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OPTICAL STUDIES OF EXCITATION–CONTRACTION COUPLING IN THE EARLY EMBRYONIC CHICK HEART

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SUMMARY

1. Excitation–contraction coupling at the onset of beating in the 9–10-somite embryonic chick heart was studied by means of an optical method together with a voltage-sensitive merocyanine–rhodanine dye. Spontaneous optical signals were recorded simultaneously from many areas of the embryonic heart, using a square photodiode matrix array.

2. At time of initiation of the heartbeat, spontaneous optical signals consisting of two components were often detected. The first component (1st signal) is a dye-related absorption change due to the action potential, and the second component (2nd signal) is a light scattering change due to contraction.

3. When Ca^{2+} in the bathing solution was partly replaced by Mg^{2+}, the peak size of both signals was reduced. The correlation between the 1st and 2nd signals corresponded to the relationship between excitation and contraction.

4. The formation of excitation–contraction coupling exhibited a regional non-uniformity in the developing 9–10-somite embryonic hearts: contraction was first generated in the right ventricular region, and then the contractile area spread widely over the whole of the heart. The curves of the excitation-related 1st signal *vs.* the contraction-related 2nd signal obtained from different areas were not superimposable.

5. Decoupling of excitation from contraction was produced by raising the Ca^{2+} concentration in the bathing solution, by lowering the Na^{2+} concentration or by inclusion of a Ca^{2+} ionophore (A23187). Replacement of the bathing solution with D_2O or hypertonic solution also suppressed excitation–contraction coupling.

6. The results suggest that in the early embryonic initial beating chick heart, the contractile system is activated by Ca^{2+} influx across the sarcolemma accompanying the action potential, and that a Na^{+}–Ca^{2+} exchange mechanism participates in the relaxation phase of the heartbeat.

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INTRODUCTION

During the early phases of cardiogenesis in the chick embryo, the right and left cardiac primordia are brought together at the mid line and progressively fuse with each other caudally. This process results in the formation of the primitive tubular beating heart. The initial heartbeat in the early developmental stages has long been noted (Patten & Kramer, 1933; Goss, 1938; Copenhaver, 1939; Bary, 1942; also for classical reviews see Eyster & Meek, 1921; Patten, 1949). However, studies on the excitation–contraction coupling process in the developing embryonic heart have been hindered because the cells are too small and frail to allow direct mechanical recording of the contraction or micro-electrode impalement.

Applying voltage-sensitive dyes to the early embryonic chick hearts, we have previously demonstrated optically that spontaneous electrical excitability is generated in the 7–8-somite stage, prior to the onset of the heartbeat (Fujii, Hirota & Kamino, 1980, 1981*a*, *b*, *c*). We established optically that the primitive tubular heart of chick embryos begins to contract at the middle period of the 9-somite developmental stage (Fujii *et al.* 1981*b*). In optical measurements of electrical activity in contractile tissues, a movement-related optical change is usually also present (Baylor & Oetliker, 1977; Baylor & Chandler, 1978; Sawanobori, Hirota, Fujii & Kamino, 1983). On the basis of such characteristic optical changes, we have monitored optically the excitation–contraction coupling in the early embryonic heart stained with a voltage-sensitive dye (for reviews see Cohen & Salzberg, 1978; Salzberg, 1983). We report here the characteristics of excitation–contraction coupling in the 9–10-somite embryonic heart at the time of initiation of the heartbeat. A preliminary report of this work has been published (Hirota, Sakai, Komuro, Yada & Kamino, 1984).

METHODS

Preparations. Fertilized chicken (white Leghorn) eggs were incubated usually for 30–40 h in a forced-draft incubator (Type P-03, Showa Incubator Lab. 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 9- and 10-somite embryos were used. Some control experiments were performed on 4–5-day-old embryos. The isolated embryos were kept in a bathing solution with the following composition (in mM): NaCl, 138; KCl, 5·4; $CaCl_2$, 1·8; $MgCl_2$, 0·5; and Tris HCl buffer (pH 7·2), 10. 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 dissecting microscope.

Potential-sensitive dye staining. The isolated embryos were incubated for 10 min in a bathing solution containing 0·1 mg/ml of a merocyanine–rhodanine dye (NK 2761, Kamino, Hirota & Fujii, 1981) obtained from Nippon Kankoh-Shikiso Kenkyusho Co., Okayama, Japan. The preparation was then washed with several changes of normal bathing solution.

Optical recording. Several components of the apparatus were previously described (Fujii *et al.* 1980, 1981*a*, *b*, *c*), and similar to those described by Salzberg, Grinvald, Cohen, Davila & Ross (1977) or Grinvald, Cohen, Lesher & Boyle (1981). 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 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., Tokyo, Japan), an interference filter (Type 1F-S, Vacuum Optics Co. of Japan, Tokyo, Japan) at 700 ± 11 nm for measuring of action-potential-related change or 610 ± 9 nm or 620 ± 9 nm for

contraction-related change, 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 (40 or 60 times) real image of the embryonic heart.

The transmitted light intensity at the image plane of the objective and photographic eyepiece was detected using a 10×10 square array of silicon photodiodes (MD-100-4PV, Centronic, Ltd., Croydon). The image of the preparation 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 96-elements of the detector were first recorded simultaneously on a 96-channel data recording system (RP-890 series with six RP-893 I/O processors, NF Electronic Instruments, Yokohama, Japan), and the signals were then displayed on two Tektronix 5113 dual beam storage oscilloscopes with two 5A18N amplifiers for analysis. In most experiments, the oscilloscopes were set to give a coupling time constant of 1·5 s and the outputs were filtered by a simple RC low-pass filter (time constant approximately 10 ms). Additionally, the signals were fed into a computer (LSI-11/23, Digital Equipment Corp., Tewksbury, MA, U.S.A.) from the recording system, and then displayed.

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, Fujii, Sakai & Kamino, 1983).

RESULTS

Optical changes accompanying contraction

Generally, optical signals derived from beating hearts include intensity changes due to mechanical movement (Fujii *et al.* 1980, 1981*a*; Hirota, Sakai, Fujii & Kamino, 1981; Hill & Courtney, 1982; Sawanobori *et al.* 1983). In ideal conditions, the change in transmitted light intensity can be proportional to tension development (Kass, 1981).

Fig. 1*A* shows optical changes accompanying beating movement in a 5-day-old embryonic heart without staining. In the standard bathing solution, the heartbeat was large enough to be observed by eye under a microscope, and the beating-related optical signal was usually large in amplitude and showed a complex pattern. Empirically, we have found that in the early embryonic heart, the beating was markedly suppressed by increasing the NaCl and $CaCl_2$ concentrations in the bathing solution. After the standard bathing solution was replaced by a solution containing 414 mM-NaCl and 9·0 mM-$CaCl_2$, the contraction gradually decreased, the amplitude of the optical signal decreased, and the shape gradually became simpler. The optical signal was reduced to a monophasic pattern and subsequently, often disappeared. Such a beating-related optical signal was detectable at various wave-lengths of visible light and by illumination with white light.

Using 4–5-day-old embryonic heart stained with the potential-sensitive dye (NK 2761), we also compared the time course of optical change accompanying spontaneous action potentials to direct electrical measurement (Fig. 1*B*). The measurements were made after the contraction was minimized in a bathing solution containing 414 mM-NaCl and 9·0 mM-$CaCl_2$. The optical change was biphasic at 700 nm: the optical signal consisted of two components (the 1st and 2nd signals), and the direction of the 2nd signal was opposite to that of the 1st signal. The time course of the 1st optical signal was very similar to the action potential measured by a micro-electrode. The 1st component varied with the wave-length of incident light, and was reduced when white light or a 620 ± 9 nm interference filter was used, whereas

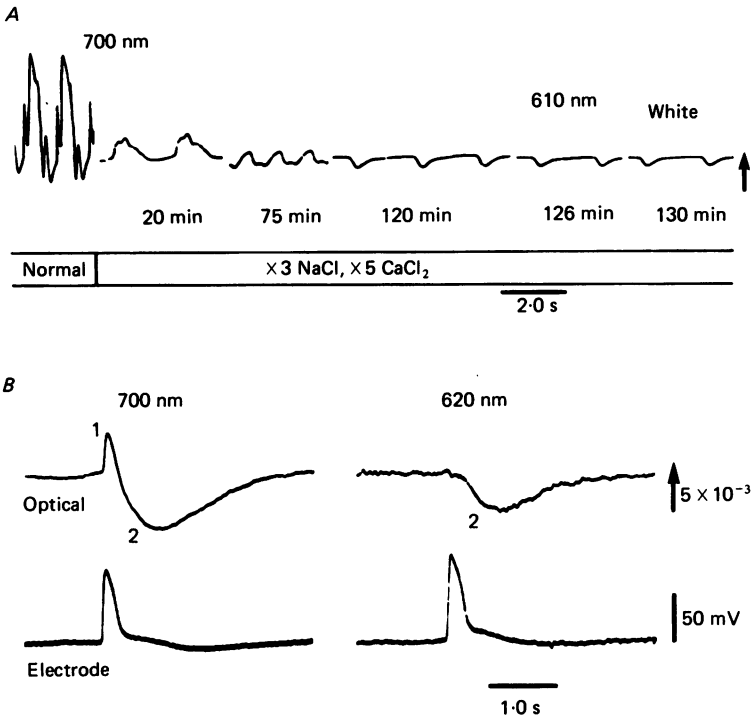

Fig. 1. *A*, demonstration of changes in transmitted light accompanying a decrease in beating of the ventricular preparation isolated from a 5-day-old embryonic heart, in a hypertonic bathing solution containing 414 mM-NaCl and 9·0 mM-$CaCl_2$. The preparation was unstained. The measurements were made at 700 nm for the normal condition, 20, 75 and 120 min after incubation in the high-NaCl and high-$CaCl_2$ solution; at 610 nm at 126 min; and with white light at 130 min. All measurements were carried out at room temperature (about 28 °C). The arrow to the right of the optical traces indicates the direction of a decrease in the intensity reaching the photodiode. The length of the arrow represents changes in fractional intensity (intensity change divided by the d.c. background intensity): $1{\cdot}5 \times 10^{-1}$ for normal; $2{\cdot}0 \times 10^{-2}$ for 20 min; $2{\cdot}2 \times 10^{-2}$ for 75 min; $3{\cdot}2 \times 10^{-3}$ for 120 min; $3{\cdot}3 \times 10^{-3}$ for 610 min; and $4{\cdot}3 \times 10^{-3}$ for white light. *B*, an optical change accompanying an action potential in a 5-day-old embryonic chick heart stained with merocyanine–rhodanine dye NK 2761. The optical changes were detected with a 700 ± 11 or 620 ± 9 nm interference filter. The action potentials were simultaneously measured with an intracellular micro-electrode filled with 3 M-KCl and of resistance about 30 MΩ, at room temperature. In order to reduce the contraction, the measurements were made in the high-NaCl, high-$CaCl_2$ solution as in *A*. At 700 nm, the 1st signal (indicated by 1) was closely correlated with the action potential. At 620 nm, the 1st signal was completely eliminated, while the 2nd signal (indicated by 2) was detected. The vertical arrow at the right indicates the direction of a decrease in transmitted light intensity.

the 2nd component remained at 620–610 nm or with white light. These characteristics of the 2nd component were similar to those of the optical change observed in unstained beating preparations.

These results suggest that the 1st component is an absorption change due to the action potential, and that the 2nd component is a change of light-scattering originating from the contraction movement (also see the next section). The

merocyanine–rhodanine dyes are impermeable to plasma membrane (Cohen & Salzberg, 1978), and thus, it is unlikely that the 2nd component corresponds to an absorption change related to intracellular dye penetration.

Early development of embryonic heartbeat

Fig. 2 illustrates the spontaneously generated optical signals from stained early embryonic chick hearts at the 9–10-somite developmental stages. The hearts were bathed in normal NaCl and $CaCl_2$ solutions. The optical signal was often biphasic and

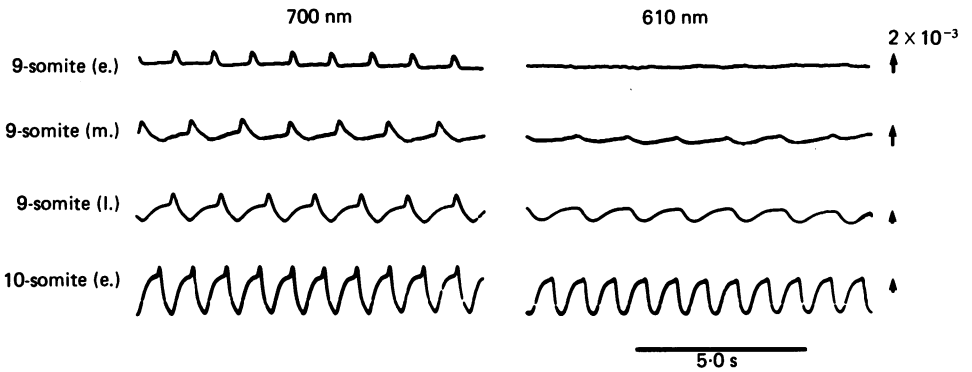

Fig. 2. Developmental changes in the biphasic type optical signals (1st and 2nd components) recorded from embryonic hearts at the early (e.), middle (m.) and later (l.) 9-somite developmental stages and the early (e.) 10-somite stage. The hearts were stained with NK 2761. All the traces were obtained from the right cono-ventricular region at 36·7–37·2 °C, the optical recording field was estimated to be $37{\cdot}5 \times 37{\cdot}5\ \mu m^2$. Note that the 1st components are all eliminated at 610 nm, and that the signals in the early period of 9-somite stage consist of the 1st component only. The action-potential-related 1st signal depends on the wave-length of the incident light, while the contraction-related 2nd signal is independent of the wave-length (also see Fig. 3).

consisted of two components as Fig. 1*B*. The direction of the 2nd signal was opposite to that of the 1st signal, The amplitude of the 1st signal varied with the wave-length of incident light, while the 2nd signal was independent of wave-length (Fig. 3). The 2nd signal was detectable with white light, at a wave-length where the spectrum of the absorption signal had a null (e.g. 610–620 nm, Fig. 3), or in unstained preparations. Accordingly, it is evident that the 1st signal corresponds to a dye-related absorption signal accompanying the action potential, and the 2nd signal is due to a small contraction.

In Fig. 2, at the early period of the 9-somite developmental stage, the 1st signals reflecting the spontaneous action potentials were detected at a wave-length of 700 nm, and they were absent at 610 nm. However, the optical signals recorded from hearts at the middle 9-somite stage to the early 10-somite stage showed a biphasic pattern in shape at 700 nm: the 1st signal was followed by a 2nd signal which was also evident at 610 nm. These optical recordings confirm that the spontaneous electrical action potential is first coupled with contraction at the middle period of the 9-somite stage (Fujii *et al.* 1981*b*).

The magnitude of the optical signal due to the heartbeat increased as development

proceeded. This result suggests an increase in the number of contracting myofibrils and progressive development of the contractility during the early phases of cardiogenesis. With further development, the shape of the optical signals from contraction became complex. Signals with reversed direction, triphasic patterns, and additional time courses were observed. It is thus possible that the large contraction-related optical signal accompanying the larger contraction of the myofibrils includes light-scattering changes due to mechanical movement of cells, intercellular components, cardiac jelly and/or fluid in the cardiac cavity.

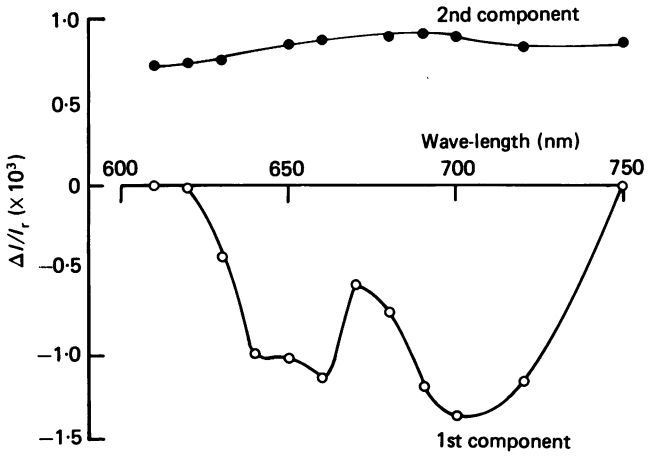

Fig. 3. Wave-length dependence of the 1st and 2nd signals recorded from a 9-somite embryonic heart stained with NK 2761. The ordinate is the fractional change in the transmitted light intensity, the change in intensity (ΔI) divided by the resting intensity (I_r). Note that the 1st signal is null at 610–620 nm.

Contractile area. In a short note (Hirota *et al.* 1981), we previously reported that the initial contractions of embryonic chick heart are localized in the right side of the cono-ventricular region and that the area of the contraction spreads as development proceeds. In the present work, using a 10 × 10 element photodiode matrix array, we have tried to assess in more detail the area where the contraction is generated in the 9–10-somite embryonic heart.

Fig. 4*A* shows spontaneous optical signals simultaneously detected from eighty-three separate areas of an embryonic heart, at the early period of the 9-somite stage. The signals appeared over the entire region of the heart at 700 nm, although there were regional differences in the signal size. It is assumed that this difference in the signal size reflects the distribution of active cells in the heart. These signals were all completely reduced at 610 nm. It was evident, therefore, that this preparation was situated at the pre-contractile stage. Fig. 4*B* shows a typical recording obtained from a 9-somite embryonic heart just at the time of initiation of contraction. Very small signals (indicated by arrowheads) were significantly detected at 610 nm by elements 53, 63, 73, 82 and 83 positioned over the image of the right margin of the right cono-ventricular region. These small signals are more clearly shown in the enlarged, representative recording shown in the lower left-hand corner. They appeared

rhythmically and were synchronized with each other, suggesting that they reflect the initial small contraction of myofibrils in this heart.

In a heart at a slightly later 9-somite stage (Fig. 4*C*), typical biphasic signals were detected from the right margin of the right cono-ventricular region by elements 31, 41, 51, 61 and 71, at 700 nm. In these biphasic signals the initial upward deflexion is taken to indicate the action potential and the following downward deflexion indicates contraction. In the recording at 610 nm, the contraction-related signals

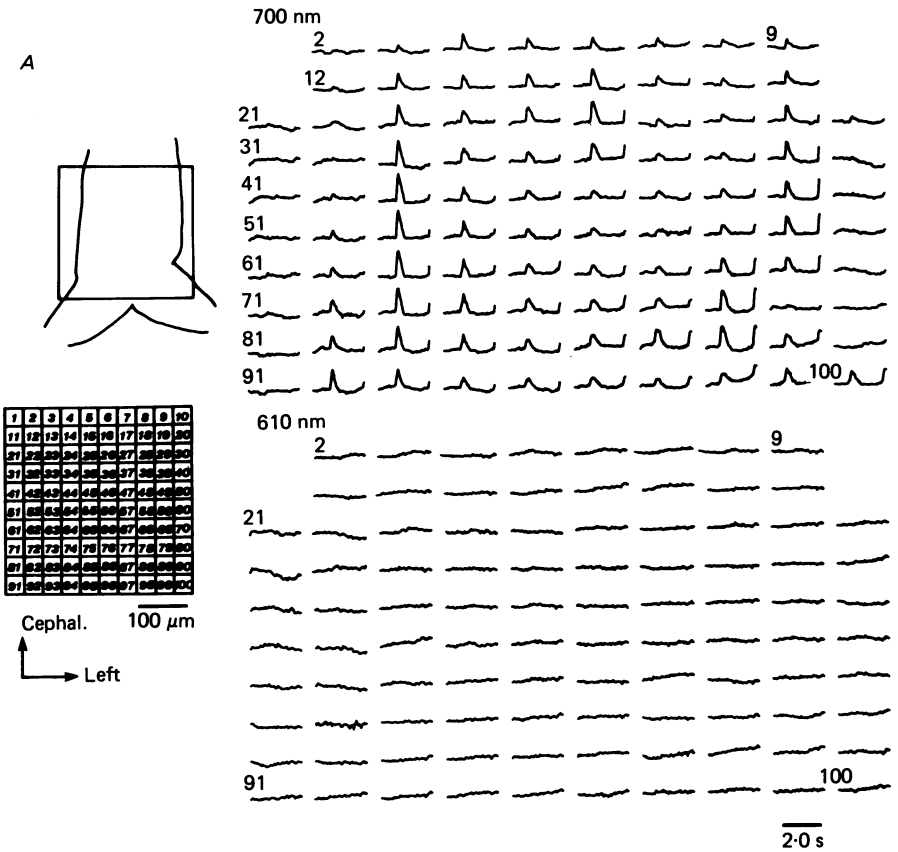

Fig. 4*A*. For legend see p. 98.

were larger and markedly prolonged. They appeared not only in the right cono-ventricular region but in the central part and in the left cono-ventricular region. The signals detected by elements 42, 52, 26, 36, 49, 59 and some other elements at 610 nm showed the same direction in the changes as that in the action-potential-related signal at 700 nm. The signals obtained at 700 nm in the corresponding elements (e.g. 12, 22, 32, 42, 52 and 62) were modified by the upward contraction-related signal. These results reflect the development of the contractility of primitive myofibrils.

Generally, as development proceeds to the 10-somite stage, the contraction-related signals become larger and the area of contraction spreads throughout the entire heart. Typical recordings from an early 10-somite embryonic heart are shown in Fig. 4*D*.

In this preparation, large signals were detected from the entire region, at both 700 and 610 nm, and there were regional differences in the size and shape of the signals. In the recording at 700 nm, biphasic signals were clearly detected from the margins of the right and left cono-ventricular regions, by elements 21, 31, 41, 51, 61, 71, 81, 91, 28 and 38. The signals detected by elements 12, 22, 32, 42, 52, 62, 72, 82, 27, 37, 47, 57, 77 and some other elements overlapped the action-potential-related 1st

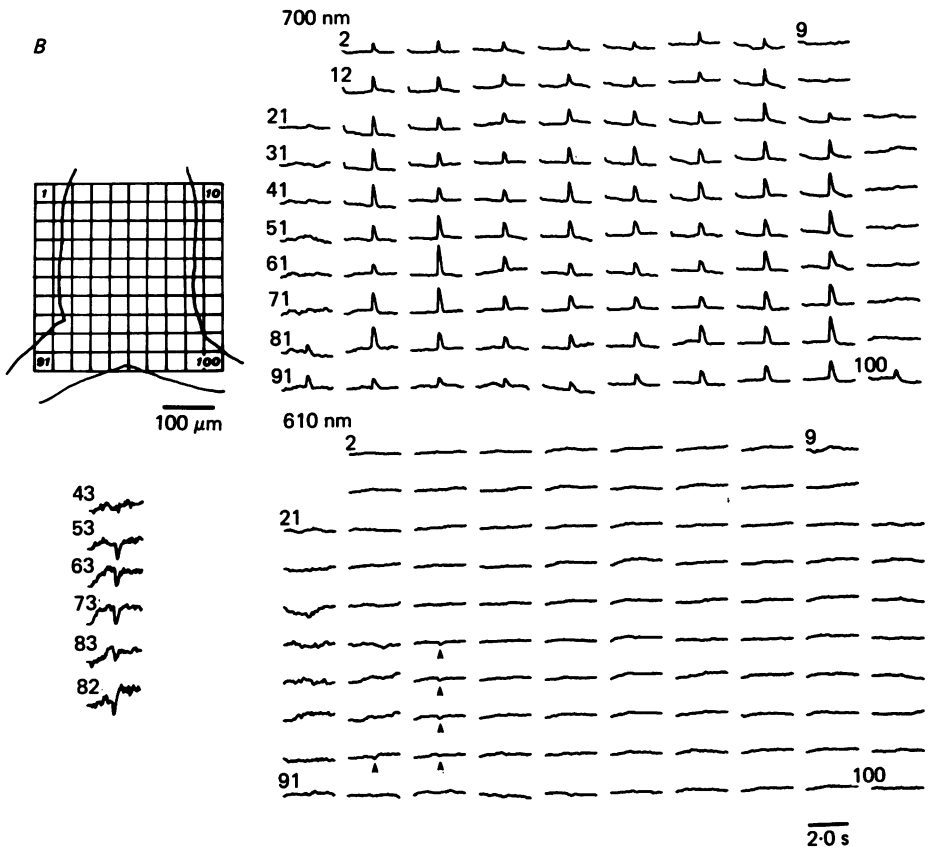

Fig. 4*B*. For legend see p. 98.

signals, suggesting that the recordings from these areas involved changes in light transmission due to movements of components other than contraction of the myofibrils.

Excitation–contraction coupling

Another series of experiments was designed to assess the nature of excitation–contraction coupling in the initial beating embryonic heart. In previous work (Sakai, Fujii, Hirota & Kamino, 1983), we demonstrated that the spontaneous action potential in the early embryonic chick heart is Ca^{2+} dependent. Reducing the external Ca^{2+} concentration results in decrease in the amplitude of the optical signals related to the action potentials. Therefore, for analysis of the excitation–contraction coupling

process in the early embryonic heart, we have studied the amplitudes of the 1st and 2nd signals at reduced bathing Ca^{2+} concentration (Fig. 5).

When the external Ca^{2+} was partly replaced by Mg^{2+}, the amplitude of both the 1st and 2nd signals decreased. This finding strongly suggests that the contractility is reduced with a decrease in the amplitude of the action potential. It is likely that the peak size of the 2nd signal is correlated with the peak size of the contraction

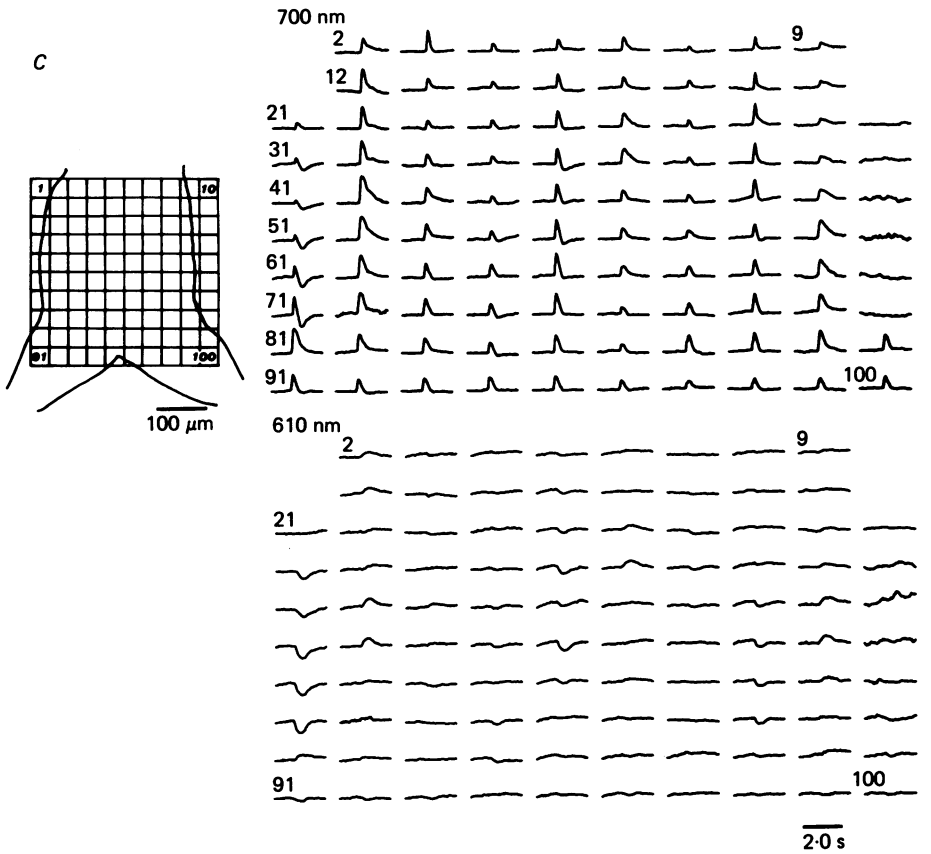

Fig. 4*C*. For legend see p. 98.

response and/or change in tension. On the basis of this idea, the peak size of the 2nd signal was plotted against the size of the 1st signal (Fig. 6). Data were obtained in the recordings of optical signals from three or four different positions of a 9- or a 10-somite embryonic heart in various external Ca^{2+} concentrations. After the size of the 1st signal exceeded a certain value corresponding to the mechanical threshold, the 2nd signal occurred and then the peak size of the 2nd signal increased sigmoidally with increases in the 1st signal. This Figure also shows that there are regional differences in the relationship between 1st and 2nd signals.

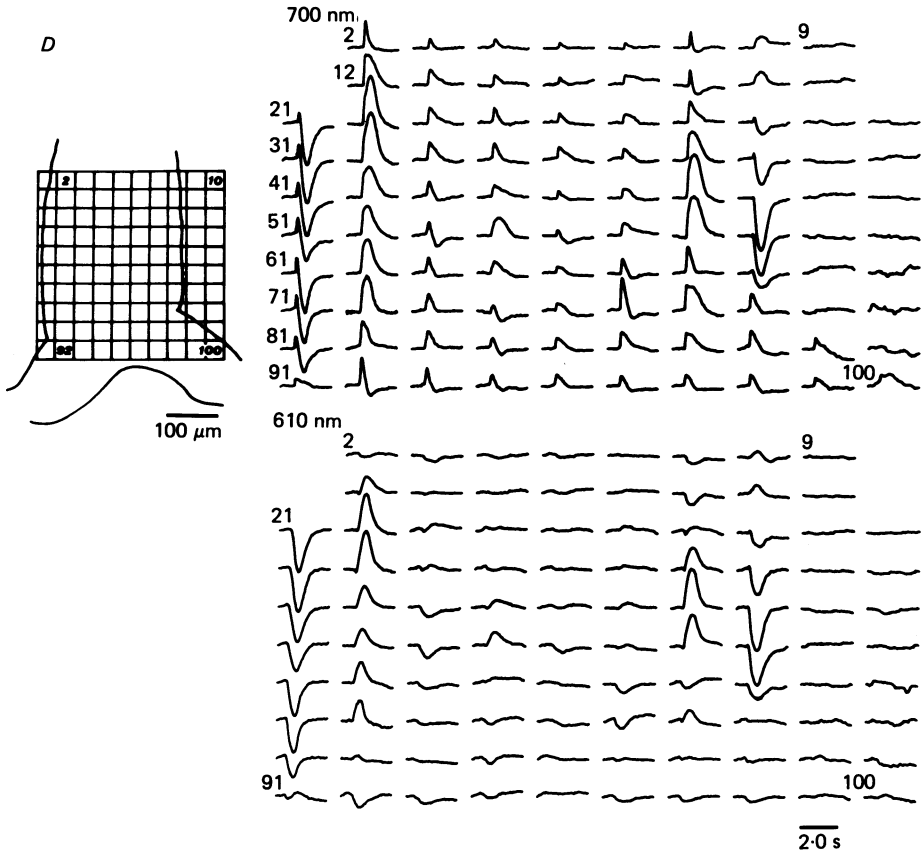

Fig. 4. Simultaneous recording of spontaneous optical signals with a 10×10 element photodiode matrix array from multiple loci in the 9–10-somite embryonic chick heart. *A*, pre-contractile heart at the early 9-somite stage; *B*, 9-somite embryonic heart just at the time of initiation of contraction; *C*, later period of the 9-somite stage; and *D*, the early 10-somite stage. The hearts had been stained with NK 2761. Measurements were made with a 700 ± 11 or 610 ± 9 nm interference filter, at 36·4–37·8 °C. The insets on the left of the traces in *A*, *B*, *C* and *D* illustrate the locations of the images of the hearts on the array elements. The drawings of the hearts are ventral views. The field detected by one element is estimated to be $37{\cdot}5 \times 37{\cdot}5\ \mu m^2$. Arrangements of the traces correspond to those of the elements shown in the left side in each recording. In *B*, enlargements of some of the traces with the corresponding detector numbers are shown in the lower left corner. The fractional intensity change of these signals was estimated to be about 3×10^{-4} and signal-to-noise ratio was estimated to be about 4.

Uncoupling of excitation and contraction

High Ca^{2+}. Fig. 7*A* shows original recordings of the optical signals at 700 and 610 nm from a 9-somite embryonic heart in a bathing solution with 18 mM-Ca^{2+}. When NaCl was partly replaced by $CaCl_2$ under conditions of a constant osmolarity of the solution, the size of the 1st signal was not altered, but the peak size of the 2nd signal dramatically decreased.

The peak sizes of the 1st and 2nd signals as a function of time (minutes) after the

normal bathing solution was replaced by a solution containing 18 mM-Ca^{2+} are plotted in Fig. 7*B* for a 9-somite embryonic heart. The peak size of the 2nd signal decreased exponentially, and reached a plateau level after about 2 min, while the size of the 1st signal remained at a constant value.

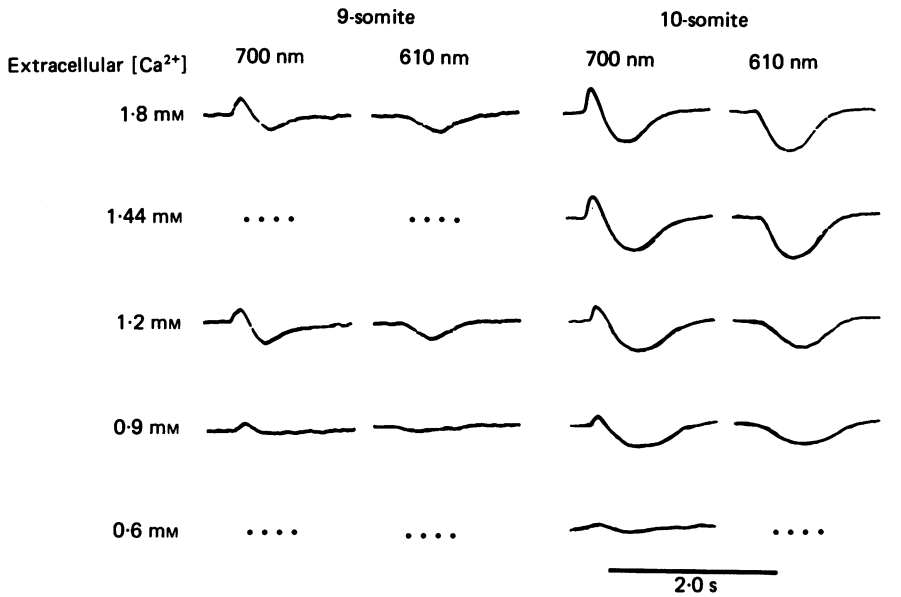

Fig. 5. Changes in the optical signals recorded from 9- and 10-somite embryonic hearts in various Ca^{2+} concentrations. Measurements were made with a 700 ± 11 or 610 ± 9 nm interference filter, at 37·2–37·6 °C for the 9-somite and 37·4–37·7 °C for the 10-somite heart. The solutions were made by replacement of a part of the Ca^{2+} in the bathing solution with Mg^{2+}. Both the excitation-related 1st and the contraction-related 2nd signals are gradually reduced with lowering of the Ca^{2+} concentration in the bathing solution. In the 9-somite embryonic heart, both the 1st and 2nd signals disappeared when the Ca^{2+} concentration was lowered to 0·6 mM.

Ionophore. It was possible that the above effects resulted from elevation of intracellular Ca^{2+} accompanying an increase in the influx of Ca^{2+}. Another way to elevate the intracellular Ca^{2+} is to use a Ca^{2+} ionophore (Loewenstein, 1981). Fig. 8 shows original recordings of the optical signals from a 9-somite embryonic heart in the presence of Ca^{2+} ionophore A23187 (1 μM). Although the size of the 1st signal was somewhat decreased, the peak size of the 2nd signal was dramatically reduced. This effect was similar to that observed in the experiment using external high-Ca^{2+} concentrations.

D_2O and hypertonicity. In skeletal muscle, D_2O is known to reduce the magnitude of the mechanical response during a twitch (Svensmark, 1961). Another type of solution used to reduce the mechanical activity in skeletal muscle, while leaving the surface action potential intact, is hypertonic NaCl Ringer solution (Hodgkin & Horowicz, 1957). It was thus of interest to determine the effects of D_2O and hypertonicity on contraction of the early embryonic heart. Results obtained from the

9-somite embryonic hearts in D_2O bathing solution (*A*) or hypertonic NaCl solution (*B*) are shown in Fig. 9. In both solutions, the 2nd signal was reduced. These results provide additional support for the idea that the 2nd signals are related to contraction.

External low Na^+. Fig. 10 illustrates optical recordings from a 10-somite embryonic heart in a low-Na^+ bathing solution. When half of the NaCl in the bathing solution

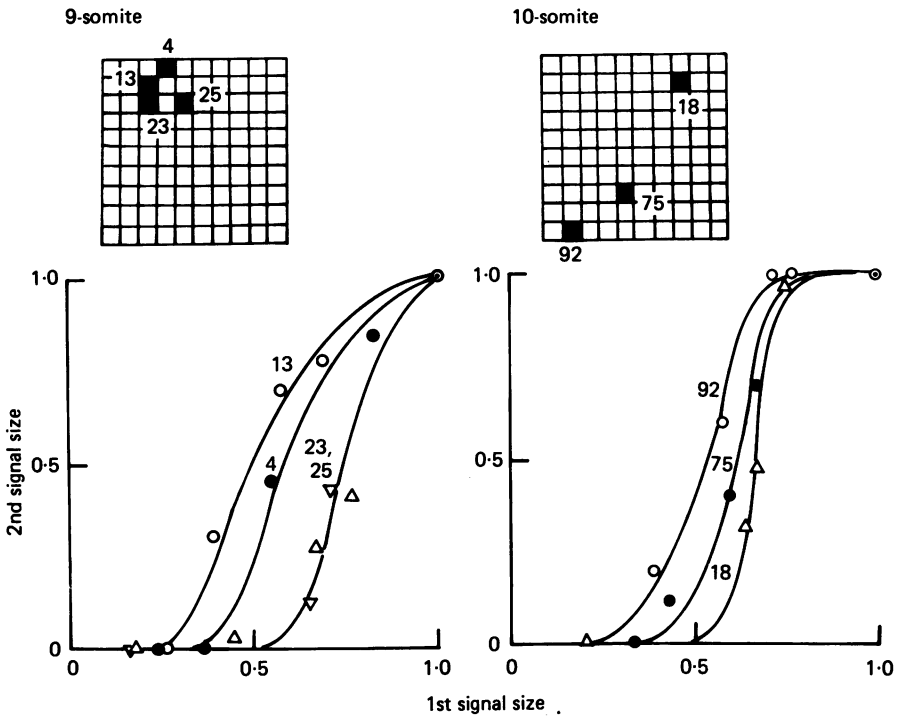

Fig. 6. Correlation between the size of the 1st signal and that of the 2nd signal obtained from a 9-somite (left) and a 10-somite (right) heart stained with NK 2761. Data were from the optical signals detected by the elements indicated above each graph. Measurements were made in normal (1·8 mM); 4/5 (1·44 mM); 2/3 (1·2 mM); 1/2 (0·9 mM); and 1/3 (0·6 mM) Ca^{2+} concentrations in the bathing solution. The ordinate is the size of the 2nd signal; the abscissa is the size of the 1st signal. Signal sizes are normalized to the size in the normal Ca^{2+} concentration. The relative position of the matrix array photodiode on the image of the heart was similar to that shown in Fig. 4.

was replaced with 69 mM-choline chloride, the size of the 2nd signal decreased dramatically, although the size of the 1st signal was not altered. During a twitch the decay of the 2nd signal was significantly prolonged, but the time-to-peak was not altered.

DISCUSSION

Because mechanical measurements cannot be feasibly used in the early embryonic heart for reasons of the small size and frailty of the cells, it is impossible to directly examine the relationship between optical change and the contraction in the early

embryonic heart. Kass (1981) compared records of tension with change in transmitted light intensity during a series of voltage-clamp steps in the calf cardiac Purkinje fibre, and obtained a linear relationship between the tension and optical change. The initial contractions in the early embryonic heart are very small, so that the peak size of the

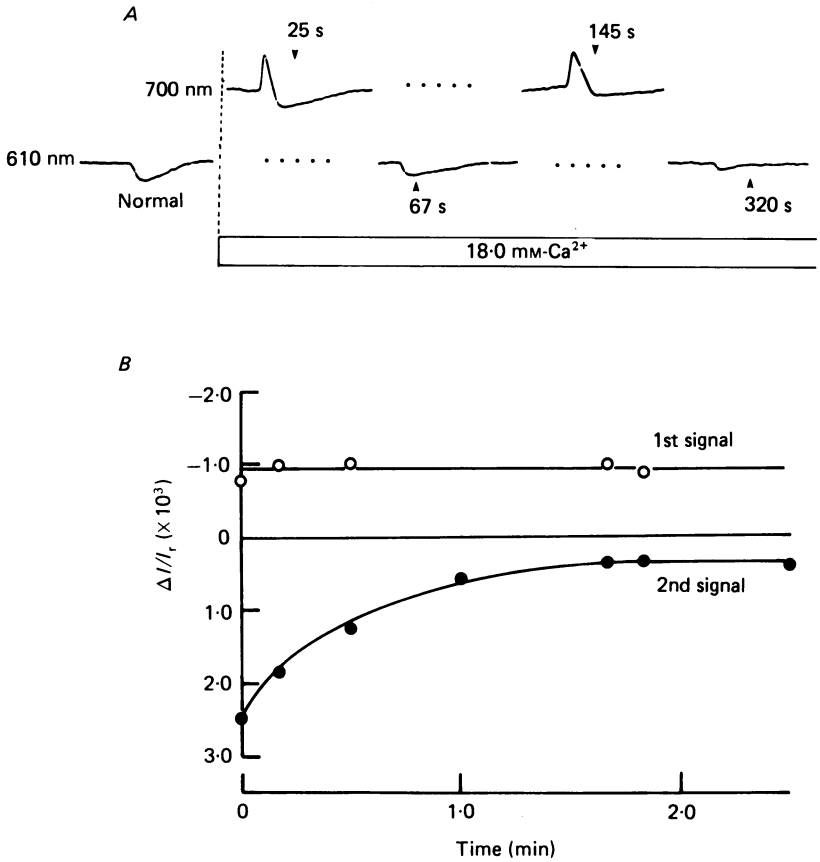

Fig. 7. Optical signals from a 9-somite embryonic heart in a bathing solution containing 18·0 mM-Ca^{2+}. *A*, during the measurements, 700±11 and 610±9 nm interference filters were alternately used. The solution used in this experiment was made by replacement of NaCl in the normal bathing solution with $CaCl_2$, and the osmolarity was maintained at an isotonic value. Arrows indicate the stated time after the heart was put in the high-Ca^{2+} bathing solution. *B*, time course of changes in the peak size of the 1st and 2nd signals in the high-Ca^{2+} solution. The ordinate is the fractional change of transmitted intensity. Downward deflexion indicates an increase in the intensity. Measurements were made using a 9-somite embryonic heart at 37·2–37·9 °C. Note that the size of the 1st signal is almost constant, while the size of the 2nd signal decreases monotonously to a plateau level.

optical change due to the contraction is in the order of a 10^{-3} change in intensity. Thus, it seems likely that the signals in this range are related to localized contractions of the myofibrils. However, the contractions become stronger during development and then the complexity of the optical signals increases. The direction can even reverse (Figs. 1*A* and 4*C* and *D*). Under these conditions, movement of the cells

and/or intercellular components may alter the signals. Thus, in the present experiments we have quantitatively assessed only the very early, small contractions leading to a biphasic optical signal at 700 nm, with an increase in light transmission during contraction.

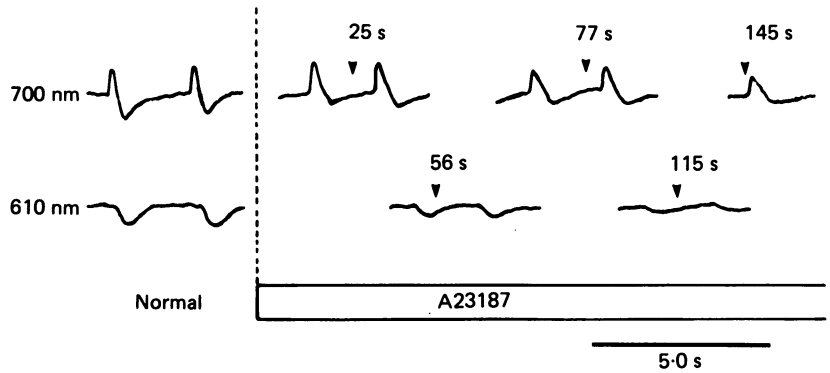

Fig. 8. Effect of a Ca^{2+} ionophore (A23187; 1·0 μM) on the optical signal. Optical signals were detected at a wave-length of 700 or 610 nm from the right cono-ventricular region in a 9-somite embryonic heart, at 36·2–37·6 °C. Arrows indicate the stated time after the heart was put into the bathing solution containing A23187. Note that the 2nd signals decrease rapidly.

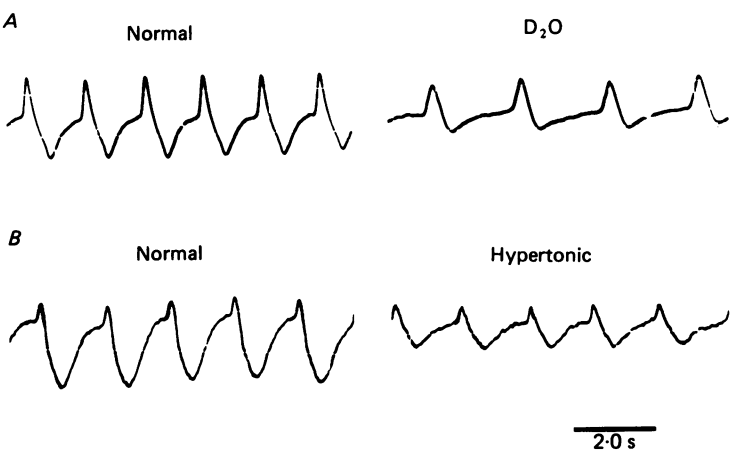

Fig. 9. Effect of a D_2O bathing solution (*A*) or NaCl hypertonic solution (*B*) on the optical signals. The D_2O solution was made by replacement of H_2O in the normal bathing solution with 95% D_2O. the NaCl hypertonic solution contained 207·0 mM-NaCl. Measurements were made using a 9-somite embryonic heart at 36·8–37·6 °C for D_2O and a 10-somite embryonic heart at 36·5–37·0 °C for hypertonicity.

A sigmoidal relationship was found between the 1st signal due to the action potential and the 2nd signal related to contraction (Figs. 5 and 6) as the Ca^{2+} concentration was varied. Thus, in the early embryonic heart, the spontaneous action potential strongly depends on the external Ca^{2+} (see also Sakai *et al.* 1983). In the low concentrations, the contraction coupled with the action potential decreased.

These results suggest two kinds of sarcolemma mechanism related to the excitation–contraction coupling process in the early embryonic heart: (1) an intracellular Ca^{2+} store is triggered by Ca^{2+} influx accompanying the action potential to release activator Ca^{2+} or (2) Ca^{2+} influx across the sarcolemma associated with the action

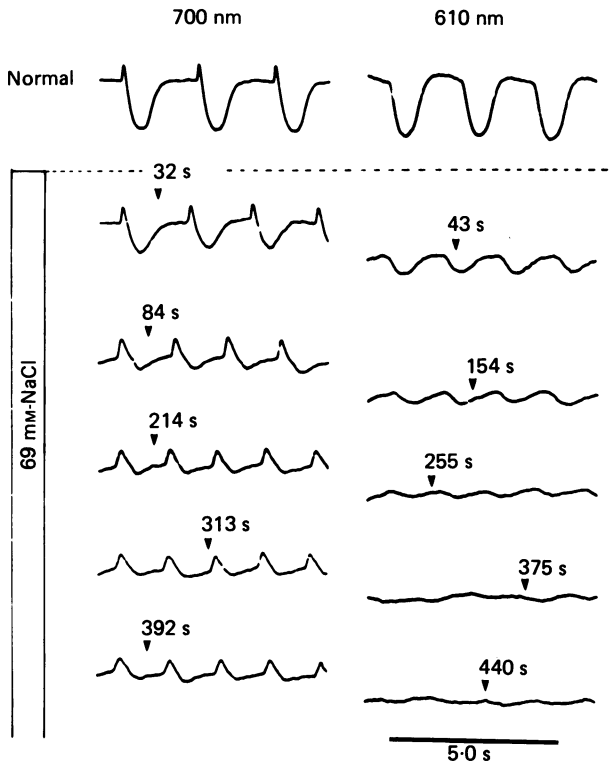

Fig. 10. Optical signals from a 10-somite embryonic heart in a low-Na^+ solution made by replacement of 69 mM-NaCl in the normal bathing solution with 69 mM-choline chloride. During the measurement, the 700 ± 11 and 610 ± 9 nm interference filters were used alternately. Temperature, 36·4–37·1 °C. Arrows indicate the stated times after the heart was put in the low-Na^+ solution. Note that in the beginning of the recording, because the 2nd signal was relatively large, the 1st signal was distorted by the 2nd signal.

potential activates the myofibrils directly and then tension is generated. The very small diameter and poorly developed intracellular membrane structure in early embryonic heart cells may easily permit Ca^{2+} diffusion to the central myofibrils.

In electron-microscopic investigations, it has been shown that neither the transverse tubular system (T-system) nor sarcoplasmic reticulum are differentiated in the early embryonic chick heart (Manasek, 1968; Ishikawa & Yamada, 1975; also for review see Manasek, 1979). Therefore, the second hypothesis seems more likely than the first.

Morad & Orkand (1971) found that in the adult frog ventricle, the time-to-peak tension was linearly related to the duration of the depolarization, if the plateau of the action potential was terminated prematurely by voltage-clamp repolarization. We also examined the correlation between the time-to-peak of the 2nd signals and

the action potential shape. In our preparation, however, significant change in the time-to-peak with changes in the external Ca^{2+} concentration was not clear. Thus, it is possible that relaxation of a beat is limited by removal of intracellular free Ca^{2+} and that this process is swamped in the experiments using high-Ca^{2+} concentrations or the Ca^{2+} ionophore.

Reuter & Seitz (1968) demonstrated that the Ca^{2+} efflux rate in adult cat myocardium was sensitive to the concentration gradient for Na^+ across the sarcolemma. Vassort (1973) studied tension responses elicited by depolarizing pulses in frog atrium as affected by alteration of the external Na^+ concentration. He found that as the external Na^+ concentration was decreased at a fixed external Ca^{2+} concentration, peak tonic tension increased transiently by as much as 50% before it declined.

Similarly, from results obtained in the present experiments using low-Na^+ bathing solutions, it is tentatively suggested that the Na^+–Ca^{2+} exchange mechanism participates in the relaxation process in the early embryonic heart. The progressive reduction of the contraction in the low-Na^+ solution shown in Fig. 10 may be produced by the following process: reduction in the Na^+–Ca^{2+} exchange rate → decrease in the Ca^{2+} efflux rate → accumulation of intracellular free Ca^{2+} → reduction of the rate of Ca^{2+} release from myofibrils → limitation of the relaxation → reduction of contraction. Chapman (1974) also suggested that the low-Na^+ contracture in frog atrial trabeculae is produced by an influx of Ca^{2+} via a surface membrane Na^+–Ca^{2+} exchange. While we cannot rule out the possibility that the Ca^{2+} pump is linked with the relaxation process, we have no data to support this idea.

It has been suggested that hypertonic solutions and D_2O Ringer solution affect the contractile elements or the excitation–contraction coupling in skeletal muscle (Svensmark, 1961; Gordon & Godt, 1970). In the early embryonic chick hearts in which both the T-system and sarcoplasmic reticulum are undifferentiated, hypertonic and D_2O Ringer solutions decreased contraction without severely reducing amplitude of the Ca^{2+}-dependent action potentials. Therefore the suppression of contraction is most likely due to a decrease in mechanochemical response of the contractile system.

The results shown in Fig. 4 demonstrate the area where the contractility and/or Ca^{2+} sensitivity of myofibrils are first developed at the time of initiation of the heartbeat. Additionally, the maps of contractile areas during development, indicate that the contractile system is generated regionally in the early phases of cardiogenesis. The different contraction *vs.* Ca^{2+} influx relationships shown in Fig. 6 indicate that there are regional differences in the sensitivity of the contractile system to Ca^{2+} in the early developing embryonic heart.

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