Development of Mechanisms Regulating Intracellular Ca\(^{2+}\) Concentration in Cardiac Muscle Cells of Early Chick Embryos

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The development of mechanisms for the regulation of intracellular-free calcium ion concentration ([Ca\(^{2+}\)]\(_i\)) was investigated in precardiac mesodermal cells (PMC) and cardiac muscle cells (CMC) from early chick embryos by microfluorometry using a Ca\(^{2+}\)-sensitive fluorescent probe, fura-2, and transmission electron microscopy. Microfluorometry indicated that two types of regulatory mechanisms, involving the dihydropyridine receptor (DHPR) and the ryanodine receptor (RYR), are present in CMC when the heartbeat begins at the 8–9 somite stages. Nifedipine completely suppressed the beating of hearts isolated from embryos on Days 1.5 and 2. Ryanodine had no effect on the beating of hearts isolated from embryos on Day 1.5, though it completely suppressed beating in hearts from Embryonic Day 2. Microfluorometry revealed that a change occured in the Ca\(^{2+}\)–regulating mechanisms of CMC on Day 2. Transmission electron microscopy showed the appearance in CMC, also on Day 2, of peripheral couplings with feet structures, and SR adjacent to the Z-line of myofibrils. These findings suggest that the calcium-induced calcium-release (CICR) mechanism appears in the CMC of the chick on the second day of embryonic development.

INTRODUCTION

The cyclic increase in the intracellular-free calcium ion concentration ([Ca\(^{2+}\)]\(_i\)) seen in cardiac muscle cells (CMC) is regulated by the coupling of Ca\(^{2+}\) influx and Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR). The entry of extracellular Ca\(^{2+}\) into the cytoplasm is achieved via the dihydropyridine receptor (DHPR; a voltage-dependent Ca\(^{2+}\) channel, which is selectively blocked by dihydropyridine) in the plasma membrane. A local increase in [Ca\(^{2+}\)]\(_i\) triggers the opening of the ryanodine receptor (RYR; a Ca\(^{2+}\)-activated Ca\(^{2+}\) channel that selectively binds ryanodine, a plant alkaloid) in the SR membrane. The opening of the RYRs releases a large amount of stored Ca\(^{2+}\) from the SR, and the increased [Ca\(^{2+}\)]\(_i\) then induces cell contraction (for review see Chapman, 1979; Fabiato, 1983; Wier, 1990; Callewaert, 1992; Stern, 1992; Niggli and Lipp, 1995). These regulatory mechanisms for [Ca\(^{2+}\)]\(_i\) in CMC are termed the calcium-induced calcium-release (CICR) mechanism (Fabiato and Fabiato, 1979).

During the course of the heart development, the precardiac mesodermal cells (PMC) acquire the regulatory mechanisms that produce cyclic increases in [Ca\(^{2+}\)]\(_i\) as well as the apparatus needed for cell contraction, such as myofibrils. During heart development in chick embryos, components of the myofibrils such as titin (Tokuyasu and Maher, 1987) and isomyosin (De Jong et al., 1987) appear before the heart has begun to beat. The myofibrils themselves are first detected at the 8 somite stage, when the heartbeat begins (Hiruma and Hirakow, 1985). Thus, a close correlation between myofibrillogenesis and the initiation of the heartbeat has been demonstrated. On the other hand, the correlation between the development of [Ca\(^{2+}\)]-regulating mechanisms and the initiation of the heartbeat is not clearly understood. Immunohistochemistry has shown that the Ca\(^{2+}\) pump (Ca\(^{2+}\), Mg\(^{2+}\)-ATPase) in the SR membrane appears at 9–11 somites, just after the heart has begun to beat (Jorgensen and Bashir, 1984). However, L-type Ca\(^{2+}\) channels/DHPRs and RYRs have not been detected in CMC until Day 3 (Tohse et al., 1992) or Day 4 (Dutro et al., 1993), respectively. Thus, it is unclear how the [Ca\(^{2+}\)]-regulating mechanisms develop and how their development correlates with the initiation of the heartbeat.

In the present study, the development of [Ca\(^{2+}\)]-regulating mechanisms in the CMC of chick embryos was investigated in relation to the initiation of the heartbeat. We followed the development of these regulatory mechanisms in isolated PMC and CMC using microfluorometry with fura-2. In addition, the development of the SR and of peripheral couplings was examined in chick embryos by transmission electron microscopy.
MATERIALS AND METHODS

Embryos. Fertile eggs from white leghorn chickens (Gallus gallus) were obtained from Saitama Prefectural Poultry Experiment Station (Saitama, Japan). They were incubated at 38 ± 0.5°C and at a constant high humidity. The embryos were staged in terms of the number of somites and the number of days of incubation. These stages were defined with Hamburger and Hamilton (1951) stages as follows: Stage 6; 3–5 somites; stage 9; 6–8 somites; stage 10; 9–11 somites and Day 1.5; stage 11; 12–14 somites; stages 12–13; Day 2; stage 17; Day 2.5; stage 20; Day 3; stage 24; Day 4; stage 31; Day 7; stage 34; Day 8; stage 35; Day 9; stage 38; Day 12. Where appropriate, and in the interests of brevity, isolated PMC, CMC, and hearts are given a prefix to indicate the somite stage and the stage of embryonic day at which they were obtained. For example, 4SS:PMC indicates a PMC from an embryo at the 4 somite stage, whereas 1SD:CMC indicates a CMC from an embryo at Day 1.5. Somite stage and day were abbreviated to SS and D, respectively.

Microfluorometry of fura-2-loaded cells. PMC and hearts were isolated from embryos in basal salt solution for chick embryos (BSS; 140 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 3 mM HEPES, pH 7.4). The BSS is a modification of Philips’ (1941) solution buffer and postfixed in 1% OsO₄ and 0.1 MgCl₂, pH 7.4 containing 0.1% trypsin (1:250, Difco Lab.) and 0.1% collagenase (Sigma). During the measurements, the flow-rate of the solution through the chamber was kept at 300±400 μl/min. The [Ca²⁺]i of the fura-2-loaded cells responded to SSD and showed an increase in [Ca²⁺]i larger than 0.02 (F340/F380) following treatment with the SSD, we defined that the cells responded to the stimuli of depolarization. Because it is not easy to distinguish a [Ca²⁺]i response to SSD smaller than 0.02 from an artificial change in [Ca²⁺]i, we used the SSD containing 50 mM nifedipine (Wako Pure Chemical, Japan) and 100 μM ryanodine (Wako Pure Chemical) and the beating of the heart was monitored for 30 min at 37°C. In control experiments, isolated hearts were incubated in BSS without these drugs.

Transmission electron microscopy. Specific cardiogenic regions containing PMC and ventricle regions of hearts were removed from embryos and cut in pieces (smaller than 1 mm in size) in the BSS-filled chamber (200 μl in volume, the bottom and the top being made of 0.12- to 0.17-mm-thick cover glass) and kept there at 37°C for the early chick embryos. PMC was digested in Ca²⁺/-free BSS [140 mM NaCl, 4 mM KCl, 0.2 mM GEDTA (Dojindo, Japan), 3 mM HEPES, pH 7.4] containing 0.1% trypsin (1:250, Difco Lab.) and 0.1% type II collagenase (Sigma Chemical) for 30–60 min at room temperature (20–25°C), so as to produce small fragments of tissue (50 × 50–100 × 150 μm² in size and 20–30 μm in thickness). Fragments of PMC isolated from each embryo before the 7SS were used for one experiment in each case. Fragments of PMC and CMC isolated from each embryo after the BSS were separated into two portions and used for two experiments in each case. After digestion, the fragments were transferred into a BSS-filled chamber (200 μl in volume, the bottom and the top being made of 0.12- to 0.17-mm-thick cover glass) and kept there for 5 min until the fragments adhered to the glass bottom. Then, BSS was flushed through the chamber for 3–5 min to wash out the digestive enzymes. Next, the BSS in the chamber was replaced by perfusion with 500 μl of BSS containing 5 μg/ml fura-2-AM (Dojindo, Japan). The fragments were incubated for 1–2 hr at 20°C in the BSS containing fura-2-AM. We confirmed that PMC and CMC were viable in the BSS containing fura-2-AM for more than 5 hr. Because no change in morphology of cells and in [Ca²⁺]i response was noticed during the 5-hr incubation. After incubation, BSS was flushed through the chamber for about 10 min to wash out the extracellular fura-2-AM. The [Ca²⁺]i of the fura-2-loaded cells was estimated by the dual-beam (340 and 380 nm) excitation method using an ARGUS-50/CA system (Hamamatsu Photonix, Japan). During the measurements, the flow-rate of the solution through the chamber was kept at 300–400 μl/min. The composition of the salt solution used to induce depolarization (SSD) was: 50 mM KCl, 10 mM NaCl, 2 mM CaCl₂, 1 mM MgSO₄, 3 mM HEPES, pH 7.4. The composition of the Ca²⁺/-free SSD was: 50 mM KCl, 10 mM NaCl, 0.2 mM GEDTA, 3 mM HEPES, pH 7.4. So far as we know from our preliminary experiments, higher concentration of KCl more than 50 mM was somewhat harmful for PMC. Therefore, we used the SSD containing 50 mM KCl. Although the SSD was more hypotonic than the BSS, repeated treatment with the SSD did not cause any changes in morphology and [Ca²⁺]i response (data not shown). According to the data of Spere-Lakis and Shigenobu (1972), 50 mM KCl caused increase of resting potential at about −21 mV in 3D:CMC of chick embryo and the critical [K⁺], level at which elicited membrane excitability and contractions were abolished was about 25 mM in 2D:CMC.

RESULTS

In PMC and CMC, changes in [Ca²⁺]i induced by a depolarization of the membrane potential were measured by microfluorometry to allow us to trace the development of voltage-dependent Ca²⁺ channels/DHPRs in the plasma membrane during the early development of the chick heart. PMC and CMC were depolarized by exposure to SSD which contained a high concentration (50 mM) of KCl. When cells showed a transient increase in [Ca²⁺]i larger than 0.02 (F340/F380) following treatment with the SSD, we defined that the cells responded to the stimuli of depolarization. Because it is not easy to distinguish a [Ca²⁺]i response to SSD smaller than 0.02 from an artificial change in [Ca²⁺]i, we used the 3-4SS:PMC showed no response to SSD (Fig. 1a), but a small number (13% of a total of 14 experiments) of such PMC showed an increase in [Ca²⁺]i in response to the stimulus. The [Ca²⁺]i response showed a slow increase in the ratio by treatment with SSD and then a slow decrease after removal of SSD. Most (75% of a total of 20 experiments) of the 5–6SS:PMC responded to SSD and showed an increase in [Ca²⁺]i during the stimulation (Fig. 1b). At these stages, the PMC are migrating bilaterally toward the heart-forming region, gathering together at the midline, and progressively fusing with each other to form the tubular heart (Stalsberg and DeHaan, 1969). All 8–9SS:CMC (8 experiments) and 10–13SS:CMC (18 experiments) showed an increase in
[Ca$$^{2+}$$] in response to SSD. At these stages, the tubular heart formation is completed and the heartbeat has begun (Patten and Kramer, 1933). Next, the response to SSD was examined during the period shown by the bars (50K$^+$). (a) Application of SSD caused no change in [Ca$$^{2+}$$] in a 4SS:PMC. (b) An induced [Ca$$^{2+}$$] increase is shown in a 6SS:PMC during the application of SSD.

**FIG. 1.** [Ca$$^{2+}$$] responses in PMC induced by depolarization with SSD (which contained 50 mM KCl). The SSD was applied for the period shown by the bars (50K$^+$). (a) Application of SSD caused no change in [Ca$$^{2+}$$] in a 4SS:PMC. (b) An induced [Ca$$^{2+}$$] increase is shown in a 6SS:PMC during the application of SSD.

TABLE 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. (%) of experiments showing</th>
<th>[Ca$$^{2+}$$] increased$^a$</th>
<th>No effect</th>
</tr>
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<tbody>
<tr>
<td>SSD</td>
<td>15 (75%)</td>
<td>5 (25%)</td>
<td></td>
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<tr>
<td>Ca$$^{2+}$$, Mg$$^{2+}$$-free SSD</td>
<td>3 (20%)</td>
<td>12 (80%)</td>
<td></td>
</tr>
<tr>
<td>SSD containing 50 $$\mu$$M nifedipine</td>
<td>2 (12%)</td>
<td>15 (88%)</td>
<td></td>
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$^a$When PMC showed a transient increase in [Ca$$^{2+}$$] larger than 0.02 (F340/F380) following treatment, we defined that the cells responded to the stimuli of depolarization.

Next, the effects of caffeine on [Ca$$^{2+}$$] in PMC and CMC were studied to trace the development of Ca$$^{2+}$$-release channels (RYRs) in the SR membrane during the early development of the chick heart. The response to 10 mM caffeine was examined under BSS and under Ca$$^{2+}$$, Mg$$^{2+}$$-free BSS to reveal the extent of the Ca$$^{2+}$$ release from the intracellular storage sites that was induced by caffeine. Caffeine induced a rapid and transient increase in [Ca$$^{2+}$$] just after the treatment and the ratio (F340/F380) of [Ca$$^{2+}$$] response was larger than 0.1. Therefore, we defined that cells responded to caffeine when the cells showed a rapid and transient increase in [Ca$$^{2+}$$] larger than 0.1. No response was observed in the 4-5SS:PMC (16 experiments) on exposure to caffeine (Fig. 2a). A [Ca$$^{2+}$$] response to caffeine was first observed in some (17% of a total of 24 experiments) of the 6-7SS:PMC. All the 8-9SS:CMC (8 experiments) (Fig. 2b) and 10-13SS:CMC (5 experiments) showed a [Ca$$^{2+}$$] response following treatment with caffeine both in normal BSS and in Ca$$^{2+}$$, Mg$$^{2+}$$-free BSS. The [Ca$$^{2+}$$] response to caffeine was smaller in Ca$$^{2+}$$, Mg$$^{2+}$$-free BSS than in normal BSS. Next, the effect of ryanodine on the [Ca$$^{2+}$$] response to caffeine was examined in the BSS:CMC. In such cells, treatment with 100 $$\mu$$M ryanodine completely suppressed the [Ca$$^{2+}$$] response to caffeine under Ca$$^{2+}$$, Mg$$^{2+}$$-free BSS (Fig. 2c). The appearance, during the development of the chick heart, of [Ca$$^{2+}$$] responses in PMC and CMC to depolarization and caffeine is summarized in Fig. 3.

Having traced the appearance of the two types of regulatory mechanisms involving DHPRs and RYRs, respectively, in the developing chick heart, we next investigated the time at which these two types of regulatory mechanism form a functional coupling. In other words, we investigated the time at which the CICR mechanism appears in CMC during the development of the chick heart. First, the suppressive effects of nifedipine and ryanodine on the heartbeat were investigated in isolated hearts from 1.5D and 2D embryos. Nifedipine (5 $$\mu$$M) completely suppressed, within 1 min, the beating of all the 1.5D isolated hearts (N = 11) and all the 2D isolated hearts (N = 5). Ryanodine (100 $$\mu$$M) had no effect on the beating of the 1.5D isolated hearts (N = 13) after 30-min incubations, but it completely suppressed the beating of all of the 2D iso-
FIG. 3. Development in PMC and CMC of [Ca\(^{2+}\)] responses to 10 mM caffeine (closed circles) and to depolarization by SSD containing 50 mM KCl (open circles). We defined that cells responded when the cells showed a transient increase in [Ca\(^{2+}\)] larger than 0.02 and 0.1 (F340/F380) following treatment with SSD and caffeine, respectively. Numbers in parentheses indicate the numbers of experiments. Numbers on the vertical axis represent the percentage of experiments in which a [Ca\(^{2+}\)] response was observed by the stimuli.

FIG. 2. [Ca\(^{2+}\)] responses in PMC and CMC induced by 10 mM caffeine and the effect of 100 µM ryanodine on the [Ca\(^{2+}\)] response to caffeine. Caffeine or caffeine plus ryanodine were applied for the periods shown by the bars (Caff and Caff + Rya, respectively). (a) No change in [Ca\(^{2+}\)] is induced in a 5SS:PMC under BSS or Ca\(^{2+}\), Mg\(^{2+}\)-free BSS. (b) [Ca\(^{2+}\)] responses to caffeine are induced in an BSS:PMC under BSS and Ca\(^{2+}\), Mg\(^{2+}\)-free BSS. (c) Treatment of an BSS:CMC with ryanodine abolished the [Ca\(^{2+}\)] response to caffeine under Ca\(^{2+}\), Mg\(^{2+}\)-free BSS.

lated hearts (N = 11) within 1–5 min. In BSS without these drugs, 1.5D isolated hearts (N = 3) and 2D isolated hearts (N = 4) all continued active beating for 30 min. Next, the effects of SSD and caffeine on [Ca\(^{2+}\)] in 1.5D:CMC and 2D:CMC were examined using microfluorometry. Small amplitude [Ca\(^{2+}\)] responses [less than 0.2 (F340/F380)] to SSD and a large amplitude [Ca\(^{2+}\)] response [more than 0.5 (F340/F380)] to caffeine were recorded in 1.5D:CMC (Fig. 4a). In contrast, both high KCl and caffeine caused large amplitude [Ca\(^{2+}\)] responses in 2D:CMC (Fig. 4b).

Finally, the development of the SR and of peripheral couplings in PMC and CMC was examined by transmission electron microscopy. The following results were concluded from observations on 3 embryos at 5–7SS, 8 embryos at 8–9SS, 3 embryos at 1.5D, 9 embryos at 2D, and 16 embryos at 3–13D. In 5–7SS:PMC, a very small number of SR-like segments was found close to the plasma membrane (Fig. 5a), but feet were never observed in the junctional gap between the SR-like segments and the plasma membrane. At the 8–9SS, thin myofibrils appear and the heart begins to beat. In CMC from this stage, a small number of SR-like segments was found to be closely attached (9- to 11-nm gap space) to the plasma membrane and, very rarely, feet-like
plasma membrane at this stage (Fig. 5d). Junctional complexes made between the SR and the plasma membrane and involving feet structures are thought to represent peripheral couplings, a type of functional coupling between the SR and the plasma membrane which acts as a focus for the formation of a structural complex between DHPRs and RYRs. The SR extending from such a peripheral coupling ended close to the Z-line of a myofibril (Fig. 5e). The SR increasingly grew to form networks surrounding the myofibrils after the 6–7D embryos. Indeed, well-developed networks of SR surrounding the myofibrils and many peripheral couplings with feet structures were observed in the 9–12D:CMC (not shown). The development of the SR and of the peripheral couplings in the PMC and CMC of early chick embryos is summarized schematically in Fig. 6.

**DISCUSSION**

The present results show that a [Ca\(^{2+}\)] increase in response to a high concentration of KCl appears in PMC and CMC after the 5–6SS in chick embryos. The [Ca\(^{2+}\)] increase was strongly suppressed by treatment with nifedipine (a dihydropyridine) and by removal of Ca\(^{2+}\) and Mg\(^{2+}\) from the outer medium. These results suggest that DHPRs appear in 5–6SS:PMC, preceding the beginning of the heartbeat. Spontaneous action potentials have been shown in the PMC of chick embryos as early as the 5–6SS by means of an optical recording method using a voltage-sensitive merocianine-rhodamine dye (Hirota et al., 1987). Such action potentials do not depend upon a component due to a fast Na current and are characterized as Ca\(^{2+}\)-action potentials, generated by a slow inward Ca\(^{2+}\) current (Hirota et al., 1985). Taken together, these observations indicate that DHPRs in the plasma membrane may play an important role in the generation of action potentials in chick PMC. Previous studies have shown the presence of DHPRs/L-type Ca\(^{2+}\)/channels in CMC of chick embryos after 3D (Tohse et al., 1992) or 7D (Protasi et al., 1996). However, the presence of DHPRs/L-type Ca\(^{2+}\) channels in PMC and CMC before 3D has not been clearly shown. This may be because of technical difficulties or because these channels are very few and scattered in the plasma membrane of PMC and CMC before 3–7D.

In the present study, both treatment with nifedipine and the removal of Ca\(^{2+}\) and Mg\(^{2+}\) from the outer medium strongly suppressed the high KCl-evoked [Ca\(^{2+}\)] increase in all 5–6SS:PMC. However, they could not completely suppress the high KCl-evoked [Ca\(^{2+}\)] increase. This could be because treatment with high KCl caused the release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) storage sites, such as SR and/or mitochondria. However, the mechanism underlying such a release of Ca\(^{2+}\) at this stage is unknown.

Application of caffeine caused a rapid and transient [Ca\(^{2+}\)] increase in CMC after the 8–9SS. The removal of Ca\(^{2+}\) and Mg\(^{2+}\) from the outer medium did not suppress the [Ca\(^{2+}\)] response to caffeine, but pretreatment with ryanodine completely suppressed it. Caffeine is known to activate RYRs by increasing the frequency and the duration of open events.

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**FIG. 4.** [Ca\(^{2+}\)] responses in CMC to 10 mM caffeine and to depolarization by SSD containing 50 mM KCl. SSD or caffeine were applied for the periods shown by the bars (50K\(^{+}\) and Caff, respectively). (a) Small amplitude [Ca\(^{2+}\)] responses to depolarization and a large amplitude [Ca\(^{2+}\)] response to caffeine are shown in a 1.5D:CMC. (b) A large amplitude [Ca\(^{2+}\)] response to caffeine and a large amplitude [Ca\(^{2+}\)] response to depolarization are shown in a 2D:CMC.
FIG. 5. Electron micrographs showing the development of the SR and of peripheral couplings in PMC and CMC. (a) An SR-like segment (arrow) is close to the plasma membrane (PM) in a 5SS:PMC. (b) Some of the SR-like segments (arrows) are adjacent to a myofibril (Mf) in a 9SS:PMC. (c) Two large SR (arrows) spanning the gap between the plasma membrane (PM) and a myofibril (Mf) are shown in a 2D:CMC. Large arrows indicate peripheral couplings between the SR and PM. (d) A peripheral coupling in a 2D:CMC. Feet structures (arrowheads) are shown in the junctional gap between the SR and the plasma membrane (PM). (e) Two SR (arrows) close to the Z-line of a myofibril (Mf) are shown in a 2D:CMC. Bars indicate 0.3 μm.
FIG. 6. The development of the SR and of peripheral couplings in PMC and CMC of the developing chick heart is shown schematically. (a) A small number of SR-like segments are shown close to the plasma membrane (PM) in a 5-SS:PMC. (b) In addition to the SR-like segments close to the plasma membrane, some SR-like segments adjacent the myofibril (M) have appeared in the CMC by 1.5D. (c) SR spanning the gap between the plasma membrane and the Z-line of the myofibril have appeared in the CMC by 2D. One end of the SR forms a junctional complex (peripheral coupling, PC) with the plasma membrane, and the opposite end of the SR is close to the Z-line of a myofibril. Feet structures appear in the junctional gaps of the peripheral couplings. (d) A meshwork of SR surrounding the myofibril has been formed in the CMC by 9–12D. The SR has peripheral couplings with the plasma membrane.

(Rousseau and Meissner, 1986). Ryanodine binds to RYRs in the SR membrane, keeps them in an open state at low concentration (<10 μM), and closes them at high concentration (100–300 μM), thus disturbing Ca2+ release from the SR (Fabiato, 1985; Feher and Lipford, 1985; Fleischer et al., 1985; Meissner, 1986; Lattanzio et al., 1987). Consequently, ryanodine treatment abolishes caffeine-sensitive Ca2+ release from the SR. In the light of these facts, our results suggest that RYRs appear in CMC by the 8–9SS, when the heartbeat begins. The presence of RYR molecules has been demonstrated in the developing chick heart after 4D (Dutro et al., 1993) or 7D (Protasi et al., 1996), but the presence before 4D is unclear. This is probably due to the low level of RYR expression or to the small size of RYR aggregations in the membrane of the SR before 4D. However, treatment with ryanodine was found to disrupt the heartbeat on 3D (Dutro et al., 1993), and the appearance of feet/RYRs in peripheral couplings has been said to be detectable in 2.5D:CMC (Protasi et al., 1996). These findings indicate that RYRs may be present in CMC in early chick embryos on or before 2.5D, and are thus in accord with our results. The present study suggests that the two types of Ca2+ channel, DHPRs and RYRs, are not present together in PMC and CMC of the chick embryo until the time at which the heartbeat begins at 8–9SS. Ryanodine treatment did not disrupt the beating of the isolated heart from a 1.5D embryo, though it completely suppressed the beating of the isolated heart from a 2D embryo. Microfluorometry showed that

application of SSD to 2D:CMC caused a large amplitude [Ca2+]i response characteristic of [Ca2+]i response to caffeine, though it could not induce such [Ca2+]i response in 1.5D:CMC. These results suggest that DHPRs and RYRs in the CMC would not form a functional coupling; the CICR mechanism, until 2D. On this basis, we can predict that the heartbeat before 2D will be mainly dependent on an influx of Ca2+ from the outer medium through the Ca2+ channels/DHPRs in the plasma membrane and that the contribution of the SR as a source of the [Ca2+]i increase underlying contraction will become important after 2D.

During the development of the mammalian heart, contraction of CMC during the fetal period is mostly dependent on the extracellular Ca2+, whereas the contribution of the SR may be larger after birth (Nakanishi et al., 1988). Thus, the processes underlying the development of [Ca2+]i regulation in the chick heart may be basically the same as those occurring in the mammalian heart, except that the CICR mechanism may start to contribute to the heartbeat at an earlier stage in the chick embryo than in the mammalian embryo.

In mammalian CMC, DHPRs and RYRs are clustered and colocalized in the triad, a junctional complex formed by the transverse-tubule and terminal cisternae (Yuan et al., 1991; Jorgensen et al., 1993). In contrast, avian CMC do not form transverse-tubules. In the CMC of the chick embryo, the SR is closely attached to the plasma membrane, with which it forms junctional complexes (peripheral couplings), and the DHPRs and RYRs are clustered and colocalized in these peripheral couplings (Sun et al., 1995). The RYRs in the peripheral couplings are visible in electron micrographs as feet structures within the junctional gap (Ferguson et al., 1984; Saito et al., 1988). The present transmission electron microscopy showed that peripheral couplings with feet structures appeared in the 2D:CMC. This observation supports the above assertion that the CICR mechanism appears in the 2D:CMC of chick embryos. We suggest that the CICR mechanism will continue to develop throughout 2–8D, becoming more and more complete until 9–12D, since well-developed meshworks of SR surrounding the myofibrils and many peripheral couplings with feet structures were observed in 9–12D:CMC.

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