Effects of Calcium on Electrical Propagation in Early Embryonic Precontractile Heart as Revealed by Multiple-Site Optical Recording of Action Potentials

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ABSTRACT The effects of Ca^{2+} on electrical propagation in early embryonic precontractile chick hearts were studied optically using a voltage-sensitive merocyanine-rhodanine dye. Spontaneous optical signals, corresponding to action potentials, were recorded simultaneously from 25 separate regions of the eight-to-nine–somite embryonic primitive heart, using a square photodiode array. Electrical propagation was assessed by analyzing the timing of the signals obtained from different regions. Electrical propagation in the heart was suppressed by either lowering or raising extracellular Ca^{2+}. Similar effects were produced by a Ca^{2+} ionophore (A23187). We have also found that electrical propagation across the primordial fusion line at the midline of the heart was enhanced by increasing, and depressed by lowering, external Ca^{2+}. One possible interpretation is that intercellular communication in the embryonic precontractile heart is regulated by the level of the intracellular Ca^{2+} concentration, and it is suggested that intercellular communication across the primordial fusion line strongly depends on external Ca^{2+}.

INTRODUCTION

Knowledge of the properties of electrical intercellular communication in the early phases of cardiogenesis is important in understanding the mechanism(s) of the functional organization of the heart. Generally, cell-to-cell coupling is measured by impaling each cell with two microelectrodes (for a review, see Loewenstein, 1979). However, in early embryonic hearts, the small size (3–5 μm in diameter) of these cells has limited the utility of the conventional intracellular

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microelectrode technique. As a result of these technical difficulties, investigations on electrical coupling in embryonic hearts during the very early stages of development have been severely hampered.

Optical methods for monitoring membrane potential have been developed and offer a powerful tool for recording electrical activity from otherwise inaccessible cells (Salzberg et al., 1977, 1983; Grinvald et al., 1981, 1982; Senseman et al., 1983; Orbach and Cohen, 1983; Sakai et al., 1983*b*; for a review, see Cohen and Salzberg, 1978). These methods permitted us to monitor, for the first time, the regional distribution of electrical activity in the early embryonic chick heart (Hirota et al., 1979; Fujii et al., 1980, 1981*a–c*; Sakai et al., 1983*b*). In addition, simultaneous optical recording from several different areas allowed us to demonstrate the conduction patterns of excitation in early embryonic hearts (Kamino et al., 1981; Sakai et al., 1983*b*; Hirota et al., 1983*a*).

In the present work, we investigated the effects of Ca^{2+} on electrical propagation in the early embryonic precontractile chick heart, using a voltage-sensitive potentiometric probe together with a multiple-site optical recording system to monitor spontaneous action potentials simultaneously in 25 different heart regions. Our findings include information about the nature of the intercellular electrical coupling within the primordial fusion line of the embryonic hearts. A preliminary report of this work has been published (Hirota et al., 1983*b*).

METHODS

Preparations

Fertilized chicken eggs (white Leghorn), weighing ~60 g, were incubated in a forced-draft incubator (model P-03, Showa Incubator Laboratories, Urawa, Japan) at 37°C and 60% humidity, and were turned once each hour. The embryos were removed at developmental stages between 25 and 35 h. For optical measurements in these experiments, the seven-to-nine–somite embryos were used. The isolated embryos were kept in the normal bathing solution. Most of the egg yolk and vitelline membrane attached to the embryo were removed in the bathing solution, under a dissecting microscope.

Staining

After the splanchnopleure was carefully peeled off, the isolated embryos were incubated for 15 min in a bathing solution containing 0.1 mg/ml of a merocyanine-rhodanine dye. After the incubation period, the dye solution was washed out. The dye was purchased from Nippon Kankoh Shikiso Kenkyusho (Okayama, Japan) as NK2761.

Bathing Solution

The solution used to bathe the embryos was as follows: 138 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 10 mM Tris-HCl buffer (pH 7.2). The solution was equilibrated with air and allowed to warm to 37°C.

Optical Recording Methods

Most of the methods were essentially as described previously (Fujii et al., 1980, 1981*a*, *b*). The preparation chamber was mounted on the stage of an Olympus Vanox microscope (model AHB-LB-1, Olympus Optical Co., Ltd., Tokyo, Japan). Bright field illumination was provided by a 300-W halogen-tungsten lamp (JC24V, Kondo Sylvania Ltd., Tokyo,

Japan) driven by a stable DC power supply (model PAD 35-20L, 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-nm (±11 nm full width at half-maximum) interference filter (model 1F-S, Vacuum Optics Co. of Japan, Tokyo, Japan), and focused onto the preparation by means of an aplanatic/achromatic condenser. A long-working-distance objective collected light transmitted by the preparation and formed a real image on a photodiode array located in the real-image plane. The magnification was usually 50 in the present work.

A 5- × 5-element silicon photodiode matrix array (model MD-25-0, Integrated Photo-Matrix Ltd., Centronix, Mountainside, NJ) was used. The array was positioned on the image of the area of interest in the preparation.

The outputs from the 25 elements of the photodiode array were fed to individual amplifiers via individual current-to-voltage converters. The amplified outputs were first recorded simultaneously on a 16-channel data recording system (RP-890 series, NF Electronic Instruments, Yokohama, Japan). The signals were then displayed on two dual-beam storage oscilloscopes (model 5113, Tektronix, Inc., Beaverton, OR) with two 5A18N amplifiers (Tektronix, Inc.) for data analysis. In most experiments, the oscilloscopes were set to give a coupling time constant of 1.5 s and the outputs were finally filtered by a simple RC low-pass filter (time constant ~10 ms).

RESULTS

Conduction Velocity

Using simultaneous optical recording of spontaneous action potentials from multiple sites, we assessed the conduction velocity of spontaneous excitatory waves in early embryonic precontractile chick hearts. Fig. 1*A* demonstrates an example of simultaneous recording of optical signals due to spontaneous action potentials from 16 different areas of a nine-somite precontractile embryonic heart in a normal bathing solution. The embryonic heart was stained with a merocyanine-rhodanine dye (NK2761), and the optical signals were recorded from 16 elements of a 5- × 5-element photodiode array. In such a recording, there are generally short delays between the feet of the signals at a higher sweep speed, and these delays reflect the conduction time of excitation.

EFFECTS OF LOW Ca^{2+} In a previous report (Sakai et al., 1983*a*), we demonstrated that both the size and frequency of spontaneous action potentials in seven-to-nine–somite embryonic precontractile chick hearts depended upon external Ca ions, and that the action potentials were reduced by lowering the Ca^{2+} concentration in the bathing solution. Also, the action potentials were reduced by Mn^{2+}, Co^{2+}, or Ni^{2+}. For these reasons, it was relatively difficult to carry out the experiments under conditions of low Ca^{2+} concentrations. Therefore, in order to analyze the effects of lowering the external Ca^{2+} concentration, the experiments were carried out in a solution in which the amount of Ca^{2+} was decreased only slightly.

Fig. 1*B* shows action potentials recorded optically in a bathing solution containing 1.44 mM Ca^{2+} (four-fifths of the normal concentration), with Mg^{2+} replacing Ca^{2+}. The signal size decreased, and the delays between the signals were prolonged.

In Fig. 2, the delays observed on the left side of the heart in the normal and low Ca^{2+}-containing solutions are plotted against the distance from a reference position, which corresponds to the pacemaking area in which the spontaneous action potential first occurred, for an eight-somite and a nine-somite embryonic precontractile heart. In the seven-to-nine–somite embryonic precontractile chick hearts, a linear relationship was obtained between the delay and distance, and

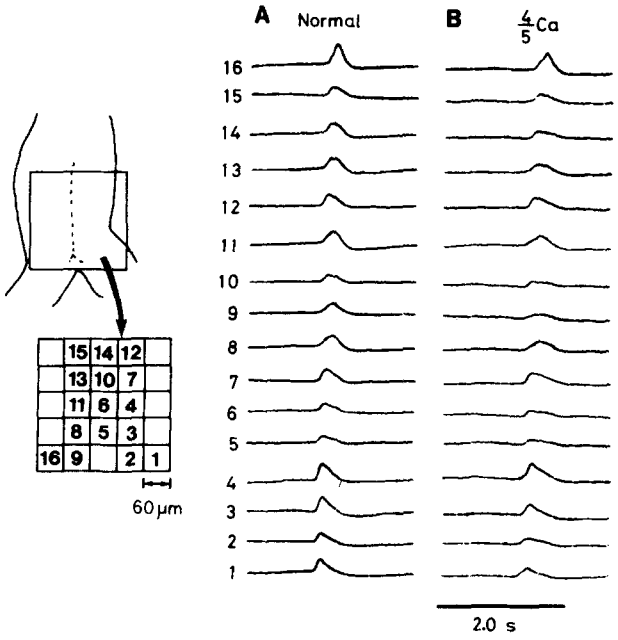

FIGURE 1. Simultaneous optical recording of action potentials from 16 separate regions of a nine-somite embryonic precontractile heart stained with a merocyanine-rhodanine dye (NK2761), in a normal bathing solution and in a solution containing 1.44 mM Ca^{2+} (four-fifths of the normal concentration), using 16 elements of a 5 × 5 photodiode array. A square superimposed on the drawing (ventral view) of the heart indicates the portion of the preparation imaged onto the detector. The field of optical recording by one element is estimated to be 0.0036 mm^2 on the heart. Each trace shows the change in transmission detected with a 700 ± 11-nm interference filter, and all traces were recorded in a single sweep at 37.1–37.6°C. Note that the signal sizes in the low Ca^{2+} solution are somewhat smaller and the delay in the low Ca^{2+} solution is slightly larger than that in the normal solution. The low Ca^{2+} solution was made by replacement of $CaCl_2$ (0.36 mM) with $MgCl_2$ (0.36 mM).

the reciprocal of the slope of the graph corresponds to the conduction velocity of the excitatory wave. From the linear relationships obtained in Fig. 2, we conclude that the spontaneous excitation spread radially at a uniform rate over one side of the eight-to-nine–somite embryonic heart, in both the normal and low Ca^{2+} bathing solutions, and that the radial conduction velocity was reduced by reducing the external Ca^{2+} concentration. Similar results were observed with

bathing solutions containing EGTA (1.0–1.5 mM). Furthermore, the conduction velocity was also slowed in the presence of Ca^{2+} blockers such as Mn^{2+}, Co^{2+}, and D600 (data not shown).

EFFECTS OF HIGH Ca^{2+} Measurements similar to those shown in Fig. 1 were also carried out under conditions of elevated external Ca^{2+}. The signals demonstrated in Fig. 3*B* were recorded simultaneously from eight different areas of a

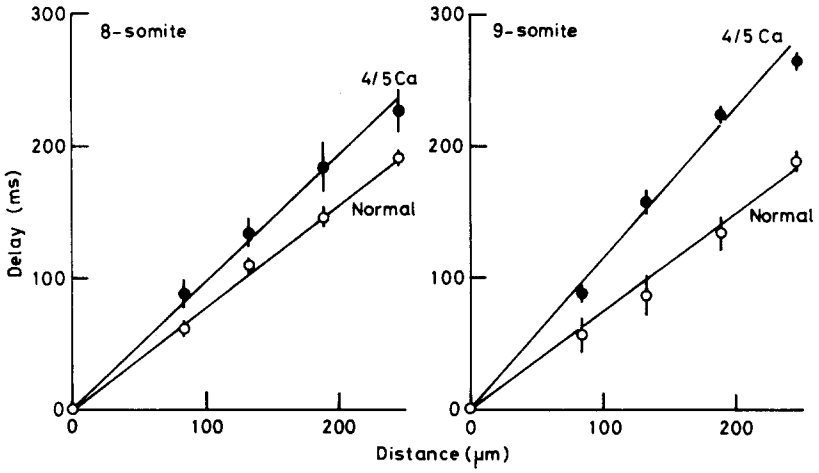

FIGURE 2. Progressive delay of the firing times of optical action signals as a function of the distance from the pacemaking area in an eight-somite and a nine-somite embryonic chick heart in normal or 1.44 mM Ca^{2+} (four-fifths of normal) solution. Optical signals were recorded simultaneously from separate areas on the left side of the hearts, corresponding to elements 1, 3, 4, 7, and 12, shown in Fig. 1, at 37.4–37.8°C (for eight somites) or 36.4–37.2°C (for nine somites). The delay between the foot of the action potential in the pacemaking area and the foot of the action potential in other areas is plotted on the ordinate; the abscissa represents the distance from the pacemaking area. The distances between the centers of the areas imaged onto each element and the pacemaking region were measured. The data obtained from five groups of the conducted signals were averaged: points give mean values ± SD. The figure shows that in early embryonic precontractile hearts, the excitatory waves propagate at a uniform rate in both normal and reduced Ca^{2+} solutions, and the conduction velocities were estimated to be 1.3 mm/s (in the normal solution) and 1.06 mm/s (in the four-fifths Ca^{2+} solution) for the eight-somite embryonic heart, and 1.36 mm/s (in the normal solution) and 0.88 mm/s (in the four-fifths Ca^{2+} solution) for the nine-somite embryonic heart.

stained nine-somite embryonic precontractile heart, in a solution containing five times the normal concentration of Ca^{2+}. The basic spatial pattern of the spread of spontaneous action potentials was present in high Ca^{2+} concentrations, but, as shown in Fig. 4, the conduction velocity decreased in a manner similar to that found in low Ca^{2+} concentrations. This effect may be related to an elevation of the intracellular Ca^{2+} concentration induced by an increase in Ca^{2+} permeation into the cells and in Ca^{2+} influx during the action potential.

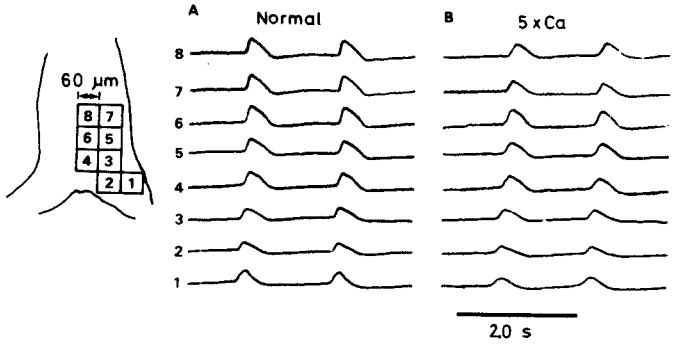

FIGURE 3. Simultaneous recording of optical action signals from eight separate regions of a nine-somite embryonic heart, in normal (*A*) and in 5× normal Ca^{2+} concentrations (*B*). The signals were recorded from eight elements positioned over the image of the left area of the heart, at 36.7–37.7°C. Other experimental conditions were as shown in Fig. 1. The propagation delays in the high Ca^{2+} concentration are larger than that in the normal solution.

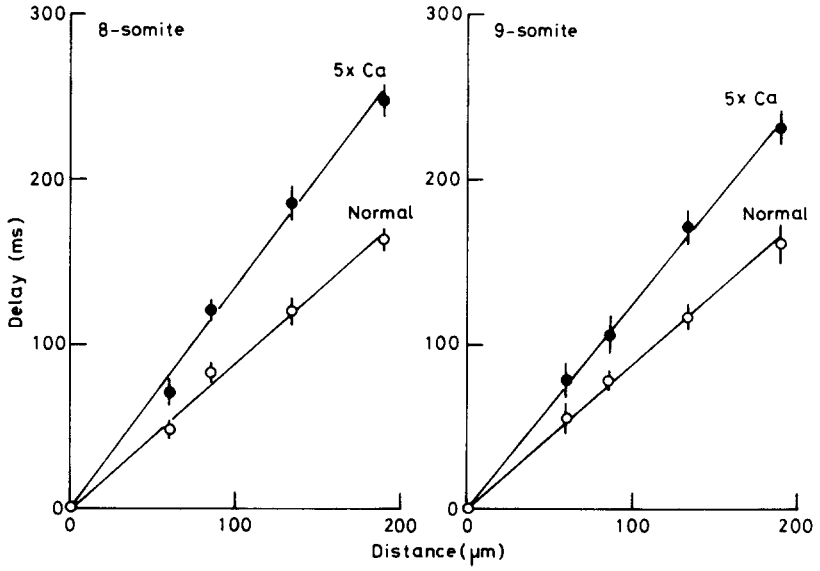

FIGURE 4. Graphic illustration of the effect of elevated (5×) Ca^{2+} concentration on the conduction of electrical excitation in an eight-somite or a nine-somite embryonic heart. Data were obtained from recordings of the signals in the areas corresponding to elements 1–3, 5, and 7, as shown in Fig. 3. The data obtained from five groups of the conducted signals were averaged: points give mean values ± SD. In these cases, conduction velocities were estimated to be 1.17 mm/s (in the normal solution) and 0.75 mm/s (in the high Ca^{2+} solution) for the eight-somite embryonic heart, and 1.18 mm/s (in the normal solution) and 0.80 mm/s (in the high Ca^{2+} solution) for the nine-somite embryonic heart.

Ca^{2+} IONOPHORE It is known that the Ca^{2+} ionophore A23187 increases the permeation of Ca^{2+} across the cell membrane. Therefore, to examine whether an increase in intracellular Ca^{2+} would lead to a decrease in the conduction velocity of the excitation, we investigated the effects of A23187.

The results of a typical experiment are shown in Fig. 5. In a bathing solution containing 5×10^{-6} M A23187 and normal Ca^{2+}, the conduction velocity of the

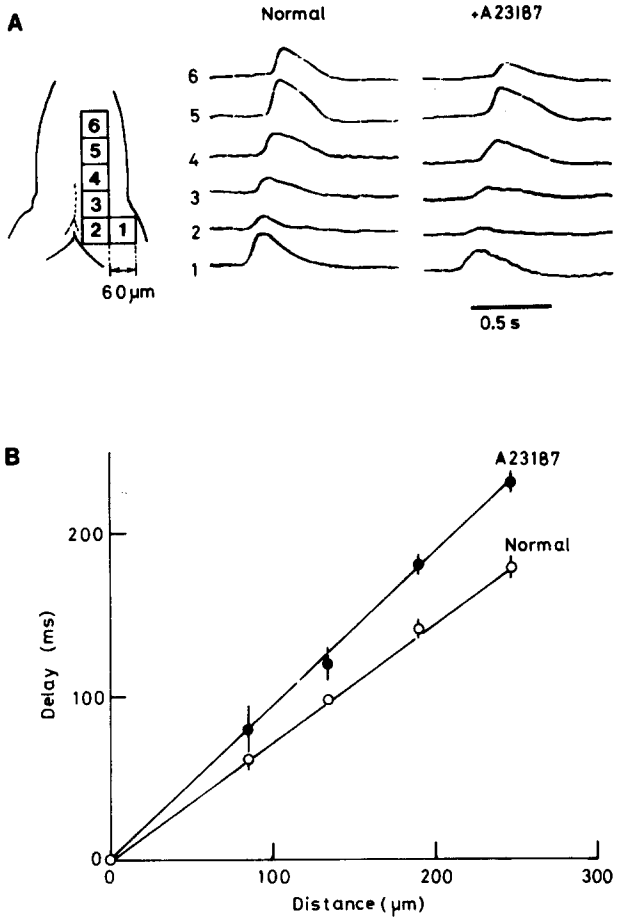

FIGURE 5. Effect of a Ca^{2+} ionophore (A23187) on the spread of electrical excitation in an eight-somite embryonic heart. (*A*) Simultaneous recording of optical action signals from six separate areas on the left side of the heart corresponding to elements 1–6, in normal bathing solution and in the presence of 5.0 μM A23187 at 37.1–37.7°C. (*B*) Graphic representation of the optical recording shown in *A*. The delay between the firing time (foot) of the action signal recorded from area 1 (corresponding to element 1) and that from areas 3–6 (corresponding to elements 3–6) was measured. Other conditions were as shown in Fig. 2. The conduction velocities were estimated to be 1.4 mm/s in the normal solution and 1.08 mm/s in the presence of A23187.

action potential was decreased. This effect was very similar to that observed in the case of elevated external Ca^{2+} concentrations. This result strongly supports the idea that an increase in the intracellular Ca^{2+} concentration reduces the conduction velocity of the excitation of early embryonic precontractile hearts (see Discussion).

Sr^{2+} Since Sr^{2+} is also an effective ion for intracellular communication in the salivary gland (Oliveira-Castro and Loewenstein, 1971) and adult heart (De Mello, 1975), we examined the effects of this ion. In a bathing solution containing $SrCl_2$ instead of $CaCl_2$, the conduction velocity of the excitation was not significantly altered. As in the Ca^{2+} experiments, the conduction velocity decreased when Sr^{2+} was partly replaced by Mg^{2+}. A decrease in velocity was also seen with an elevated extracellular Sr^{2+} concentration.

Effects on the Primordial Fusion Line

During the seven-to-nine–somite stage of development of the chick embryo, the right and left cardiac primordia approach each other and fuse in the midline. This process results in the formation of the primitive tubular heart (Patten, 1971). Earlier, we reported that in the later period of eight somites to the beginning of the nine-somite stage, the radially spreading excitatory waves slowed considerably within the primordial fusion line at the midline of the heart. This delay disappeared at the later period of the 9-somite stage to the 10-somite stage (Hirota et al., 1983*a*).

In Fig. 6 (top), we have plotted the delay in the optical action signals recorded simultaneously from 15 different adjacent areas in an eight-somite embryonic heart against the distance from a pacemaking area. In this figure, the delays in the signals recorded from positions 1–8 on the left area of the heart were linearly related to the distance from position 2, an area that corresponds to the pacemaking area. However, the delays measured at positions 9–15 on the right side of the heart deviated from this linear relationship. This result clearly indicates that the uniform radial propagation of the excitatory waves is slowed within the primordial fusion line (indicated by "fl") at the midline in the heart, and suggests that functional cell-to-cell communication is relatively poor within the primordial fusion line (Hirota et al., 1983*a*).

On the other hand, in the presence of a fivefold increase in Ca^{2+} in the bathing solution, the values measured on the right side (positions 9–15) of the heart approximated the linear relationship obtained on the left side (positions 1–8), as shown in Fig. 6 (bottom). It is possible that the degree of electrical coupling among the cells within the fusion line is enhanced when the external concentration of Ca^{2+} is increased.

A similar effect is demonstrated in Fig. 7. This experiment was carried out using an embryonic precontractile heart at the early period of the nine-somite developmental stage. In this heart, a small conduction delay was also observed along the fusion line, and when the external Ca^{2+} concentration was reduced to 1.44 mM (four-fifths of the normal concentration), the delays measured optically in areas 7–11 on the right side of the heart deviated further from a linear relationship than did the equivalent measurement on the left side. This result

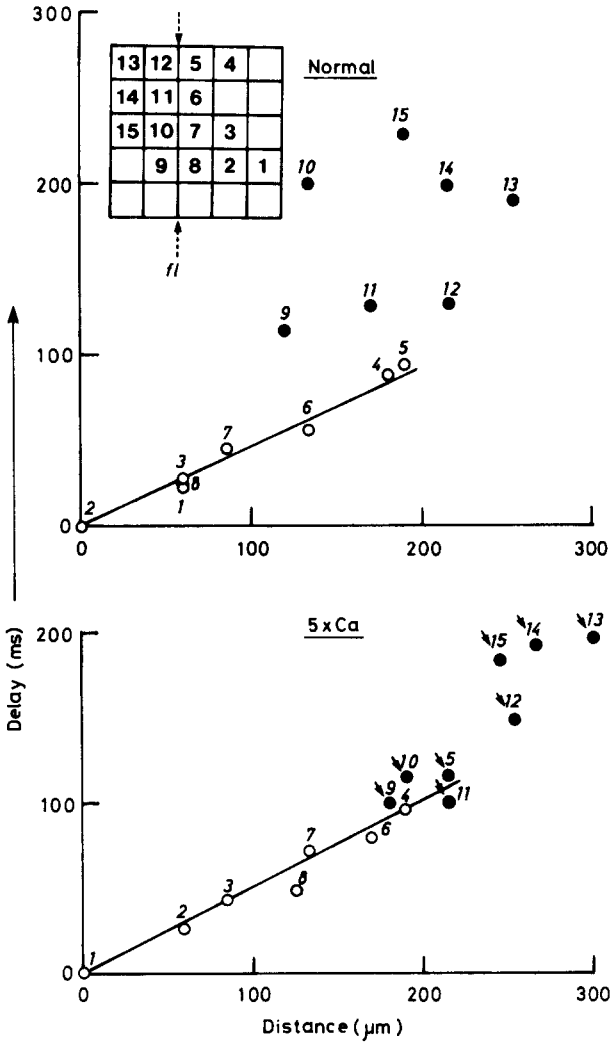

FIGURE 6. A typical example of the effect of the high external Ca^{2+} on the conduction of excitation within the primordial fusion line. The delay in the initiation of the action potential is plotted against the distance from the pacemaking area. The delay was measured in simultaneous recordings from 15 separate areas of an eight-somite embryonic heart, corresponding to elements 1–15 shown in the upper left corner: elements 1–8 were positioned over the image of the left side of the heart and elements 9–15 were positioned over the image of the right side of the heart. In normal bathing solution, area 2 corresponds to the pacemaking area in which spontaneous action potentials occurred first; in the high (5×) Ca^{2+}, the pacemaking area shifted to area 1. Note that in normal solution, the values obtained from areas 9–15 (filled circles) deviate greatly from the linear relationship obtained from the values on the left side of the heart, and this deviation of the values obtained on the right side of the heart became smaller, as indicated by the arrows, in the solution containing a high Ca^{2+} concentration.

suggests that the formation of intercellular communication within the fusion line was particularly sensitive to a reduction in the extracellular Ca^{2+} concentration.

Correction of Blocking on the Fusion Line

In some hearts, conduction block was observed within the primordial fusion line. A typical example is shown in Fig. 8. Preparations of this type were observed occasionally in the seven-to-eight–somite embryonic hearts.

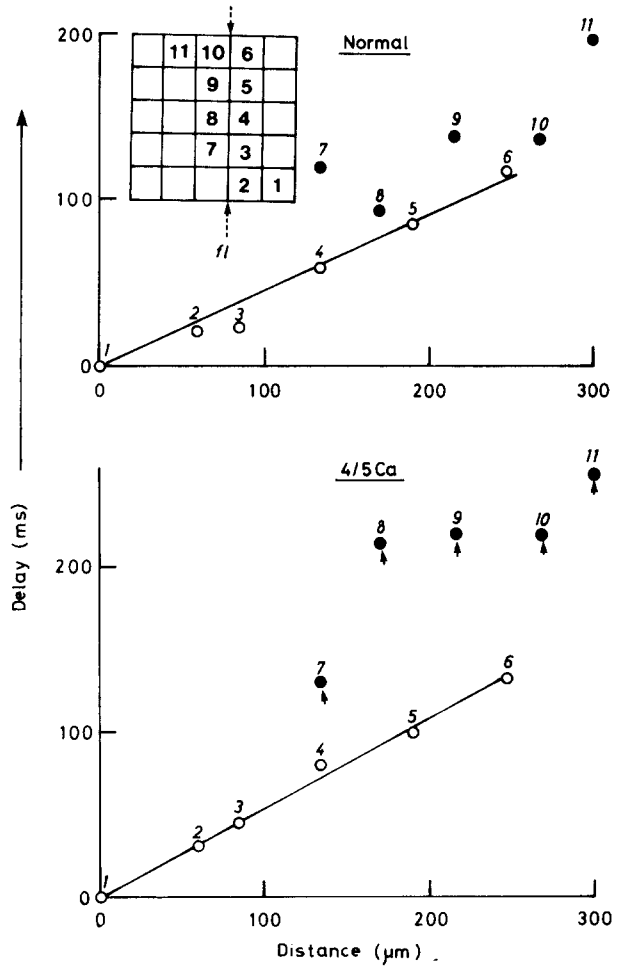

FIGURE 7. A typical example of the effect of reduced external Ca^{2+} on the conduction of excitation within the primordial fusion line. Data were obtained from a nine-somite embryonic heart, at 36.9–37.3°C. Elements 1–6 were positioned over the left side of the heart; elements 7–11 were positioned over the right side of the heart. Note that the deviation of the values obtained on the right side of the heart from the linear relationship obtained on the left side increased in the low Ca^{2+} concentration, as indicated by the arrows.

In the experiment shown in Fig. 8, elements 6, 7, and 8 of the photodiode array were positioned over the image of the primordial fusion line of an eight-somite embryonic heart. In this heart, two different kinds of groups of excitatory waves were clearly identified: groups I, II, and III were detected on the left side of the heart; groups IV and V were detected on the right side; elements 6, 7, and 8 detected both of the groups. On the left side of the heart, the optical

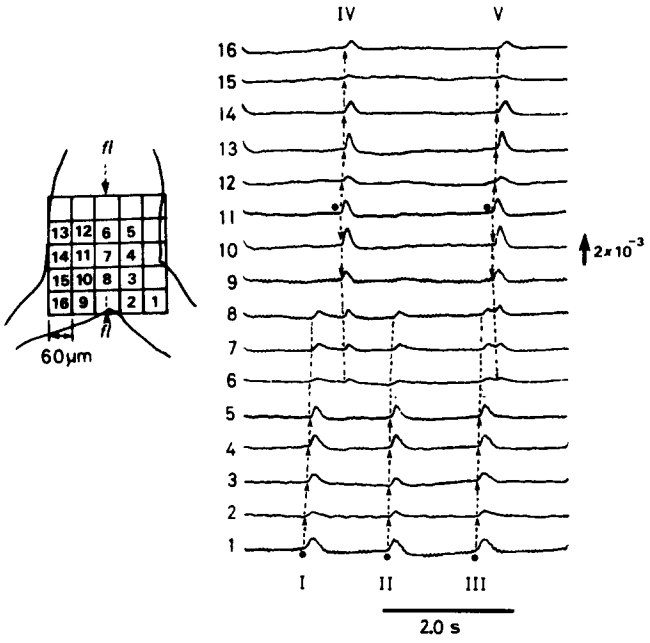

FIGURE 8. Illustration of an eight-somite embryonic heart in which conduction is blocked at the primordial fusion line. Optical action signals were recorded simultaneously from 16 separate areas, in the normal bathing solution at 36.7–37.1°C. The inset on the left side of the recordings illustrates the location of the image of the heart on the array elements. Elements 6, 7, and 8 were positioned over the fusion line (indicated by "fl") of the heart. Groups I, II, and III of the excitatory waves spread from area 1 (corresponding to element 1), and groups IV and V of the excitatory waves spread from area 11. Elements 6, 7, and 8 detect the excitatory waves from both the right and left sides.

action signal first occurred in area 1, which corresponds to element 1, and on the right side, the signal first occurred in area 11, which corresponds to element 11. This result indicates that two different pacemaking areas were situated independently on the right and left sides of this heart, and that conduction of the excitatory waves from each pacemaking area is blocked within the primordial fusion line on the midline of the heart.

In order to examine in greater detail the conduction pattern of excitation on both the right and left sides of the heart, the delay in the optical signals in group I and group IV shown in Fig. 8 is plotted against the distance from area 1 (for

group I) or area 11 (for the group IV) in Fig. 9. The delays obtained from areas 1–6 on the left side are linearly related to the distance. Similarly, in areas 9–16 on the right, an approximately linear relationship was obtained between the delays and the distance. The center of area 1 or of area 11 was the zero-point reference for the timing of the propagation of excitation on each side of the heart, and the distances from the center of area 1 or of area 11 were plotted. In this experiment, the centers of elements 6, 7, and 8 were situated just on the

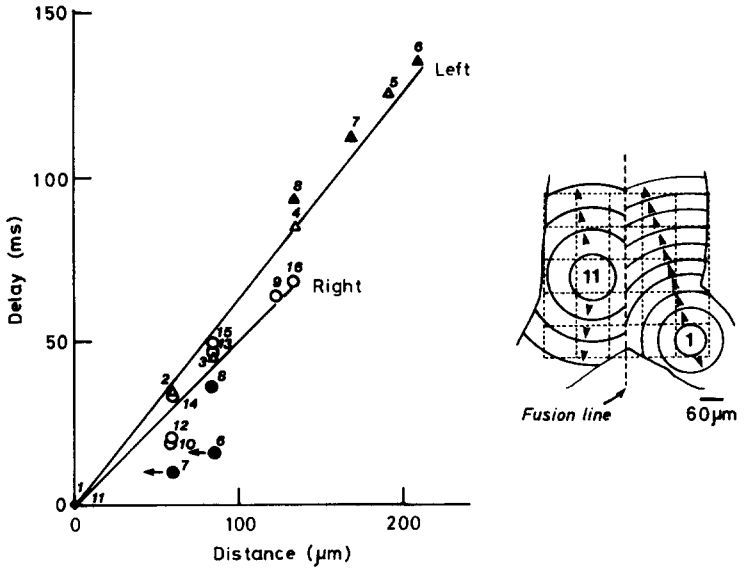

FIGURE 9. Graphic representation of groups I and IV of the excitatory waves in the optical recording shown in Fig. 8. Delay in occurrence of action signals plotted against distance from the pacemaking area: area 1 for group I (triangles) and area 11 for group IV (circles). Area 1 corresponds to the left pacemaking area, and area 11 corresponds to the right pacemaking area. The conduction pattern assessed from this graphical analysis is also shown on the right of the graph. The spacing of the contour lines displays the conduction velocity, with one-contour intervals representing approximate values of the propagation distance per 20-ms time interval. See text for additional details.

primordial fusion line. Therefore, in Fig. 9, the points obtained from areas 6, 7, and 8 should shift slightly to the left (for group IV; indicated by filled circles) along the abscissa.

From this result, it is most likely that in the case of the heart shown in Fig. 8, the excitatory waves conducted radially and at a uniform rate over the right or the left side from each pacemaking area, as shown in Fig. 9. In this case, the conduction velocity was estimated to be 1.6 mm/s on the left side and 2.0 mm/s on the right side.

In the next experiment, we examined the effect of high external concentrations of Ca^{2+} on this preparation. When the Ca^{2+} concentration was increased fivefold,

conduction block along the primordial fusion line was relieved, and excitation propagated progressively over the whole heart, starting from area 1, which behaved as a single pacemaker (Fig. 10). This finding tentatively suggests that in the primordial fusion line, electrical coupling can be induced by elevation of the external Ca^{2+} concentration (see Discussion).

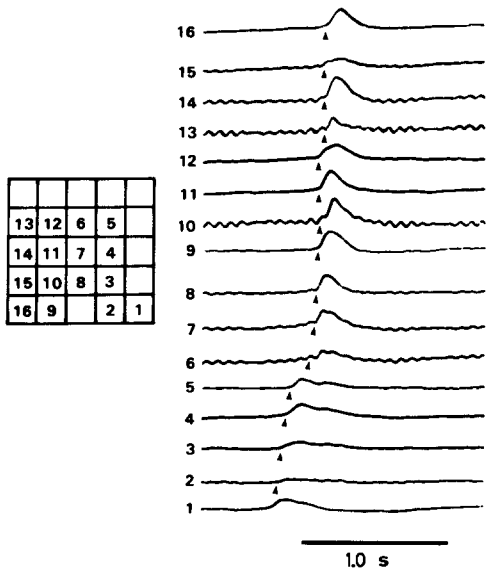

FIGURE 10. Demonstration of relief of conduction block in the fusion line by high external Ca^{2+}. In this experiment, the Ca^{2+} concentration in the bathing solution was raised from 1.8 (standard concentration) to 9 mM. The excitatory wave spread progressively over the whole area of the heart under these conditions.

DISCUSSION

Optical techniques for monitoring changes in membrane potential have offered critical advantages over conventional measurements in the investigation of cardiac functions in the early phases of cardiogenesis. Here, multiple-site optical recording of action potentials has demonstrated the effects of Ca^{2+} on the propagation pattern of spontaneous electrical activity in the early developing heart.

We have already shown that in the majority of early embryonic precontractile hearts, excitatory waves propagate in a uniform radial fashion over the heart from the pacemaking area (Hirota et al., 1983*a*). The results demonstrated in Figs. 1–4 tentatively suggest that Ca^{2+} plays an important role in modulating electrical propagation in the early phases of cardiogenesis.

In adult hearts, electrophysiological evidence has established that the propagation velocity of the action potential is determined by several factors, such as (*a*) the rate of the initial rapid depolarization and (*b*) the magnitude of the action potential, and that excitation is spread by local circuit flow (Weidmann, 1970).

Furthermore, there is much evidence to suggest that a low-resistance intercellular pathway is required for propagation of the action potential, and that gap junctions are the anatomical sites of these low-resistance pathways (for reviews, see De Mello, 1982; Spach and Kootsey, 1983).

The involvement of gap junctions in cell-to-cell coupling in developing embryonic hearts was demonstrated by the observation of induced synchrony in spontaneously beating mouse myocardial cells in culture (Goshima, 1969, 1970). DeHaan and Hirakow (1972), studying the acquisition of synchrony between pulsating cultured chick embryonic myocardial cells, also demonstrated gap junctions in thin sections of synchronously beating pairs of cells. Also, Ypey et al. (1979), using cultured embryonic chick heart cells, showed that electronic junctions formed between paired heart cell aggregates, causing a gradual development of action potential synchrony. Manasek (1968) observed by electron microscopy an outer junctional complex between cells in the 10-somite embryonic chick heart. Although there have been few morphological investigations dealing with intercellular communication in the early phases of cardiogenesis, it is tempting to suppose that similar junctional structures are formed in the seven-to-nine–somite embryonic heart.

As reported in an earlier paper (Sakai et al., 1983*a*), the spontaneous action potential in the seven-to-nine–somite embryonic heart has the characteristics of a Ca^{2+}-dependent action potential: when the Ca^{2+} in the bathing solution was partly replaced by Mg^{2+}, the amplitude, the rate of rise, and the duration of the action potential decreased. On the basis of this finding, for experiments using a low external Ca^{2+} concentration, we suggest that (*a*) the Ca^{2+} current accompanying the action potentials is small, (*b*) current flow through gap junction channels (Loewenstein, 1981) between the cell interiors is decreased, and (*c*) these processes result in a reduction in the conduction velocity of the excitation. The inhibitory effect of Ca^{2+} channel blockers on the conduction velocity also tentatively confirms this idea. Incubation of cardiac muscle in Ca^{2+}-free solution led to a rupture of the fasciae adherens and desmosomes (Muir, 1967; De Mello et al., 1969), which in turn led ultimately to a complete separation of the cells (De Mello et al., 1969). Nakas et al. (1966) also reported that removal of external Ca^{2+} resulted in functional uncoupling of *Chironomus* salivary gland cells. This evidence suggests that in low Ca^{2+} concentrations, the structure of the gap junction might deteriorate. However, as the external Ca^{2+} concentration was decreased by only 20% in the present experiment, this possibility can probably be ruled out.

In salivary glands of *Chironomus*, when Ca^{2+} was microinjected into the cells, the junctional conductance decreased (Loewenstein, 1966; Loewenstein et al., 1967; Nakas et al., 1966). On the basis of this result, Loewenstein proposed the Ca^{2+} hypothesis for the regulation of gap junction permeability. De Mello (1972, 1975) has also demonstrated that intracellular injection of Ca^{2+} causes electrical uncoupling in normal cardiac cells, as well as in cardiac Purkinje cells. Accordingly, it seems likely that the effect of the external high Ca^{2+} concentration shown in Figs. 3 and 4 resulted from the elevation of intracellular Ca^{2+} induced by increasing the diffusion of Ca^{2+} into cells. The effect of the ionophore

(A23187) on electrical propagation shown in Fig. 5 strongly supports the idea that the elevation of intracellular Ca^{2+} concentration reduced the electrical coupling among the early embryonic heart cells. Similarly, Rose and Loewenstein (1976) have reported that in the *Chironomus* salivary gland, treatment with ionophores X537A or A23187 produced diffuse elevation of intracellular Ca^{2+} associated with depression in junctional conduction.

It is technically impossible to quantitatively estimate the increment of the intracellular Ca^{2+} concentration under conditions of high external Ca^{2+} concentrations. Oliveira-Castro and Loewenstein (1971) have reported that the minimum effective concentration of the internal Ca^{2+} concentration that elicited uncoupling of the gap junction was $4–8 \times 10^{-5}$ M in *Chironomus* salivary gland cells. Recently, Spray et al. (1982) have also demonstrated that the amount of Ca^{2+} required to change the junctional conductance was at least 50 μM (pCa = 4.3) in *Fundulus* blastomeres. On the other hand, from the findings in skinned frog ventricular strips (Winegrad, 1971), these amounts should evoke irreversible contracture in adult cardiac muscle. Similarly, in the experiment using the 10-somite embryonic beating chick hearts, it was shown that contraction is reduced by raising the Ca^{2+} concentration in the bathing solution to two to five times the normal concentration, although action potentials remain (Hirota et al., 1984). Thus, it seems likely that when the external Ca^{2+} concentration is raised to five times the normal concentration in the early embryonic heart, the increment of the intracellular Ca^{2+} concentration is sufficient to alter the junctional conductance. Furthermore, it should be pointed out that although changes in both the signal size and the rate of rise were very small, the conduction velocity was markedly decreased, as shown in Fig. 4.

De Mello (1975) reported that other cations, such as Sr^{2+} or Mn^{2+}, injected into the cytosol also uncouple cells in the adult heart, and it was suggested that the site controlling the junctional conductance is not specific for Ca^{2+}. Accordingly, it is likely that the inhibitory effect of a high external Sr^{2+} concentration on the electrical propagation observed in the early embryonic precontractile heart results from the same mechanism(s) as that underlying the elevation of cytoplasmic Ca^{2+} concentration.

In addition, we should consider some important factors affecting conduction velocity in excitable cells. It is possible (*a*) that the changes observed with variations in the Ca^{2+} concentration are due to modulation of excitability either by changing excitability itself or by passive properties of nonjunctional membrane; (*b*) that a decrease in the amplitude and/or duration of the action potential results in a decrease in current through the junction; and (*c*) that high external Ca^{2+} decreases input resistance. However, most of those factors cannot be measured separately by our optical technique.

Coupling in the Fusion Line

A separate problem is the formation of functional cell-to-cell communication in the primordial fusion line in the seven-to-nine–somite embryonic primitive tubular heart. It seems difficult at the present stage, where morphological and structural bases are not established, to make any definite pronouncements on

the mechanism(s) of functional coupling in the fusion line. However, the junctional unit concept (Loewenstein, 1966) and the following results are helpful in considering the possible mechanism(s). (*a*) Electrical excitation is nearly synchronized between the right and left areas of the seven-somite embryonic heart at the initiation of fusion between the right and the left cardiac primordia (Fujii et al., 1981*c*). (*b*) From the seven-somite stage to the beginning of the nine-somite stage, the radially spreading electrical excitatory wave slowed within the primordial fusion line at the midline of the heart, and this delay disappeared in the later period of the 9–10-somite stage (Hirota et al., 1983*a*). Accordingly, it seems reasonable to suggest that (*a*) formation of the gap junction begins in the fusion line at the 7-somite stage; and (*b*) the resistance of the junctional area is relatively high during the 7–8-somite stage, but falls by the 9–10-somite stage.

Thus, a possible explanation is considered for the effect of Ca^{2+} on functional coupling in the fusion line: the external Ca^{2+} stabilizes the cell-to-cell interaction, which is structually unstable, among the myocardium cells in the fusion line. The results shown in Figs. 6 and 7 could thus be interpreted in this way. Ultrastructual and/or biochemical approaches are required to support these postulations.

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REFERENCES

Cohen, L. B., and B. M. Salzberg. 1978. Optical measurement of membrane potential. *Rev. Physiol. Biochem. Pharmacol.* 78:35–88.

DeHaan, R. L., and R. Hirakow. 1972. Synchronization of pulsation rate in isolated cardiac myocytes. *Exp. Cell Res.* 70:214–226.

De Mello, W. C. 1972. The healing-over process in cardiac and other muscle fibers. *In* Electrical Phenomena in the Heart. W. C. De Mello, editor. Academic Press, Inc., New York. 323–351.

De Mello, W. C. 1975. Effect of intracellular injection of calcium and strontium on cell communication in heart. *J. Physiol. (Lond.).* 250:231–245.

De Mello, W. C. 1982. Intercellular communication in cardiac muscle. *Circ. Res.* 51:1–9.

De Mello, W. C., G. Motta, and M. Chapeau. 1969. A study of the healing-over of myocardial cells of toads. *Circ. Res.* 24:475–487.

Fujii, S., A. Hirota, and K. Kamino. 1980. Optical signals from early embryonic chick heart stained with potential sensitive dyes: evidence for electrical activity. *J. Physiol. (Lond.).* 304:503–518.

Fujii, S., A. Hirota, and K. Kamino. 1981*a*. Optical recording of development of electrical activity in embryonic chick heart during early phases of cardiogenesis. *J. Physiol. (Lond.).* 311:147–160.

Fujii, S., A. Hirota, and K. Kamino. 1981*b*. Optical indications of pacemaker potential and rhythm-generation in early embryonic chick heart. *J. Physiol. (Lond.).* 312:253–263.

Fujii, S., A. Hirota, and K. Kamino. 1981*c*. Action potential synchrony in embryonic precontractile chick heart: optical monitoring with potentiometric dyes. *J. Physiol. (Lond.)*. 319:529–541.

Goshima, K. 1969. Synchronized beating and electrotonic transmission between myocardial cells mediated by heterotypic strain cells in monolayer culture. *Exp. Cell Res.* 58:420–426.

Goshima, K. 1970. Formation of nexuses and electronic transmission between myocardial and FL cells in monolayer culture. *Exp. Cell Res.* 63:124–130.

Grinvald, A., L. B. Cohen, S. Lesher, and M. B. Boyle. 1981. Simultaneous monitoring of activity of many neurons in invertebrate ganglia using a 124 element photodiode array. *J. Neurophysiol. (Bethesda)*. 45:829–840.

Grinvald, A., A. Manker, and M. Segal. 1982. Visualization of the spread of electrical activity in rat hippocampal slices by voltage-sensitive optical probes. *J. Physiol. (Lond.)*. 333:269–291.

Hirota, A., S. Fujii, and K. Kamino. 1979. Optical monitoring of spontaneous electrical activity of 8-somite embryonic chick heart. *Jpn. J. Physiol.* 29:635–639.

Hirota, A., H. Komuro, T. Sakai, T. Yada, and K. Kamino. 1984. Optical indications of excitation-contraction coupling in early embryonic heart. *J. Physiol. Soc. Jpn.* 46:484. (Abstr.)

Hirota, A., T. Sakai, S. Fujii, and K. Kamino. 1983*a*. Initial development of conduction pattern of spontaneous action potential in early embryonic pre-contractile chick heart. *Dev. Biol.* 99:517–523.

Hirota, A., T. Sakai, T. Yada, H. Komuro, S. Fujii, and K. Kamino. 1983*b*. Effects of Ca^{2+} on excitation spread in early embryonic hearts. *Biophysics (Kyoto)*. 23:S131 (in Japanese).

Kamino, K., A. Hirota, and S. Fujii. 1981. Localization of pacemaking activity in early embryonic heart monitored using a voltage-sensitive dye. *Nature (Lond.)*. 290:595–597.

Loewenstein, W. R. 1966. Permeability of membrane junctions. *Ann. NY Acad. Sci.* 37:441–472.

Loewenstein, W. R. 1979. Junctional intercellular communication and the control of growth. *Biophys. Biochim. Acta.* 560:1–65.

Loewenstein, W. R. 1981. Junctional intercellular communication: the cell-to-cell membrane channel. *Physiol. Rev.* 61:829–913.

Loewenstein, W. R., M. Nakas, and S. J. Socolar. 1967. Junctional membrane uncoupling permeability transformations at a cell membrane junction. *J. Gen. Physiol.* 50:1865–1891.

Manasek, J. F. 1968. Embryonic development of the heart: a light and electron microscopic study of myocardial development in the early chick embryo. *J. Morphol.* 125:329–366.

Muir, A. R. 1967. Effects of divalent cations on the ultrastructure of the perfused heart. *J. Anat.* 101:239–261.

Nakas, M., S. Higashino, and W. R. Loewenstein. 1966. Uncoupling of an epithelial cell membrane junction by calcium ion removal. *Science (Wash. DC)*. 151:89–91.

Oliveira-Castro, G. M., and W. R. Loewenstein. 1971. Junctional membrane permeability: effects of divalent cations. *J. Membr. Biol.* 5:51–77.

Orbach, J. S., and L. B. Cohen. 1983. Optical monitoring of activity from many areas of the *in vitro* and *in vivo* salamander olfactory bulb: a new method for studying functional organization in the vertebrate central nervous system. *J. Neurosci.* 3:2251–2262.

Patten, B. M. 1971. Early Embryology of the Chick. McGraw-Hill Book Co., New York.

Rose, B., and W. R. Loewenstein. 1976. Permeability of a cell junction and the local cytoplasmic free ionized calcium concentration: a study with aequorin. *J. Membr. Biol.* 28:87–119.

Sakai, T., S. Fujii, A. Hirota, and K. Kamino. 1983*a*. Optical evidence for calcium-action

potentials in early embryonic precontractile chick heart using a potential-sensitive dye. *J. Membr. Biol.* 72:205–212.

Sakai, T., A. Hirota, S. Fujii, and K. Kamino. 1983*b*. Flexibility of regional pacemaking priority in early embryonic heart monitored by simultaneous optical recording of action potentials from multiple sites. *Jpn. J. Physiol.* 33:337–350.

Salzberg, B. M., A. Grinvald, L. B. Cohen, H. V. Davila, and W. N. Ross. 1977. Optical recording of neuronal activity in an invertebrate central nervous system: simultaneous monitoring of several neurons. *J. Neurophysiol. (Bethesda).* 40:1281–1291.

Salzberg, B. M., A. L. Obaid, D. N. Senseman, and H. Gainer. 1983. Optical recording of action potentials from vertebrate nerve terminals using potentiometric probes provides evidence for sodium and calcium components. *Nature (Lond.).* 306:36–40.

Senseman, D. M., H. Shimizu, I. S. Horwitz, and B. M. Salzberg. 1983. Multiple-site optical recording of membrane potential from a salivary gland. Interaction of synaptic and electrotonic excitation. *J. Gen. Physiol.* 81:887–908.

Spach, M. S., and J. M. Kootsey. 1983. The nature of electrical propagation in cardiac muscle. *Am. J. Physiol.* 13:H3–H22.

Spray, D. C., J. H. Stern, A. L. Harris, and M. V. L. Bennet. 1982. Gap-junctional conductance: component of sensitivities to H and Ca ions. *Proc. Natl. Acad. Sci. USA.* 79:441–445.

Weidmann, S. 1970. Electrical constants of trabecular muscle from mammalian heart. *J. Physiol. (Lond.).* 210:1041–1054.

Winegrad, S. 1971. Studies of cardiac muscle with a high permeability to calcium produced by treatment with ethylenediaminetetraacetic acid. *J. Gen. Physiol.* 58:71–93.

Ypey, D. L., D. E. Clapham, and R. L. DeHaan. 1979. Development of electrical coupling and action potential synchrony between paired aggregates of embryonic heart cells. *J. Membr. Biol.* 51:75–96.