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ORIGINAL PAPER

The beginning of the calcium transient in rat embryonic heart

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Abstract Although many researchers have tried to observe the beginning of the heartbeat, no study has shown the beginning of the calcium transient. Here, we evaluate the beginning of the calcium transient in the Wistar rat heart. We first tried to reveal when the heart of the Wistar rat begins to contract because no previous study has evaluated the beginning of the heartbeat in Wistar rats. Observation of embryos transferred to a small incubator mounted on a microscope revealed that the heart primordium, the so-called cardiac crescent, began to contract at embryonic day 9.99–10.13. Observation of embryos loaded with fluo-3 AM revealed that the beginning of the calcium transient precedes the initiation of contraction which precedes the appearance of the linear heart tube. Nifedipine (1 μM), but not ryanodine (1 μM), abolished the calcium transients. These results indicate that calcium transients in the early embryonic period involve exclusively calcium entry through L-type calcium channels in contrast to the situation in mature hearts. This study provides the first demonstration of the relationship between morphological changes in the heart primordium and the beginning of the calcium transient and contraction.

Keywords Cardiogenesis · Developmental biology · Excitation-contraction coupling · Calcium transient

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Introduction

The cardiovascular system is necessary for animals to transport oxygen, carbon dioxide, nutrients, and waste products to/from cells. Therefore, the heart must begin to contract early during development. For a long time, researchers have tried to observe the initiation of heart contraction in the chick [1, 2], zebrafish [3], mouse [4, 5], and rat [6–8] and have shown that the first contractions are weak, and then the contracting area enlarges gradually [1, 7].

During the usual action potential, calcium influx via L-type calcium channels triggers the release of calcium from the sarcoplasmic reticulum (SR) by activating ryanodine receptors (RyR) [9–12]. The combination of calcium influx and release raises the free intracellular calcium concentration, the so-called calcium transient. The calcium transient results in calcium binding to troponin C, the interaction between myosin and actin, and contraction [13, 14]. These reports raise the following questions: Does the calcium transient begin in conjunction with the beginning of the contraction? Are the first calcium transients weak and then enlarge gradually in the same way as the contraction? Unfortunately, these questions remain unanswered because no study has caught the beginning of the calcium transient. Therefore, we evaluated when and how the calcium transient begins.

Contraction of mature cardiomyocytes is known to depend on the release of calcium from the SR through RyR [11, 12]. In contrast, the contraction of immature cardiomyocytes depends on calcium influx through L-type calcium channels rather than calcium release from the SR [11, 15–17]. We previously reported that ryanodine (1 μM), an SR calcium release channel inhibitor, had less effect on the embryonic 10.5 (E 10.5)-day Wistar rat heart than on the E

12.5-day heart [15]. In addition, Takeshima et al. [16] showed that contraction of the RyR-2 knockout mouse heart was observed at E 9.5 days; this mutant mouse died at approximately E 10.5 days. These reports suggest that the first contractions and first calcium transients depend on trans-sarcolemmal calcium influx through L-type calcium channels. Another study, however, showed that spontaneous calcium transients in early-stage embryonic stem cell–derived cardiomyocytes were independent of trans-sarcolemmal calcium influx [18]. Therefore, we investigated the role of L-type calcium channels in the beginning of the calcium transient.

Materials and methods

Definition of embryonic period

These experiments were reviewed and approved by the Ethics Committee for Animal Experiments and carried out under the Guidelines for Animal Experimentation at Sapporo Medical University. Female Wistar rats were mated with male rats overnight for 12 h. When a vaginal plug was found in the morning, the midpoint of the previous night was designated embryonic day 0.00 (E 0.00 day).

Recording of the contraction only

The embryo was removed from a pregnant rat that had been anesthetized by pentobarbital sodium, as previously described [15]. For recording the contraction, the embryos were transferred to a small incubator (MI-IBC, Olympus, Tokyo, Japan) mounted on a microscope. The incubator contained Tyrode solution as a perfusate, at 37°C. The composition of Tyrode solution (in mmol/L) was NaCl 143, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.33, glucose 5.5, HEPES 5.0 (pH 7.4 by NaOH). The contraction of the heart primordium was recorded on a digital versatile disc (DVD) recorder (DMR-XW30, Panasonic, Osaka, Japan) via charge-coupled device (CCD) camera (CS230B, Olympus, Tokyo, Japan) at standard video rate (29.97 frames per second). The motion of the heart primordium was detected by ImageJ software (NIH, Bethesda, MD, USA).

Recording of the contraction and calcium transient

In order to record the appearance of calcium transients, the pericardium of the embryo was removed and incubated with fluo-3 AM (8.85 μM; Dojindo Laboratories) and pluronic F127 (0.25%; Molecular Probes) for 7 min. Subsequently, the embryos were transferred to a small incubator and were kept in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with fetal bovine serum (FBS) (10%, Invitrogen), streptomycin (100 μg/mL, Invitrogen), penicillin (100 units/mL, Invitrogen), and L-glutamine (4 mM, Invitrogen), equilibrated with 5% CO_2. The main composition of this mixed DMEM (in mmol/L) was Na 158, K 6.0, Ca 1.9, Mg 0.9, Cl 126, glucose 5.4. The calcium transients were recorded with imaging software (MetaMorph, Universal Imaging, Buckinghamshire, UK) by a cooled CCD camera (I-Penta-MAX, Princeton Instruments, Trenton, NJ, USA). Typical frame rate for recording the calcium transient was 49.8 frames per second. The contraction (visible light signal) and calcium transient (fluorescence signal) were recorded one after the other. The characteristics of the calcium transient were evaluated by imaging software (MetaMorph) and ImageJ software, as previously described [15]. To evaluate the effect of nifedipine and ryanodine (Wako Pure Chemical Industries), the embryos were transferred to hand-made flow chambers used in patch-clamp recording [19].

Statistical analysis

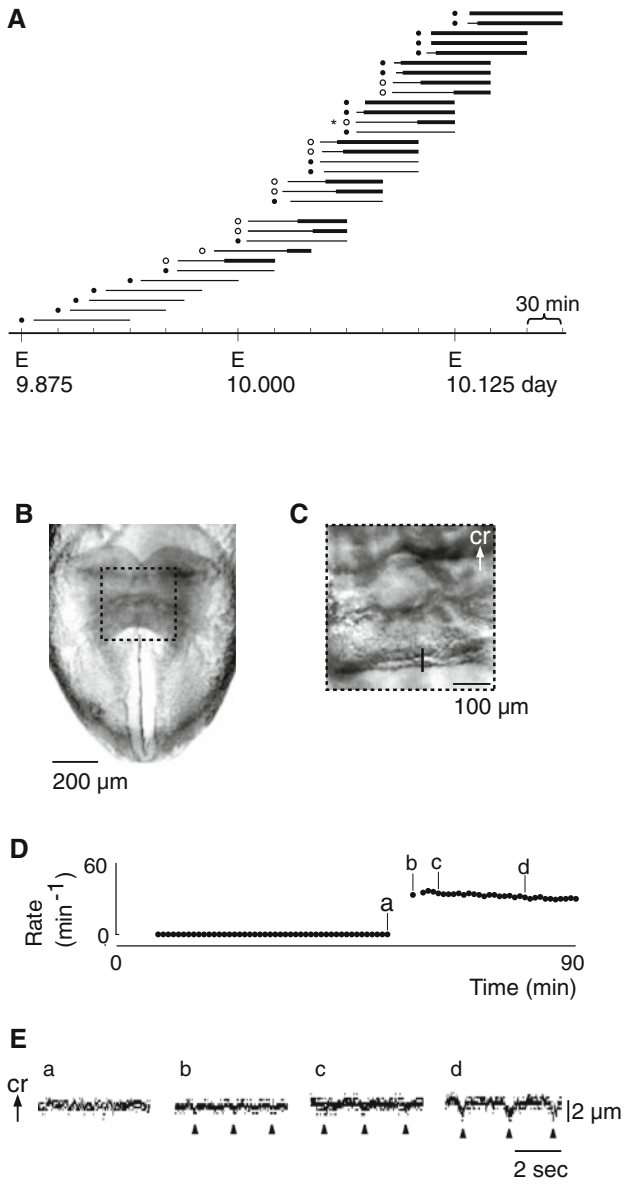
Values are expressed as mean ± SEM. The statistical significance of differences in means was evaluated by ANOVA followed by Bonferroni's test. Differences were considered significant when P values were less than 0.05.

Results

The beginning of contraction of the rat heart primordium

Goss estimated the age of rat embryos at the time of initiation of contraction as approximately E 9.5 days [7]. Our preliminary study, however, revealed that Wistar rat heartbeat was not observed at E 9.5 days. This discrepancy might be mainly due to different strains of rat, although he did not identify the strain used. Therefore, we began this study by clarifying when the Wistar rat heart begins to contract.

To observe the initiation of rat heart contraction, the embryo was removed from a pregnant rat between E 9.88 and E 10.19 days and transferred to a small incubator mounted on a microscope. Figure 1A shows the relationship between operation period and the initiation of contraction. As previously reported [7], there were variations in the period at which heart primordia began to contract. In this study, we used a small incubator, which required 16 min for the specimens to reach a constant temperature of 37°C. We cannot rule out the possibility that heart primordia could not contract temporarily because of the low temperature of the dissection solution. Therefore, we



◀ **Fig. 1** The initiation of contraction of the rat heart primordium. **A** Time courses of the beginning of contraction of heart primordia between E 9.88 and E 10.19 days ($n = 31$). Each *circle* represents the time at which an embryo was removed from one pregnant rat. The time courses with *open circles* represent data from embryos that began to contract more than 16 min after transfer to the incubator. The time courses with *closed circles* represent data from embryos that began to contract within 16 min of transfer to the incubator or that did not begin to contract. The *thin line* represents the period when the heart primordium did not contract. The *bold line* represents the period when the heart primordium exhibited beating. The *left edge* of *each bold line* indicates the time when beating became apparent. **B** Representative *ventral view* of a rat embryo just removed from the uterus. This example corresponds to the time course marked with the *asterisk* in **A**. **C** The expanded picture at the time when the embryo was observed in a small incubator. This picture corresponds to the *dotted rectangle* in **B**. **D** The change in beating rate. **E** The recording of tissue motion corresponding to the position shown as a *black line* in **C** and to the time shown in **D**. The data were obtained using ImageJ software. *Arrowheads* indicate the timing of contractions. Direction of the contraction, *cr* (cranial), corresponds to that in **C**

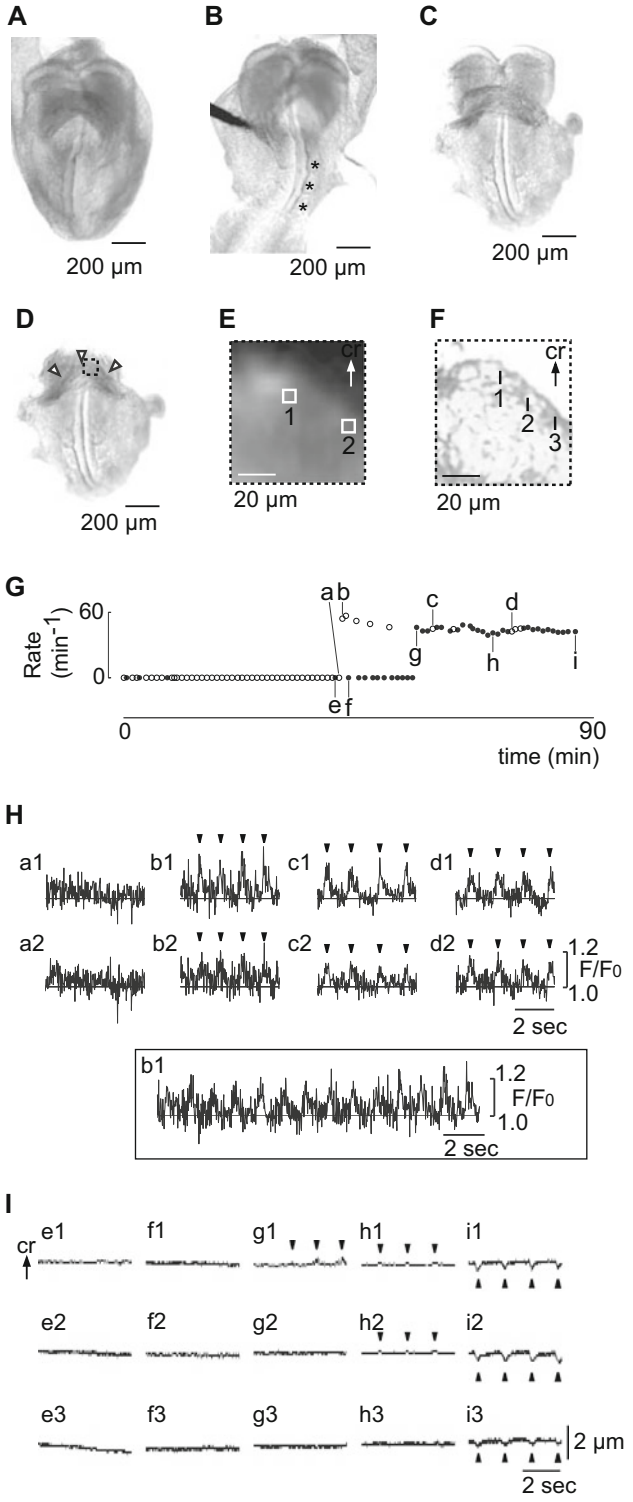
evaluated only tissues that began to contract beyond 16 min (indicated by open circles in Fig. 1A). Figure 1E and Supplementary movie 1 show representative data exhibiting the change in contraction of the heart primordium around the period of the appearance of the heartbeat. The observed first contractions were so weak that it was difficult to count the beating rate. Several minutes later, contractions became gradually stronger and counting became feasible (Fig. 1E*b* and panel b of Supplementary movie 1). When counting was feasible, heart primordia contracted regularly at a rate of 43.5 ± 5.0 beats/min ($n = 11$). Subsequently, the contractions became stronger at a constant rate (Fig. 1D, E and panels c, d of Supplementary movie 1). These results indicated that the heart began to contract at E 9.99–10.13 days (9 days 23 h 49 min to 10 days 3 h 0 min, 10.06 ± 0.01 days, $n = 11$).

The appearance of calcium transients and contraction

In order to investigate the relationship between the calcium transient and contraction, we carefully removed the pericardium of embryos aged E 10.03–10.06 days (Fig. 2A–D) and incubated the dissected tissues with fluo-3 AM. Figure 2 shows a representative experiment simultaneously recording contraction and calcium transient. Figure 2I and Supplementary movie 2 show representative data exhibiting changes in contraction. The first contraction was recognized during period g (Fig. 2I *g1* and panels g, g′ of Supplementary movie 2). The first contractions were weak and the contracting area was small. Then the contracting area enlarged gradually (Fig. 2I *h2*, *i3* and panels h, i of Supplementary movie 2) at an approximately constant rate (Fig. 2G), as observed in the embryos with the pericardium (Fig. 1). These results indicate that the number of contracting cells increased with time.

Interestingly, a spontaneous calcium transient was observed before the initiation of contraction (Fig. 2H *b1*, *b2* and panel b of Supplementary movie 2). The frequency of the calcium transients was regular, and they exhibited a small amplitude at first, which increased over several seconds (Fig. 2H *inset*, Fig. 4D *a*, panel b of Supplementary movie 2 and panel a of Supplementary movie 3). In contrast to the situation at the initiation of contraction, which appeared in a small area, the calcium transient first appeared throughout the heart primordium (Fig. 2H *b1*, *b2*, panel b of Supplementary movie 2 and panel a of Supplementary movie 3). After the beginning of contraction, the frequency of calcium transients was the same as the heart rate calculated from the contractions (Fig. 2G).

Figure 3 shows the starting points of the calcium transient and contraction at E 10.5 days and E 10.03–10.06 days for heart primordia. One heart primordium at E 10.5 days



◀ **Fig. 2** Representative data showing the relationship between the beginning of the calcium transient and contraction of the rat heart primordium loaded with fluo-3 AM. **A** Representative ventral view of a rat embryo just removed from the uterus. This sample corresponds to the data marked with the *asterisk* in Fig. 3. **B** Dorsal view of the embryo shown in **A**. *Asterisks* indicate somites. The number of somites for this embryo is 3. **C** Ventral view of the embryo. The pericardium was partially removed and the caudal part of the embryo was removed. **D** Ventral view of the embryo. The pericardium and forebrain were almost completely removed. *White arrowheads* indicate the outer margin of the heart primordium. **E**, **F** Fluorescence (**E**) and visible light (**F**) images of the embryo in the small incubator. These pictures correspond to the *dotted rectangle* in **D**. **G** The change in rate. *Open circles* represent the rate calculated from the fluorescence movie and *filled circles* represent the rate calculated from the visible light movie. **H** Fluorescence recordings of the change in the calcium transient corresponding to several parts shown in **E** and to several times shown in **G**. *Arrowheads* indicate the peak of the calcium transient. The data in the *inset panel* show a longer dataset than subpanel *b1*. **I** Recordings of motion corresponding to several parts shown in **F** and to several shown in **G**. Direction of the contraction, *cr* (cranial), corresponds to that in **F**

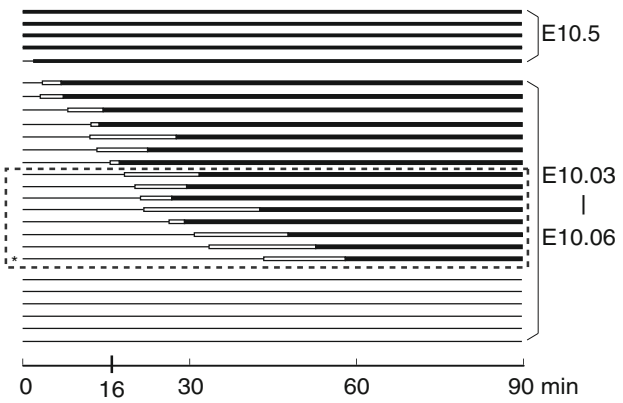


Fig. 3 Time courses of the beginning of the calcium transient and contraction of rat heart primordia loaded with fluo-3 AM at E 10.5 days ($n = 5$) and E 10.03–10.06 days ($n = 21$). The *left edge* of *each line* indicates the start time of observation. The *thin line* represents the period when the heart primordium did not contract. The *white bar* represents the period when the calcium transient was observed and the contraction was not observed. The *black bar* represents the period when the heart primordium contracted with calcium transients. In samples surrounded by a *broken line*, the calcium transient began more than 16 min after the start of observation. The frequencies of the first calcium transients and first contractions of these samples were 57.8 ± 4.9 and 54.1 ± 3.5/min, respectively ($n = 8$). Detailed data for the sample indicated with an *asterisk* are shown in Fig. 2

did not contract just after transfer to the incubator (Fig. 3) but resumed contraction soon thereafter because of the rise in temperature. On the other hand, other hearts at E 10.5 days already showed beating upon transfer to the incubator, but the rate was slow and increased with time (57.9 ± 14.9 and 98.7 ± 4.9 beats/min at 0 and 16 min after transfer to the incubator, respectively; $n = 5$). These results indicated that temperature affects the frequency of the calcium transient and contraction. In the region of eight embryos surrounded by the broken line in Fig. 3, the calcium transients began 16 min after the start of observations. Analysis of these eight primordia revealed a precise time lag (12.9 ± 2.1 min, $n = 8$) between the beginning of the calcium transient and contraction. The number of somites of these eight embryos ranged from three to five.

Table 1 Characteristics of calcium transients

	Rate (min^{-1})	Time to peak (ms)	Time constant of decay (ms)
E 10.03–E 10.06 days with only calcium transient (area surrounded by the broken line in Fig. 3, $n = 8$)	55.7 ± 3.8	165.7 ± 13.0	234.7 ± 9.9
E 10.03–E 10.06 days with calcium transient and contraction (area surrounded by the broken line in Fig. 3, $n = 8$)	52.7 ± 3.1	170.8 ± 12.0	235.1 ± 15.1
E 10.5 days (16 min after transfer to the incubator, $n = 5$)	98.7 ± 4.9*,†	124.6 ± 9.8	132.5 ± 9.7*,†

Data are mean ± SEM

* $P < 0.01$ versus E 10.03–E 10.05 days with only calcium transient, † $P < 0.01$ versus E 10.03–E 10.05 days with calcium transient and contraction

We evaluated the characteristics of the calcium transient (Table 1). There were no differences in rate, time to peak, and time constant of decay of calcium transient among these eight primordia without contraction and with contraction.

The role of L-type calcium channels in the first calcium transients

To investigate the role of L-type calcium channels in the first calcium transients, nifedipine, a selective L-type calcium channel blocker, was applied to heart primordia exhibiting a calcium transient without contraction. At a concentration of 1 μM, nifedipine abolished the calcium transients in the heart primordium (Fig. 4D *c* and panel c of Supplementary movie 3). After washout of nifedipine, calcium transients reappeared (Fig. 4D *d* and panel d of Supplementary movie 3). The nifedipine-induced abolishment of the calcium transient was observed in five different preparations.

In order to clarify the possible contribution of intracellular calcium stores, ryanodine, a specific calcium-release channel (RyR) blocker, was applied to the preparations exhibiting calcium transients. Ryanodine (1 μM) did not affect the calcium transients ($n = 3$, data not shown). These results indicate that calcium transients in the early embryonic period result exclusively from calcium entry through L-type calcium channels.

The relationship between morphological changes in the heart primordium and the beginning of the calcium transient and contraction

We also analysed the relationship between morphological (or chronological) and functional changes in the heart primordium. Before initiation of the calcium transient and contraction, the heart primordium displayed a flat shape (number of somites: 2–3, Fig. 5A). When the central part of the heart primordium rose up (number of somites: 3–5, Fig. 5B), the calcium transient appeared throughout the

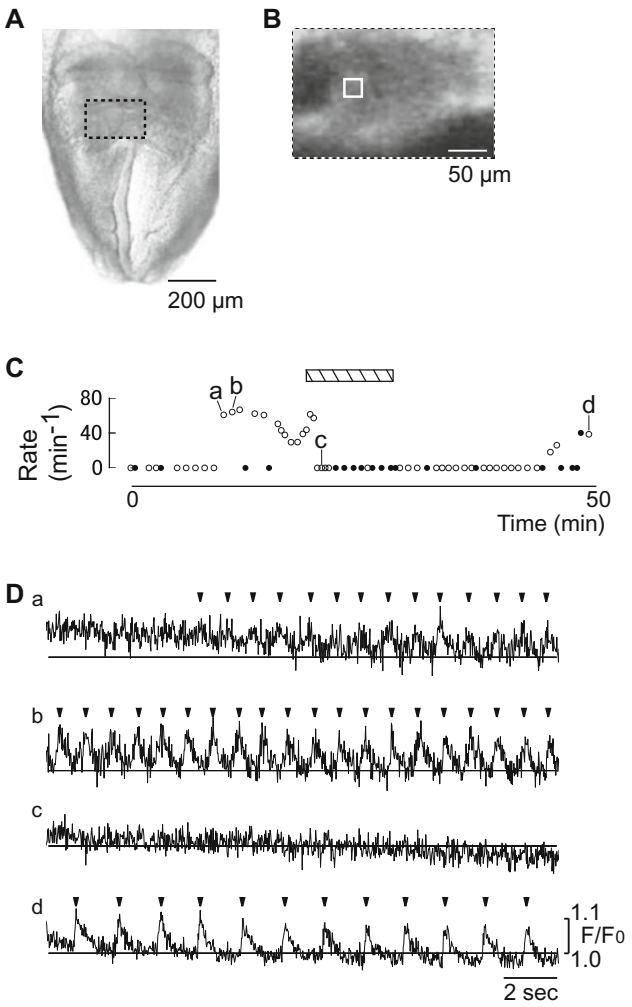


Fig. 4 The effect of nifedipine on the calcium transient. To apply and wash out nifedipine, embryos ($n = 5$) were continuously perfused with DMEM. **A** Representative ventral view of a rat embryo just removed from the uterus. **B** Fluorescence image when the embryo was observed in a small incubator. This picture corresponds to the *dotted rectangle* in **A**. **C** The change in rate. *Open circles* represent the rate calculated from the fluorescence movie, and *filled circles* represent the rate calculated from the visible light movie. The *hatched box* represents the period of nifedipine (1 μM) application. **D** Fluorescence recordings of the change in calcium transient corresponding to the *white rectangle* in **B** and to several times shown in **C**. *Arrowheads* indicate the peak of the calcium transient

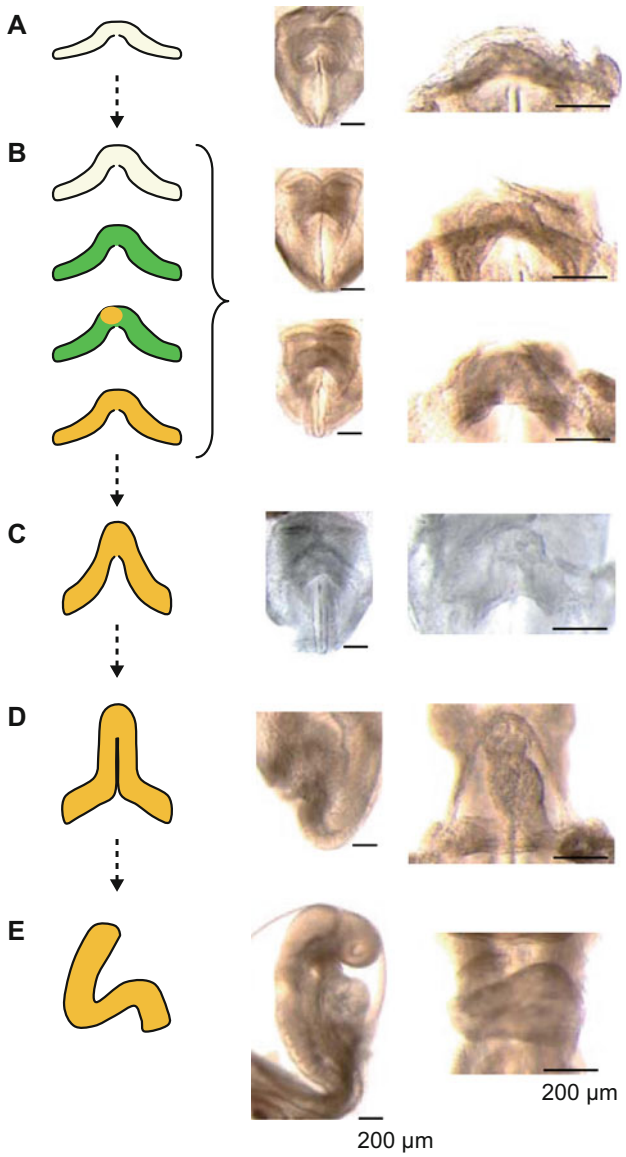


Fig. 5 The relationship between the morphological changes in the heart primordium and the beginning of the calcium transient and contraction. The *drawings* at the left of each panel show the morphological changes in the heart primordium. The *drawing* in **A** represents the heart primordium before initiation of the calcium transient and contraction. In **B**, the *green* and *yellow areas* correspond to the expression of calcium transients and contraction, respectively. The *drawing* in **C** represents the heart primordium after the calcium transient and contraction have already begun. These changes shown in **A**, **B**, and **C** were observed at approximately E 10.0 days. The *drawings* in **D** and **E** represent the heart primordium at E 10.25 days and E 10.5 days, respectively. The images in the *center of each panel* represent the whole embryos corresponding to each cartoon. The *images* at the *right of each panel* were recorded after removal of the pericardium of each embryo shown in the center

heart primordium. After the appearance of the calcium transient, contraction began in a small area of the heart primordium, which then enlarged with time. Therefore, cardiac contraction had begun before fusion of the lateral parts of the heart primordium (number of somites: 3–5, Fig. 5C), after which the heart primordium formed a linear heart tube (Fig. 5D) and finally the looped heart (Fig. 5E).

Discussion

This is the first study revealing that a spontaneous calcium transient begins before the initiation of contraction of the Wistar rat heart. In addition, the calcium transient and the contraction begin before the appearance of a linear heart tube.

Among the previous papers on this subject, only two described the beginning of the heartbeat, e.g., Goss's report showing the beginning of rat heart contraction [7]. Because embryos used in the study were cultured for 2 h before the motion was recorded, they swelled at the recording. Therefore, a wax plate reconstruction technique was applied in order to reveal the morphological features of rat embryonic hearts that began to beat. Unfortunately, the author did not particularly describe the shape of the outer margin of the heart primordium. Therefore, it was difficult to understand the relationship between morphological changes in the heart primordium and the beginning of contraction. The manuscript included the sketch of the living rat whole embryo immediately after removal from the uterus (Fig. 8 in [7]). This sketch appeared similar to our photograph shown in Fig. 2A. Therefore, we expected that Goss might have observed the first heartbeat before the appearance of a linear heart tube, similar to our result.

Another description of the beginning of the heartbeat is found in Patten's report [1]. He reported that the embryonic chick heart began to contract after formation of a linear heart tube. It was easy to understand the relationship between morphological changes in the heart and the beginning of contraction because he described the shape of the outer margin of the heart primordium not only at the beginning of contraction but also after the beginning of contraction. Therefore, several reviews have mentioned that the linear heart tube shows the first heartbeat [1, 20, 21]. Patten's result was similar to Hirota's result [2]. Although the beginning of the heartbeat was not observed, Hirota et al. intermittently recorded the changes in fluorescence of chick embryos loaded with voltage-sensitive dye and reported that (1) the electrical activity of the chick was detected in either the lateral part of the heart primordium before fusion of the heart primordium, and (2) contraction of the chick heart begins after fusion of the heart primordium. The fluorescence signal was also used for analysis of the change of contraction. The fluorescence was detected with the 100 elements of a 10 × 10 square array of silicon photodiodes. Therefore, the first contractions could be missed, since we showed that, in the rat heart, they were

very weak. For these reasons, we assume that contraction of the chick heart may begin before the appearance of a linear heart tube, as in the rat heart.

When developmental changes in embryonic tissue are investigated, somite staging is usually applied [22]. Previous reports, however, mentioned that heart development is not strictly synchronous with developmental processes such as somite formation [7, 23, 24]. For this reason, Biben et al. [23] proposed a new classification based on morphogenetic progression of the heart. At the first stage of their classification, the LS-0 stage, the heart is a linear tube. This classification had been reasonable because the linear heart tube was believed to show the first heartbeat [1, 20, 21]. In this study, however, we observed the beginning of contraction before the appearance of the linear heart tube. Therefore, we cannot apply Biben's classification to our data. In addition, there is interlitter developmental variability that does not originate from differences in timing of fertilization [25]. For all of these reasons, we believe our data clearly indicate the time point at which the heart primordium of the Wistar rat begins to contract.

In this study, we showed that the first calcium transients appeared throughout the heart primordium. In addition, there were no differences in rate, time to peak, and time constant of decay of the calcium transient between the primordia without contraction and with contraction. These results indicate that action potential conduction to the whole heart primordium may already be established in conjunction with the beginning of the calcium transient. Hirota et al. [2, 8], however, showed that the electrical activity was detectable in a small area at first and enlarged with time. One reason for this discrepancy may be species differences between mammals and avians. Another may be the different recording methods used. Although Hirota et al. [2] intermittently examined discrete embryos, we continuously recorded the same embryo. Further experiments are necessary to reveal whether or not the inconsistency in the origin of automaticity is due to differences in the beginning of membrane excitation and the calcium transient.

Previous studies examined the expression of contractile and regulatory proteins, i.e., α-actin, myosin, and troponin C, of early-stage embryonic heart [26–30]. However, it remains unclear which molecule orders the heart to contract. In this study, we showed that the appearance of calcium transient precedes the initiation of contraction at a relatively short interval. This result suggests that repetitive calcium transients first modify the activities of intracellular enzymes and then enhance phosphorylation of regulatory proteins.

Some researchers assumed that it was difficult to investigate the early-stage embryonic heart [18, 31–33]. Recently, it was reported that embryonic stem cell–derived cardiomyocytes recapitulate features of embryonic cardiac development [34, 35]. Therefore, embryonic stem cell–derived cardiomyocytes were used as a tool for the investigation of early cardiomyogenesis [18, 31–33]. Viatchenko-Karpinski et al. [18] showed that spontaneous calcium transients in embryonic stem cell–derived cardiomyocytes were independent of trans-sarcolemmal calcium influx. On the other hand, we previously reported that ryanodine (1 μM) had less effect on the E 10.5-day than the E 12.5-day rat heart [15]. E 10 and E 12 days in that study correspond to E 10.5 and E 12.5 days, respectively, in the present study. Takeshima et al. [16] showed that contraction of the RyR-2 knockout mouse heart was observed at E 9.5 days, although this mutant mouse died at approximately E 10.5 days. In the present study, we showed that nifedipine, but not ryanodine, abolished calcium transients. These results indicate that calcium transients in the early embryonic period involve exclusively calcium entry through L-type calcium channels in contrast to the situation in mature hearts.

In this study, we showed that L-type calcium channels play a pivotal role in excitation-contraction coupling before the appearance of a linear heart tube. Liu et al. showed that the 5′-flanking region of the gene encoding the α_{1C} subunit of the L-type calcium channel possesses several consensus sequences for binding of transcription factors, i.e., Nkx 2.5 and MEF2 [36]. Other studies reported that gene expression of Nkx 2.5 and MEF2 was detected in the pre-beating heart primordium of the embryonic mouse [37, 38]. Therefore, it is assumed that these transcription factors may directly induce the expression of the α_{1C} subunit. Unfortunately, no previous paper showed changes in the expression of subunits of the L-type calcium channel around the period of the appearance of the calcium transient. It is likely that Nkx2.5 or MEF2 induces transcription of L-type calcium channels and then produces the initiation of the calcium transient.

In evaluating the frequency of the calcium transient and contraction, temperature is a key factor. At the exchange of solution for nifedipine, the temperature decreased briefly because of the decrease in flow rate of the solution, as shown in Fig. 4C. In addition, one heart primordium of E 10.5 days did not contract just after transfer to the incubator (Fig. 3) but resumed contraction soon thereafter because of the rise in temperature. On the other hand, other hearts at E 10.5 days already showed beating upon transfer to the incubator, but the rate was slow and increased with time. Therefore, we analyzed only the data taken starting 16 min after the start of observation of E 9.99- to 10.13-day hearts.

In the experiment with fluo-3 AM, we firstly chose Tyrode solution as perfusate. Under these conditions, several heart primordia that had begun to contract showed

cessation of the heartbeat. This phenomenon was never observed in embryos with the pericardium. These results suggested that fluid in the pericardial cavity was important for cardiomyocytes of the heart primordium. For evaluating the calcium transient, however, the pericardium must be removed to load with fluo-3 AM. Therefore, we chose DMEM as perfusate. Accordingly, no heart primordium in DMEM showed cessation of the calcium transient and contraction. These phenomena may be due to differences in the amino acid and protein composition and/or osmolality of Tyrode solution and those of DMEM.

The present study is the first one revealing the precise relationship between the morphological changes in the heart primordium and the beginning of the calcium transient and contraction. The present study also provides moving images of the initiation of the calcium transient and contraction for the first time.

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Conflict of interest The authors declare that they have no conflict of interest.

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