP-893), a 64K word wave memory (RP-892), and a video tape recorder. The program for the computer was written in assembly language (Macro-11) under the RT-11 operating system (version 5.0).

Thermoregulation

The temperature of the bathing solution in the chamber was controlled by means of a thermoregulator fixed to the stage of the microscope used for the optical measurements as described previously (Hirota et al., 1983).

RESULTS

Looking for the Pacemaker Site

Experiments were first carried out to look for the site at which the spontaneous action potential first appeared. The 12- × 12-element photodiode array was positioned over the magnified (× 33) real image of the entire heart in the embryo, including the ventricle and the right and left pre-atrial tissues.

Fig. 1 *A* shows an example of the original recordings obtained in such an experiment. Action potential–related optical signals were recorded simultaneously from 91 contiguous areas of an early nine-somite embryonic precontractile heart stained with a merocyanine-rhodanine dye (NK 2761). The recording was made with a 702 ± 13-nm interference filter. In the lower left corner is a drawing of the heart imaged on the photodiode matrix array, indicating the relative

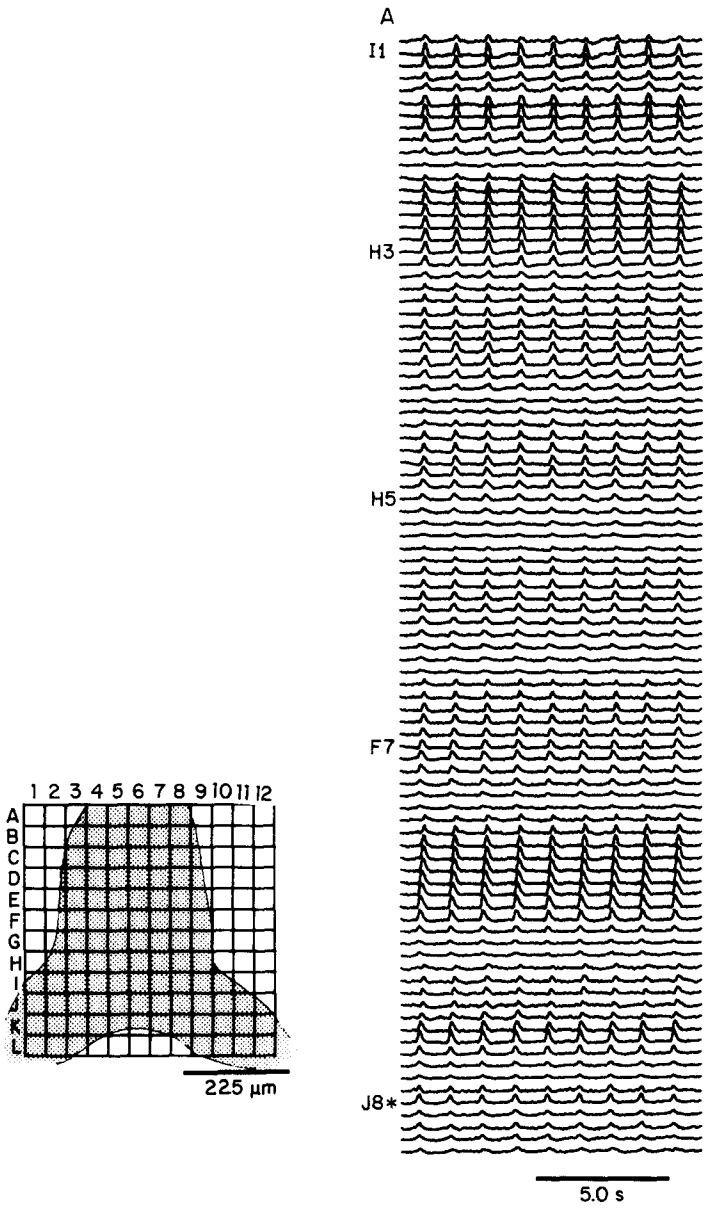
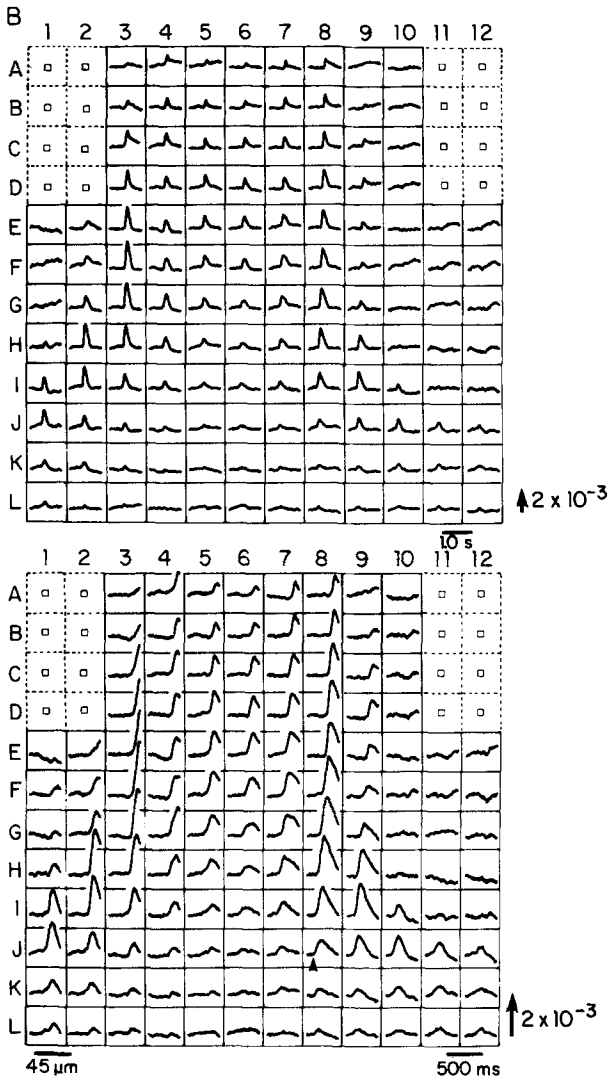


FIGURE 1. (*A*) Simultaneous optical recording of spontaneous electrical activity from 91 contiguous regions of a nine-somite embryonic precontractile chick heart, using a 12- × 12-element photodiode matrix array. The preparation was stained with a merocyanine-rhodanine dye (NK 2761). The photodiode array was positioned over a 33×-magnified image of the heart. Thus, each trace represents signals detected by one photodiode from a 42- × 42-μm^2 area of the heart: the size of each area in the grid includes the dead space owing to the insulating part of the diode array (see Methods). The outputs of the individual detectors have been divided by the resting light level (DC-background intensity = fractional change). Early embryonic preparations are usually very thin, so that effect of scattering from the preparation is not serious. The 91 traces with signals



are arranged in parallel. The relative location of the photodiode array on the image of the heart is illustrated on the lower left: the shaded part corresponds to the heart. The measurement was carried out at 36.6–37.5°C. A 702 ± 13-nm interference filter was used in this and subsequent recordings. (*B*) Real-time optical recording of spontaneous action potentials from a nine-somite embryonic heart, using 128 photodiodes. This preparation and the relative position of the photodiode array on the image were the same as those shown in *A*. The lower recording was obtained at a higher sweep speed. Note the delays in the timing among the signals. The traces of the earliest signal(s) are indicated by an asterisk in *A* and by an arrowhead on the lower recording in *B*. The displayed signals were normalized by compensating the DC-background intensity. The direction of the arrows to the right of the optical recordings indicates a decrease in transmission (increase in absorption) and the length of the arrows represents the stated value of the fractional change.

positions and size of individual detector elements. Each photodiode element detected optical signals from a 42- × 42-μm^2 area in the heart.

Applying such a multiple-site optical recording method, we have been able to determine easily the relative position of the pacemaking area in which the action potential signal first appears. In the recording shown in Fig. 1 *A*, the earliest signal was detected by element J-8 positioned on the left atrial primordium, so it is likely that the pacemaker site was located around this area. In addition, we can see that the rhythmically appearing spontaneous action potential signals spread widely over the heart from this position, and that they were nearly synchronized among the 91 different regions with short delays among the onsets of the signals. These delays represent the conduction time of excitation. Differences in the signal size among the traces would represent regional differences in electrical activity and/or active membrane area (Obaid et al., 1985; Hirota et al., 1987).

TABLE I

Comparison of Locations of the Pacemaking Areas in Seven- to Nine-Somite Embryonic Chick Hearts

	Number of preparations					
Location	7-S	8-E	8-M	8-L	9-E	9-M
Right ventricle	4	4				
RV/RA*	2	7	2	1		
Right atrium	1	4	1	1		
Left ventricle		5	4	1	1	
LV/LA‡	1	2	10	9	13	17
Left atrium		1	5	3	17	25

Data were obtained from 141 preparations sampled at random. 7-S indicates the seven-somite stage of development; 8-E, 8-M, and 8-L indicate the early, middle, and later periods of the eight-somite stage; 9-E and 9-M indicate the early and middle periods of the nine-somite stage.

* RV/RA: pacemaking area located over the right ventricle and the right atrial tissue.

‡ LV/LA: pacemaking area located over the left ventricle and the left atrial tissue.

Fig. 1 *B* also shows two trials of simultaneous optical recordings of action potentials obtained from the same embryonic heart, using 128 photodiodes. In this figure, the signals are arranged so that their relative positions correspond to the relative positions of the regions of the preparation imaged onto the diode array. This figure thus represents more clearly the regional distribution of the electrical activity and spreading of the action potential in the heart. In the recording made at a higher sweep speed (lower set), the delays among the firing times of the signals are clearly visible, and it quickly became evident that the action potential signal first appeared at position J-8 in the left atrial primordial tissue and the excitation spread over the heart from this area, which corresponds to the pacemaking site.

The relative positions of the pacemaking area observed in seven- to nine-somite embryonic hearts are summarized statistically in Table I. We have previously reported that at the seven- to eight-somite stages, the location of the pace-

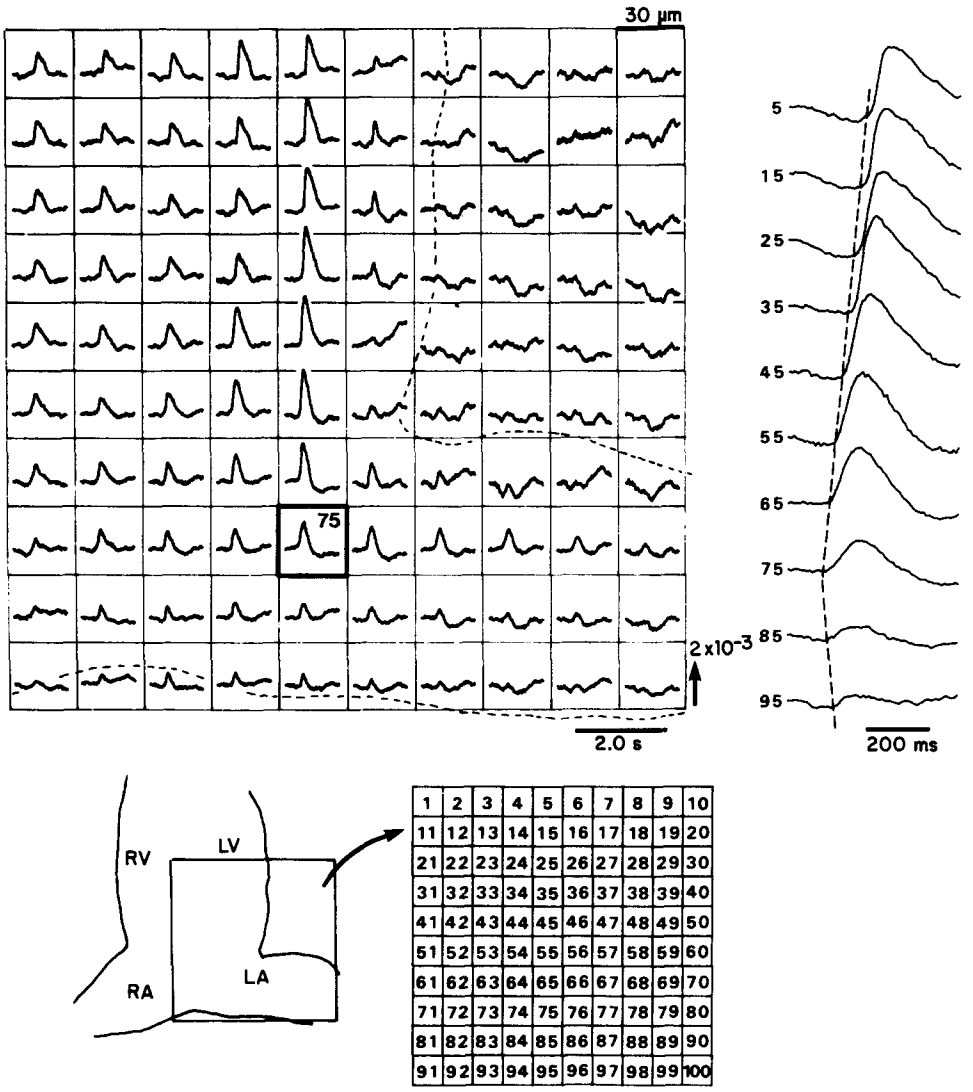


FIGURE 2. Spontaneous action potentials measured simultaneously from 80 adjacent regions of an early nine-somite embryonic chick heart, using a 10- × 10-element photodiode array. The array was positioned on the 50× -magnified image of the part of the left ventricle and atrial tissue, as shown at the bottom. Each photodiode detected the electrical activity from a 28- × 28-μm area of the heart. Note that in this recording, the earliest action signal was detected by one detector (element 75: square with thick outline), as shown on the right. The measurement was made at 37.3–37.5°C. The direction of the arrow to the right of the traces indicates a decrease in transmittance and the length of the arrow represents the stated value of the fractional change in intensity.

making area is not uniquely determined, and as development proceeds to the nine-somite stage, the pacemaking area becomes localized in the left pre-atrial tissue (Kamino et al., 1981). The results obtained in the present study provide more accurate information on the location of the pacemaker.

Assessment of the Pacemaking Area

The second series of experiments was designed to quantitatively evaluate the pacemaking site. Accordingly, the 10- × 10-element or 12- × 12-element photodiode matrix array was positioned on the magnified (× 50) image of the region on which the pacemaker site was located: the location of the pacemaker site was preliminarily examined on a less magnified image of the preparation. In such an experiment, several patterns of the recording were obtained.

Case 1. Fig. 2 shows simultaneous optical recording of spontaneous action potentials obtained from the left area involving the atrial portion of a nine-somite embryonic heart, using a 10- × 10-element photodiode array. It was preliminarily determined that in this preparation the pacemaker site was located at the left atrial region. In this recording, the earliest action potential signal was detected by the element at position 75. We thus assumed that in this heart the pacemaking area was located near this position. In the next step, using this recording, we attempted to assess graphically the size of the pacemaking area, using the following procedure.

In the first step, we measured the delays in the firing time of the signals between position 75 and other positions in which each signal was detected. The observed values are displayed in Fig. 3 *A*, with regard to the corresponding arrangement of elements that detected each signal. In this figure, the crosses indicate the center of each element and the numerals under the crosses represent the delays in milliseconds.

In the second step, the delays were plotted against the distance from the center of position 75. The results are shown in Fig. 3 *B*. In this graphical representation, despite the small scatter in the data, two simple straight lines were obtained for the plots in two areas of the cephalic and caudal sides of position 75.

In the third step, we extrapolated these two straight lines on the distance axis, and as a result, an extrapolated value of ~21 μm was obtained with both the straight lines (indicated by an arrowhead). This graphical representation indi-

FIGURE 3. (*opposite*) (*A*) Numerical representation of delays in the firing time of the spontaneous action potential–related optical signals. The data were obtained from the recording shown in Fig. 2: in the measurement of the delay (in milliseconds), the center of position 75 is regarded as the zero point reference for the timing. (*B*) A plot of the delays against distance (in micrometers) between the center of position 75 (indicated by a square in *A*) and that of other positions. The open circles were obtained from the cephalic area and the open triangles from the caudal area (marked by shading). The extrapolated value of the two straight lines on the distance axis (indicated by an arrowhead) is 21 μm. (*C*) An illustration of location, shape, and relative size of the pacemaking area and the conduction pattern of the excitation. See text for additional details.

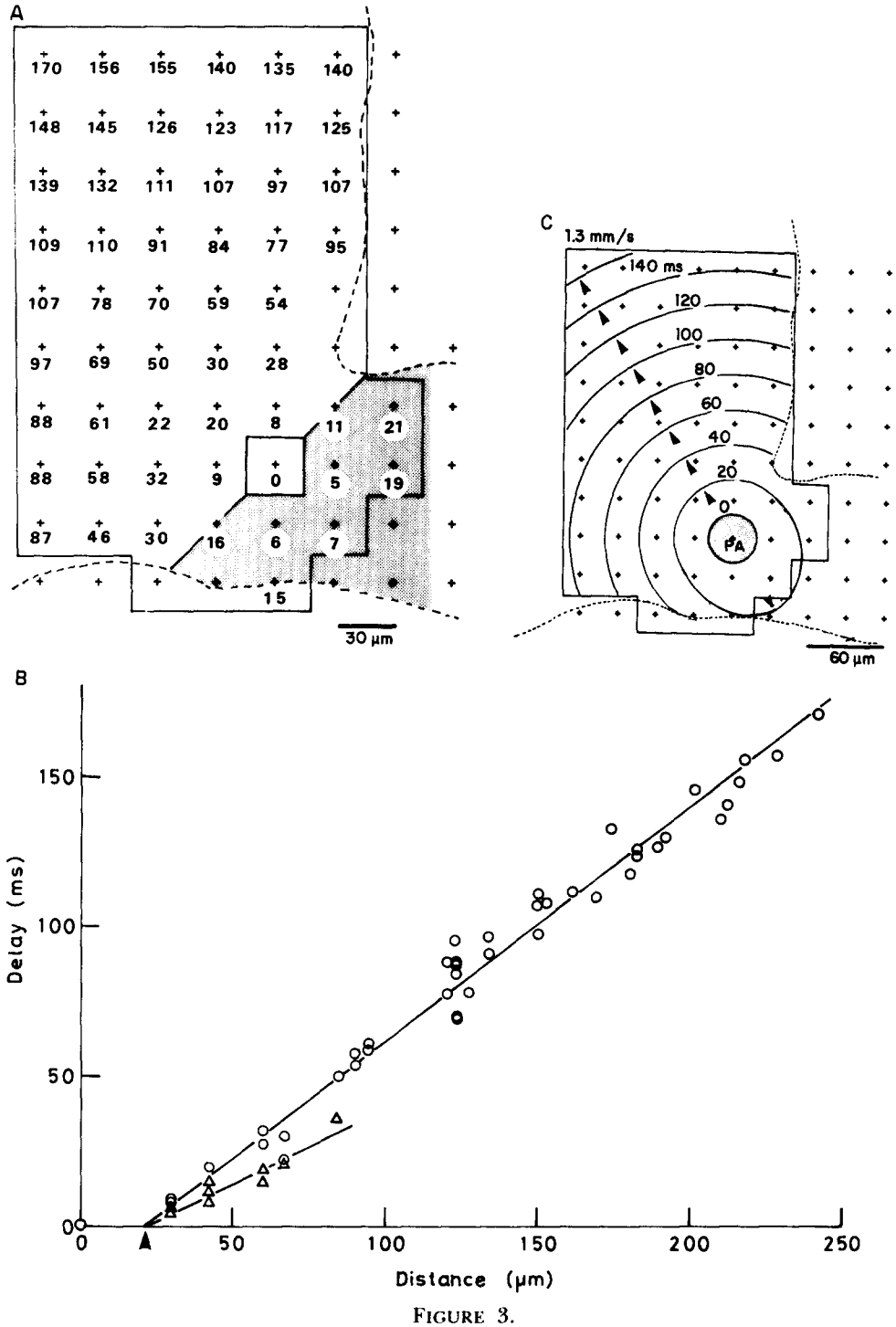

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FIGURE 3.

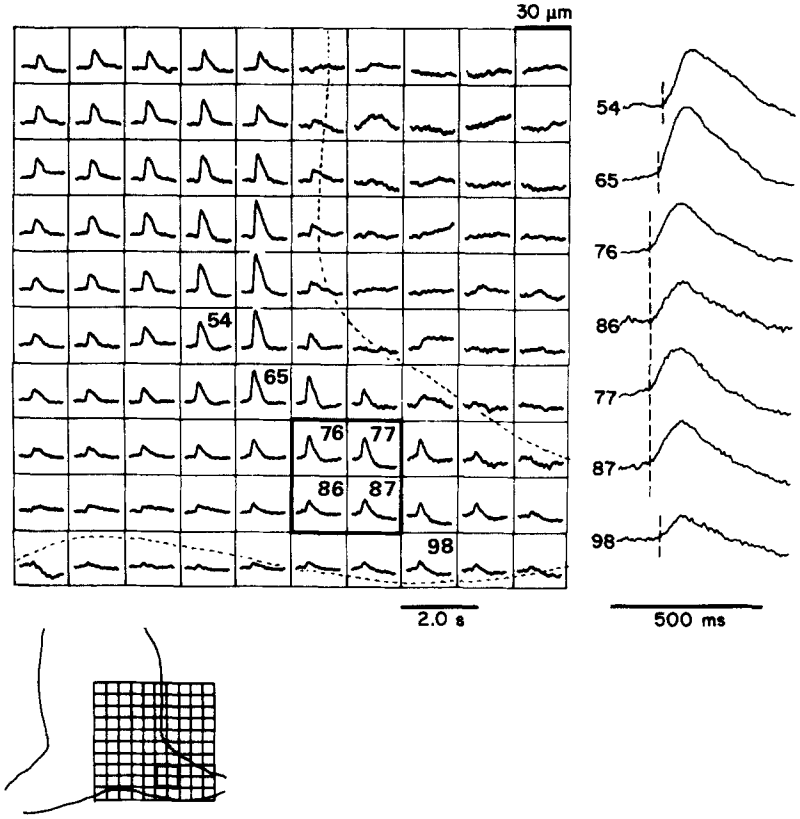

FIGURE 4. An example of multiple-site recording in which four adjacent photodiodes detected the earliest signals. As compared on the right, signals first appeared in positions 76, 77, 86, and 87 in the left atrial tissue. Corresponding areas are outlined with a heavier border in the matrix display. The numbers of the elements correspond to those shown in Fig. 2. The recording was obtained from a nine-somite embryonic heart at 36.4–36.8°C.

cates that in this heart the pacemaking area is circular in shape and that the points where the lines cross the distance axis (x axis) correspond to the radii of the circular pacemaking area. Therefore, the pacemaking area in this heart was calculated to be $\sim$1,500 μm^2. It is likely that the excitation conducts radially over the heart at a uniform rate from the circular pacemaking area, with the pattern illustrated in Fig. 3 *C*. From the slope of the straight lines, the conduction velocity was calculated to be $\sim$1.3 mm/s, in the cephalic direction. Results like these were often seen in later eight- to nine-somite embryonic hearts.

Case 2. The second case is the situation where four elements of the photodiode array detected the earliest signals. An example of such recordings is shown in Fig. 4. This recording was obtained from an early nine-somite embry-

onic heart. Four adjacent photodiode elements detected the earliest signals from positions 76, 77, 86, and 87, in the left atrial tissue.

As we did for case 1, we measured the delays in the firing time of the signals (Fig. 5 *A*) and then plotted the delays against the distance (Fig. 5 *B*). The center of positions 76, 77, 86, and 87 is regarded as the point of origin for the timing of firing of the signals and for the distance. As can be seen in Fig. 5 *B*, a single straight line could be used to represent the relationship between delay and distance. The extrapolated value of this straight line on the distance axis was ~28 μm. It is thus demonstrated that in this heart the pacemaking area has a circular shape and is ~2,500 μm^2 in size and that the excitatory waves spread radially over the heart from the pacemaking area located in the left atrial tissue, at 1.5 mm/s, as illustrated in Fig. 5 *C*. This conduction velocity also was calculated from the slope of the straight line.

Case 3. A third pattern was often obtained in recordings in which the earliest signals were detected by a row of two or three photodiode elements, in the seven- to early eight-somite embryonic hearts. A typical example is shown in Fig. 6, which was obtained from an early eight-somite heart. In this heart, the earliest signals were detected by two elements at positions 55 and 65 in the left atrial tissue. In Fig. 6 *B*, the observed delays in the firing time of the signals are plotted against the distance from the middle point between the center of positions 55 and 65, and this point also is regarded as the zero point reference for the firing time of the signals. Although the scatter in the data is large in this plot, a characteristic feature has been recognized: the delays measured at positions 5, 15, 25, 35, 45, 55, 65, and 75 (along the direction indicated *Y* axis in *A*) were linearly related to the distance, and the values observed on the right and left sides of *Y* axis (in Fig. 6 *A*) either fit to this line or only deviated upward from the straight line.

This graphical feature suggests that in this heart the pacemaking area is of elliptical shape and that the excitation spread elliptically at a uniform rate over the heart from this pacemaking area. The major axis can be estimated to extend ~30 μm along the *Y* direction. However, in such a case, it is very difficult to quantitatively evaluate the length of the minor axis in the present experiment.

In addition to these three cases, we have observed other patterns. Examples of findings often observed are classified into the six patterns shown in Table II. This table also shows the shapes of the pacemaking area evaluated from these patterns, together with the frequency of incidence of each pattern, representing the number among 150 preparations randomly sampled.

Increased Magnifications

In experiments done to evaluate the pacemaking area, the degree of magnification of the image of the preparation was closely related to the accuracy of the result. Therefore, we used larger images formed with a water-immersion surface objective. Fig. 7 is a graphic representation of data obtained in experiments using 50×- and 100×-magnified images of an early nine-somite embryonic

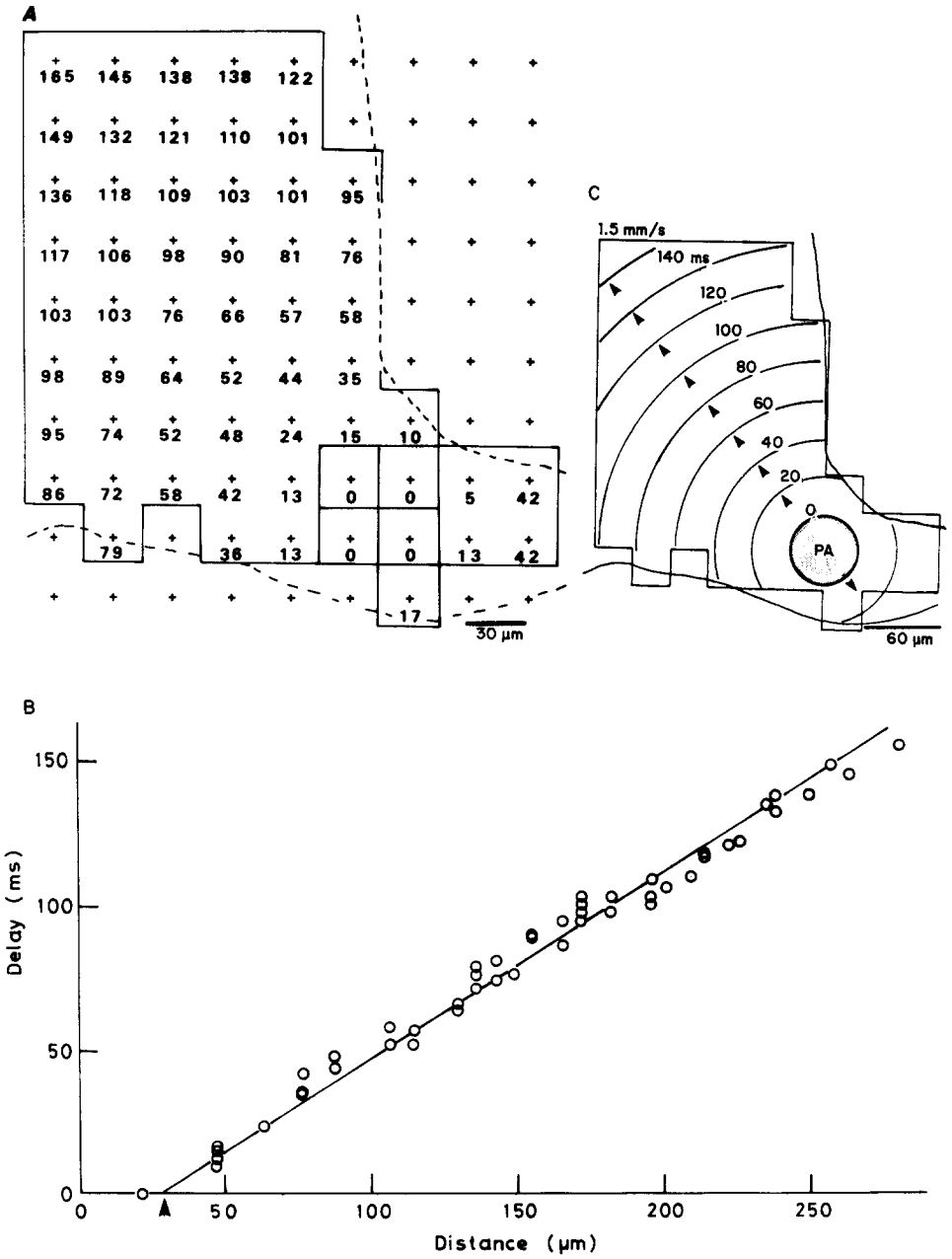

FIGURE 5. (*A*) Numerical representation of delays (in milliseconds) in the firing time of the signal shown in Fig. 4. (*B*) Plot of the delays as a function of distance. The center of positions 76, 77, 86, and 87 is regarded as the zero point reference for the timing. The delay is related to the distance with a straight line, and the extrapolated value of the distance axis is 28 µm (indicated by an arrowhead). See text for additional details.

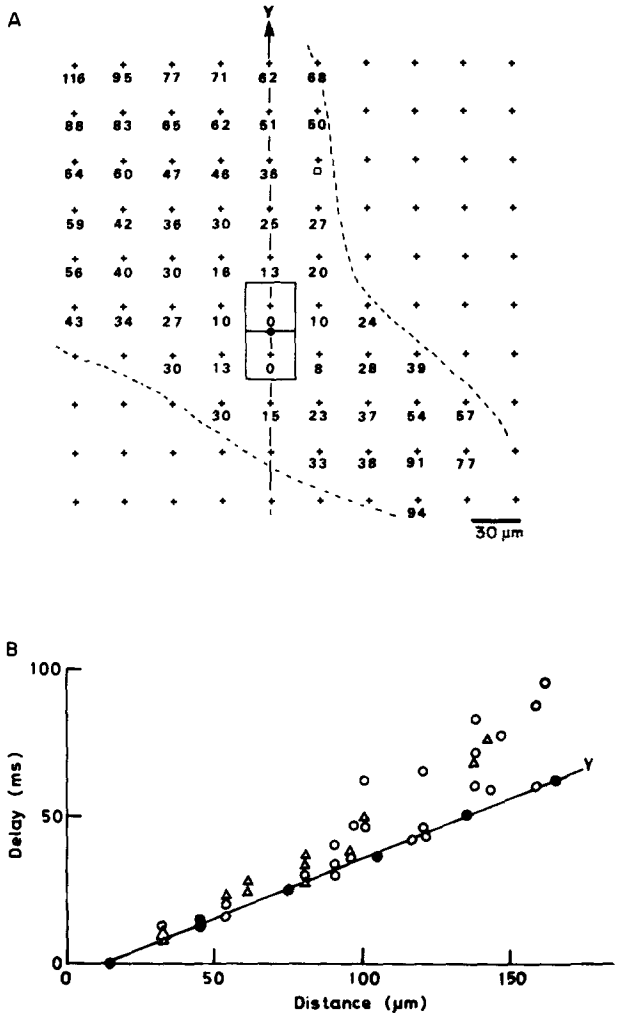

FIGURE 6. An example of the recordings in which two adjacent photodiodes detected the earliest signals. (*A*) Numerical representation of the delays (in milliseconds) in the firing times of action signals. (*B*) Plot of the delays against distance from the center of positions 55 and 65 (surrounded by two squares in *A*). Note that the values measured in the left (triangles) and right (open circles) areas of positions 5, 15, 25, 35, 45, 55, 65, and 75 (along the *Y* direction shown in *A*) mostly shift upward only from the straight line (indicated by *Y*) obtained from the values (represented by filled circles) measured along direction *Y* in *A*. Data were obtained from the recording in an eight-somite embryonic heart at 36.9–37.4°C.

TABLE II

Classification of Arrangements of Photodiode Detecting the Earliest Signals and Their Incidence

	Pattern	Number of preparations: Seven-somite	Eight-somite	Nine-somite	Shape
(I)		2	2	15	Circular
(II)			18	26	Circular
(III)	,	2	8	14	Elliptical
(IV)	,	4	8	9	Elliptical
(V)	,	1			Elliptical
(VI)	,		4	2	Elliptical

Shapes were evaluated from each pattern. The incidence represents the number of the preparations found at each stage of development.

heart. The same results were obtained with two different magnifications. Furthermore, the results obtained from eight preparations are compared in Table III; it can be seen that the two magnifications yielded similar results. In addition, experiments with further enlarged images were carried out; however, because of the small signal-to-noise ratio, it was difficult to obtain reliable data.

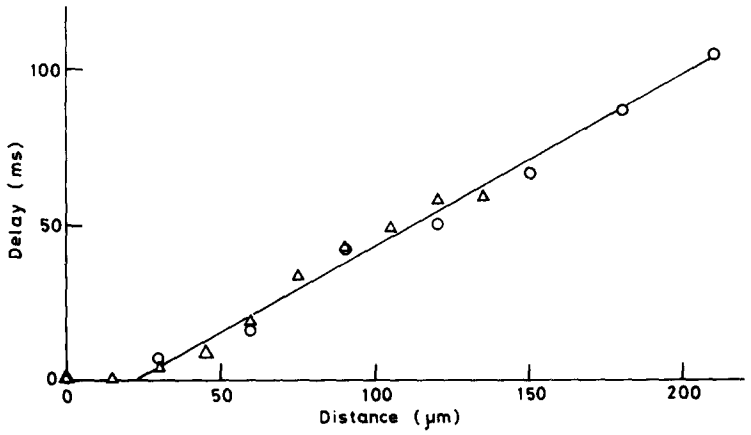

FIGURE 7. Plot of delays in the firing times of the action signals recorded using 50×- and 100×-magnified images of a nine-somite embryonic heart. The photodiode array was positioned over the image of the left area of the heart, involving the atrial tissue in which the pacemaker site was located. The measurements were carried out at 36.8–37.1°C. The circles represent data obtained from a 50×-magnified image, and triangles from a 100×-magnified image.

Variations in the Size

We also compared the sizes of the pacemaking areas among 100 preparations during the eight- to nine-somite stages. Fig. 8 illustrates histograms of the radius of circularly shaped pacemaking areas. These histograms show the variations in the size of the pacemaking area in embryonic precontractile hearts during early development. The mean radius was 25.2 ± 5.3 μm for eight-somite embryonic hearts (35 preparations) and 24.9 ± 5.1 μm for nine-somite hearts (65 preparations). These results suggest that although the size of the heart increased gradually throughout the eight- to nine-somite stages, as shown in our previous report (Hirota et al., 1987), the size of the pacemaking area did not increase.

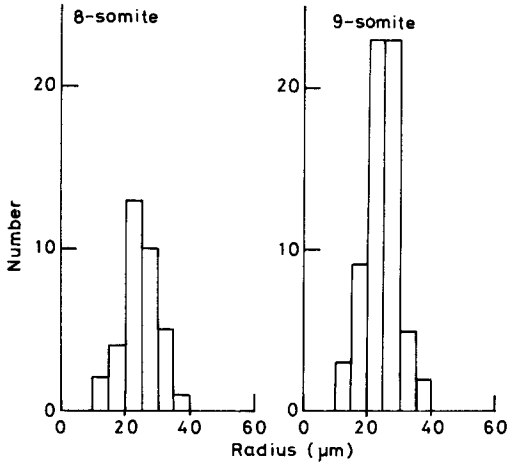

FIGURE 8. Histograms of the radius of the circular pacemaking areas in the eight-somite and nine-somite embryonic precontractile hearts. Data were obtained from 35 eight-somite and 65 nine-somite preparations sampled at random. The ordinate is the number of preparations in a given bin. The bin width is 5.0 μm.

DISCUSSION

By combining simultaneous multiple-site recording of electrical activity with simple graphical analysis, we have revealed features of the pacemaking area and its development in early embryonic chick hearts for the first time.

The first report of simultaneous optical recording of electrical activity from several sites (in an invertebrate central nervous system) employed several single photodiodes glued directly to light guides (Salzberg et al., 1977) and up to 14 sites were monitored simultaneously. Similarly, in our early work (Kamino et al., 1981), we monitored spontaneous action potentials simultaneously from five to eight discrete sites in early embryonic chick hearts and demonstrated that the pacemaking area is located in various regions of the heart at the seven- to early eight-somite stages, and that as development proceeds to the nine-somite stages it becomes localized in the left pre-atrial tissue. These early observations have been extended by the present results obtained using the multiple-element photodiode array. The sequence of these events is in good agreement with our earlier finding that the pacemaking area shifts to the left sinus primordium at the 10–11-somite stage (Kamino et al., 1981).

In experimentally produced "cardia bifida" embryos, DeHaan (1959) demonstrated that from the stage of 10–11 somites to the stage of 18–19 somites, the left heart beats faster than does the right in the great majority of embryos, and he postulated that during the earliest stages, in normal development, the left side should dominate and serve as a pacemaker for the tubular heart. Furthermore, using early embryos having congenital double hearts (Fujii et al., 1983), we found that there was a difference in the rhythmicity between the right and left sides of the heart. In addition, we also found that the heart rate in the left half-heart was faster than that in the right half-heart in the great majority of the eight- to nine-somite embryonic cultured double hearts (Yada et al., 1985). On the basis of this evidence, it is reasonable to postulate that a regional gradient of rhythmicity determines the location of the pacemaker site. We have

TABLE III

Comparison of Size of the Pacemaking Area Evaluated from Recordings with 50×- and 100×-magnified Images of the Hearts

Heart reference	Stage (somites)*	Radius	
		× 50	× 100
		μm	μm
27123–25	9-E	23	20
27057–19	9-M	23	18
27069–22	9-E	27	26
27082–14	8-L	25	24
27032–24	9-M	23	22
27006–18	9-M	23	22
27022–24	8-L	25	22
27080–12	9-M	27	25

* E, M, and L indicate the early, middle, and later periods of each stage.

previously discussed a possible mechanism for the localization of the pacemaker site (Sakai et al., 1983).

In the present study, new advances include the quantitative evaluation of the shape and size of the pacemaking area in early embryonic hearts. In the experiments with the 50× real image of the heart, we observed that the delays in the firing times of the action potential signals are often related to the distance from the area(s) in which the signals first appear by a single straight line, and that this straight line has a positive extrapolated value on the distance axis. This initially suggested that the shape and size of the pacemaking area could be graphically assessed.

Shape

Of particular interest is the finding that the shape of the pacemaking area in the early embryonic heart is basically circular: in the seven- to early eight-somite embryonic hearts, the pacemaking area is sometimes elliptical, and as development proceeds to the nine-somite stage, the pacemaking area is organized into

the circular shape. From this evidence, it is reasonable to postulate that the pacemaker originally forms a circularly shaped sheet composed of the pacemaking cells. A circular shape for the pacemaking area may be related to the optimality principle in biological systems (Rosen, 1967).

Size

In the case shown in Fig. 3, it is reasonable to consider that the extrapolated value on the distance axis of the single straight line corresponds to the radius of the circularly shaped pacemaking area. Indeed, although the graphical method employed was somewhat crude, the result does give the first quantitative evaluation of the size of the cardiac pacemaker. Through the seven- to nine-somite stages, the electrically active area of the heart increased dramatically (Hirota et al., 1987). However, as shown in Fig. 8, although the position is translocated to the left atrial tissue, the size of the pacemaking area is maintained at relatively constant size, 1,200–3,000 μm^2. Here, considering that in the seven- to nine-somite embryonic chick hearts the epimyocardial cells nearly form a monolayer and the diameter of the cells is $\sim$5 μm (Manasek, 1968; Ojeda and Hurle, 1981; Hiruma and Hirakow, 1985), the pacemaker in early embryonic chick hearts is probably composed of $\sim$60–150 cells. It is thus strongly implied that not a single cell but a group of cells having pacemaking ability serves as a rhythm generator in the heart and that these cells form a strong functional syncytium. We are now attempting to assess the population of active cells in the pacemaking area, using a higher magnification of the image of the preparation.

Accuracy

The results obtained in the present experiment should be taken only as a first approximation; it should be possible to improve the accuracy of the data and reduce the experimental errors. Although we have not analyzed them mathematically, the errors were empirically evaluated to be $\sim$10% at most. These result mainly from (*a*) lack of precision in measuring the delay, (*b*) spatial resolution of the optical recordings, and (*c*) the extent of deviation of the center of the empirically presumed pacemaking area from that of the true pacemaker site. Factor *a* may be further improved with simple statistical procedures. Concerning factor *b*, the spatial resolution of optical recording from the two-dimensional preparation depends upon the microscope resolution, together with the diffusion of the signals introduced by light-scattering. As can be seen in Fig. 7 and Table III, there was good agreement in the results obtained from the experiments using 50× and 100× magnifications. Thus, we think factor *b* is not a serious source of error. We have tried to use more magnification by using a water-immersion 40×, 0.65-NA surface objective together with 6.7× photographic eyepiece. In this situation, the signal-to-noise ratio was too small, and the precision in reading the delay of the signals was consequently poor. To overcome this limitation, a brighter illumination lamp is being designed. It thus seems that factor *c* may be the most important in the present experiment. In addition, in the optical method, the resolution along the axis perpendicular to the object plane (Salzberg et al., 1977) and light-scattering (Orbach and Cohen,

1983) should be considered. Nevertheless, as stated above, because the myocardium cells are organized in a monolayer in the seven- to nine-somite stages of development, these problems are not serious in the present experiment.

We believe that our first findings obtained using the optical method provide new insight into the functional organization of the early developing embryonic heart, and we feel that our optical method could be a powerful tool for further new approaches in developmental cardiology.

We are grateful to Drs. L. B. Cohen, R. L. DeHaan, and B. M. Salzberg for reading the manuscript and for their useful comments. We are also indebted to M. Ohara for her kind advice concerning the manuscript.

This work was supported by grants from the Ministry of Education, Science and Culture of Japan and the National Art and Science Asociation.

Original version received 20 July 1987 and accepted version received 21 September 1987.

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