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Optical Evidence for Calcium-Action Potentials in Early Embryonic Precontractile Chick Heart Using a Potential-Sensitive Dye

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Summary. Using an optical method for monitoring membrane potential, spontaneous action potentials in the 7- to 9-somite embryonic precontractile chick hearts were measured. The optical action potential in the 7- to 9-somite embryonic heart was lacking 'phase 0' and 'phase 1' attributable to the fast Na^+current. The embryonic precontractile heart continued to generate spontaneous action potentials in a Na^+-free solution or in the presence of tetrodotoxin. Such an action potential was blocked by adding Co^{2+}, Mn^{2+}, Ni^{2+}, La^{3+}, D-600 or GEDTA, and the frequency, the amplitude, and the rate of rise of the spontaneous action potentials depended closely upon the external Ca^{2+} concentration; reducing the external Ca^{2+} concentration resulted in suppression of the spontaneous excitability. From the above results, we concluded that the spontaneous action potential in the early phases of cardiogenesis is characterized as a Ca^{2+}-dependent action potential.

Key Words embryonic precontractile heart · Ca^{2+}-action potential · optical monitoring · potential-sensitive dye

Introduction

In the last few years, using an optical method (for review *see* Cohen & Salzberg, 1978) in the 7- to 9-somite embryonic precontractile hearts in which microelectrodes are not applicable because of cell size and frailty, we have demonstrated the generation of spontaneous action potentials (Hirota, Fujii & Kamino, 1979; Fujii, Hirota & Kamino, 1980, 1981*a*), the origin of the pacemaker potential, rhythm generation (Fujii, Hirota & Kamino, 1981*b*), localization of pacemaking activity (Kamino, Hirota & Fujii, 1981), synchronization of action potentials (Fujii, Hirota & Kamino, 1981*c*), and the spatial spread of the initial excitation-contraction coupling (Hirota, Sakai, Fujii & Kamino, 1981).

Throughout a series of those works, inevitably, a knowledge of the ionic properties of the spontaneous action potential would add much to an understanding of the genesis of electrical activity in the early phases of cardiogenesis.

It has been established that in the embryonic chick heart, mechanisms that underlie the generation of action potential during the second day of incubation and for the next few days are markedly different from those that characterize adult hearts (for reviews *see* DeHaan, McDonald & Sacks, 1976; DeHaan, 1980*a, b*). Hearts of embryos aged 2 to 4 days continue to generate spontaneous action potentials in the presence of concentrations of tetrodotoxin (TTX) up to 10 μg/ml. In contrast, electrical activity in hearts from embryos of 7 days or older is completely blocked with TTX in a thousand times lower dose (Ishima, 1968; Shigenobu & Sperelakis, 1971).

Similarly, we have preliminarily examined the spontaneous action potential in the 8-somite embryonic heart and found it to be insensitive to TTX (Fujii et al., 1980). The findings reported in the present paper expand this result to include a detailed analysis of the ionic properties in 7- to 9-somite embryonic precontractile chick hearts. Some of the results reported here have appeared in a preliminary report, in Japanese (Sakai, Fujii, Hirota & Kamino, 1981).

Materials and Methods

Most of the methods were as described previously (Fujii, Hirota & Kamino, 1980; 1981*a, c*). Early embryonic chick hearts, usually at the 7- to 9-somite developmental stages, were stained with a merocyanine-rhodanine dye (NK 2761; Fujii et al., 1981*c*; Kamino et al., 1981) at 0.1 mg/ml in the bathing solution for 15 min. The composition of this standard bathing solution was (mM): NaCl 138; KCl 5.4; $CaCl_2$ 1.8; $MgCl_2$ 0.5; and Tris-HCl buffer (pH 7.2) 10. The dye, NK 2761, was purchased from Nippon-Kankoh-Shikiso Kenkyusho (Okayama, Japan). For recording of spontaneous action potentials from eight different portions of an embryonic chick (white Leghorn) heart, 8 light-guides were used to carry the light from the images of eight different regions of the preparation to individual photodiodes (Bell & Howell, Type 509-10, Bridgeport, Conn.). The

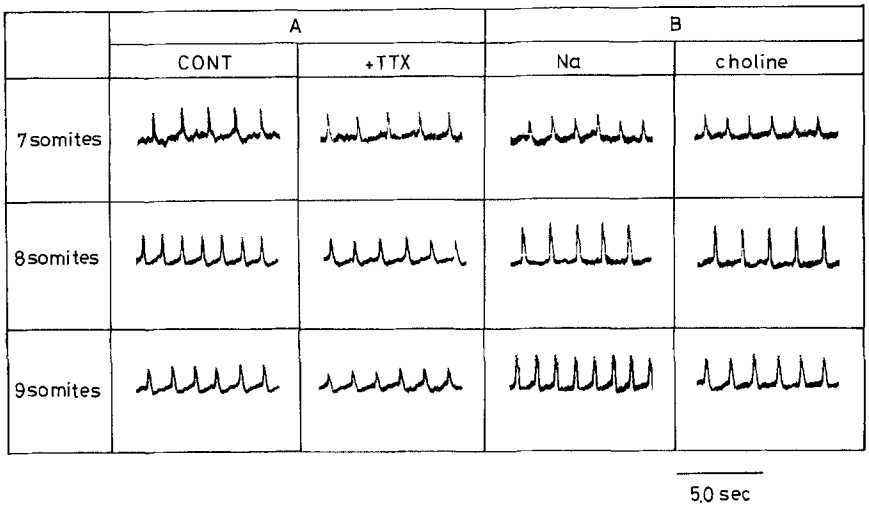

Fig. 1. Spontaneous absorption signals detected from the cono-ventricular region in 7-, 8- and 9-somite embryonic chick heart stained with a merocyanine-rhodanine dye (NK 2761). The traces were obtained with a 700 ± 11 nm interference filter, and the measurements were made in a single sweep, at $36.8 \sim 37.3$ °C. For the recording illustrated in this Figure, the oscilloscope input was AC coupled (time constant of 600 ms). *A*. Spontaneous absorption signals in normal bathing solution (left traces), and in TTX (1.0 μg/ml)-containing solution (right traces). *B*. Spontaneous absorption signals in normal bathing solution (left traces), and Na^+-free solution in which NaCl was replaced by choline chloride (right traces). In the one preparation, the slight difference was attributed to a slight difference in the temperature. In these experiments, after addition of TTX or replacement of Na^+ by choline, sometimes the frequency slightly decreased and sometimes slightly increased. Thus these slight changes were not significant

light source was a tungsten halogen lamp powered by a regulated DC supply (MODEL PAD 35-20L, 0–35 V, 20 A, Kikusui Electronics Corp., Tokyo, Japan); the incident light was usually passed through a 700 ± 11 nm interference filter (Type 1 F-S, Vacuum Optics Corporation of Japan, Tokyo, Japan). The tips of the light-guides had diameters of 4.0 mm and were positioned over different areas of the X66 magnified real image of an embryonic chick heart. The optical signals were displayed simultaneously on a Tektronix 5113 Dual Beam Storage Oscilloscope (Beaverton, Oregon). An Elcaset-Data-Recorder (FE-3000 Series, Sony, Tokyo, Japan) was also used as required. Signal averaging was not used. In most experiments, the oscilloscope was set to give a coupling time constant of 1.5 s. For the experiments illustrated in Fig. 1 the oscilloscope was also AC coupled (time constant of 600 ms). The outputs were finally filtered by a simple *RC* low-pass filter (time constant about 10 ms). The ionic basis for the spontaneous action potential was studied by replacement of Ca^{2+} with other ions in the bathing solution or adding agents in the solution. In such treatments, neither the DC-background intensity nor the spectra of the absorption change were altered. Thus, anxiety for direct effects of changes in ionic conditions or adding agents on the photometric nature of the dye was ruled out.

Results

Lack of the Fast Na Spike

Following the classical assumptions that action potentials are generated by either Na or Ca conductances (Hodgkin & Huxley, 1952; Hagiwara, 1973), the first experiments were designed to determine the contribution of Na-ions to the spontaneous action potentials.

Figure 1 *A* demonstrates spontaneous absorption signals recorded from the cono-ventricular region in the 7- to 9-somite embryonic chick hearts stained with the merocyanine-rhodanine dye (NK 2761), in a normal bathing solution or a tetrodotoxin (TTX: 1.0 μg/ml)-containing solution. These absorption signals all depended on the wavelength of the incident light, and were reduced at a wavelength of 620 nm or by illumination with white light. It is thus apparent that the absorption signals, from previous criteria (Fujii et al., 1980), resembled the spontaneous action potentials. As shown in Fig. 1 *A*, in the 7 to 9 somite embryonic chick heart, TTX had no effect on the spontaneous optical action potentials; neither signal size, nor frequency, nor shape were altered by TTX.

These results with TTX led to the suggestion that the spontaneous action potentials in the early embryonic precontractile heart are independent of the external sodium ions. Therefore, we carried out experiments in a Na-free solution. As shown in Fig. 1 *B*, replacemement of the external Na with choline resulted in no change in the size, the frequency or the shape of the spontaneous absorption signals.

From these results, it is then reasonable to conclude that the spontanous action potentials in the early phases of cardiogenesis are independent of fast sodium ion channels. Indeed, as can be seen in Fig. 5 or elsewhere, the rate of rise of the action

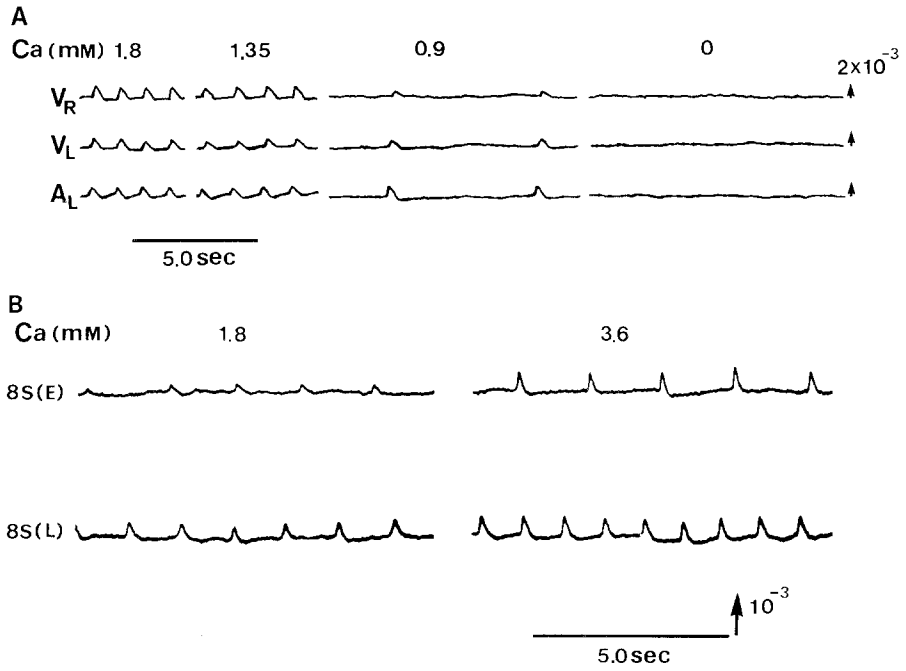

Fig. 2. *A*. Effect of low external concentrations of Ca^{2+} on the spontaneous absorption signals from a 9-somite embryonic chick heart. The signals were recorded simultaneously from the left cono-ventricular region (V_L), the right cono-ventricular region (V_R), and the left atrial level (A_L). Signals from the cono-ventricular regions (V_L, V_R) resemble typical cardiac type action potentials, and signals from the atrial level (A_L), a pacemaker type action potential with a slow diastolic depolarization (phase 4). $CaCl_2$ in the normal solution was partly or completely replaced by $MgCl_2$; traces were recorded in a normal solution containing 1.8 mM $CaCl_2$, in 1.35 mM $CaCl_2$-containing solution, in 0.9 mM $CaCl_2$-containing solution, or in Ca^{2+}-free solution. Temp. 36.9 to 37.1 °C. The fractional change in absorption ($\Delta A/A_r$) is related to the fractional change in transmitted intensity ($\Delta I/I_r$); evidence presented below indicates that the intensity decreased resulted from an absorption increase. The direction of the arrows to the right of the traces indicates a decrease in transmitted intensity and the lengths of the arrows represent the stated values of the changes in transmitted intensity divided by the resting transmitted intensity. *B*. Effect of elevation in the external Ca^{2+} concentration on spontaneous action potentials in the embryonic hearts at the early (8*E*) or the later (8*L*) period of the 8-somite stage of development. Traces were obtained from the right cono-ventricular region in the hearts. Ca^{2+} concentration was elevated by adding excess $CaCl_2$ to the standard bathing solution; the high Ca^{2+} solution contained 3.6 mM Ca^{2+}. Note that, especially in the heart at the early period of the 8-somite stage, weak signals are revived in high Ca^{2+} concentration. Traces were obtained at 37.1 °C in 8*E* or 37.0 °C in 8*L*

signals was relatively small in the 7- to 9-somite embryonic hearts; the initial rapid upstroke (phase 0), which results from fast sodium current is absent in the action signals in the embryonic precontractile heart.

Dependence upon External Ca^{2+}

Figure 2 demonstrates original recordings of spontaneous action potentials, under conditions of various external Ca-concentrations.

The signals shown in Fig. 2*A* were recorded simultaneously from three different areas: the right cono-ventricle (V_R), the left cono-ventricle (V_L) and the left atrial primordium (A_L) corresponding to the pacemaking area (Kamino et al., 1981) in a 9-somite embryonic heart just before the initiation of spontaneous contraction.

When the external Ca^{2+} was partly replaced by Mg^{2+}, the frequency of rhythmic recurrence of the spontaneous action potentials decreased strikingly. The signal size also decreased and was accompanied by a decrease in the frequency. Similar effects were caused by removal of external Ca^{2+} from the bathing solution using GEDTA (ethylene glycol diethyl ether diaminetetraacetate). Reducing external Ca^{2+} to about 1.3 mM (in 7-somite embryonic hearts), to about 0.9 mM (in 8-somite embryonic hearts) or to about 0.4 mM (in 9-somite embryonic hearts) completely abolished spontaneous action potentials.

In Fig. 3 are plotted the signal size (*A*) and the frequency (*B*), as functions of the external Ca^{2+} concentration. This Figure shows that both the signal size and frequency decrease with decreases in the external Ca^{2+} concentration in each heart at the 7- to 9-somite stage of development, and that the slopes of these response-dose curves, reflecting the resistance to the external low Ca^{2+} concentration, decrease as development proceeds.

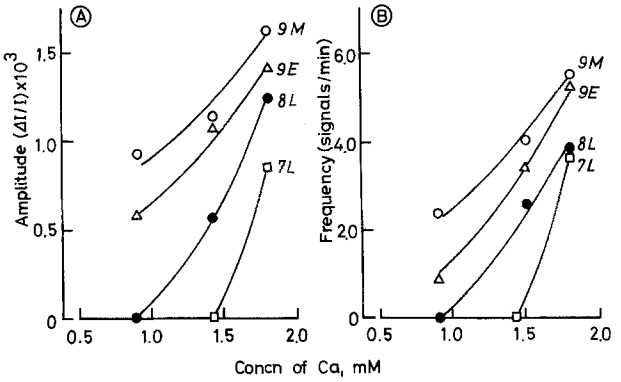

Fig. 3. Relationship between amplitude (*A*) or frequency (*B*) of spontaneous optical action signals and external Ca^{2+} concentration in early embryonic hearts at 7(□)-, 8(●)- or 9(△ or ○)-somite stages of development. Frequency is represented as the number of signals per minute. The points were obtained at 36.9 to 37.1 °C. *E*, *M* or *L* indicates the early, the middle or the later period of the stated stage. The data in *A* and *B* were obtained from the same preparation for each stage of development. The line among the points was drawn by eye

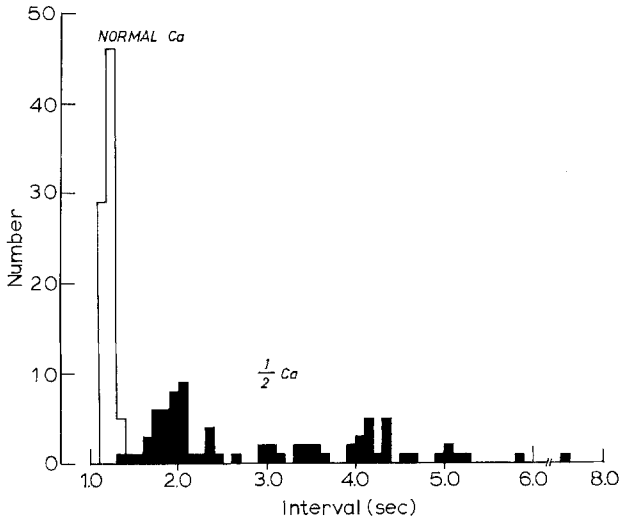

Fig. 4. Time-interval histograms of spontaneous optical action signals in a 9-somite embryonic heart in normal bathing solution (open column) or in a 1/2 (0.9 mM) Ca^{2+}-containing solution (black column). These histograms were obtained with 80 intervals. The ordinate is the number of intervals in a given bin. Bin width is 0.1 s. Temperature: 36.8 °C. In the standard bathing solution, the mean interval was 1.17 s; the standard deviation was 0.06 s, and in the 1/2 Ca^{2+} concentration, the mean interval was 2.92 s; the standard deviation was 1.29 s

On the other hand, as the external Ca^{2+} concentration is increased, both the size and frequency of the spontaneous action potentials increased gradually up to a saturation level. Especially, in the embryonic hearts at the 7-somite stage or the early period of the 8-somite stage, the optical action signals were often very small in the normal solution, but the signals improved dramatically when the external Ca^{2+} concentration was elevated. A typical example is seen in Fig. 2*B*.

In addition, in Fig. 2*A*, it is worth remarking that the action potential synchrony among the three different areas of the heart was maintained under conditions of low external Ca^{2+} concentrations. This finding indicates that action potentials initiated in the pacemaking region can propagate over the entire heart, and that the frequency of recurrence of the pacemaking action potentials is closely dependent on the external Ca^{2+} concentrations.

Rhythmicity. To analyze the effect of low concentrations of external Ca^{2+} on the stabilization of the rhythm of spontaneous action potentials, the time intervals (peak-to-peak) of the optical action potentials were measured. Figure 4 illustrates the time interval histograms of the optical action signals obtained from the right cono-ventricular region of a 9-somite embryonic heart (without contraction) in a normal bathing solution (on the left) and in low Ca^{2+} concentration solution (on the right). As can be seen in this figure, reducing external Ca^{2+} concentrations tended to disturb the rhythm of the spontaneous action potentials in the embryonic precontractile heart. These histograms were constructed from 80 intervals. In low Ca^{2+} concentration, we obtained a remarkably broad time interval histogram; the mean interval shifted to 2.92 s in 1/2Ca^{2+} concentration from 1.17 s in the normal condition, and the standard deviation of the time intervals increased to 1.29 s in 1/2 Ca^{2+} concentration from 0.06 s in the normal condition.

Shape. The initial rapid upstroke (phase 0) and the early repolarization (phase 1) are lacking in the spontaneous action potentials of the embryonic prebeating hearts during the 7- to 9-somite stage of development. Examples are shown in Fig. 5. The upper traces were recorded from the right cono-ventricular region in a 9-somite prebeating heart, and resemble a cardiac type action potential. The lower traces were obtained from the left atrial primordium, corresponding to the pacemaking area, in the same preparation. This signal resembled a pacemaker type action potential; particularly in normal solution, the slow diastolic depolarization that occurs between discharges of the action potential (phase 4) was evident.

As can be seen in Fig. 5 under conditions of lower external Ca^{2+} concentrations, the duration,

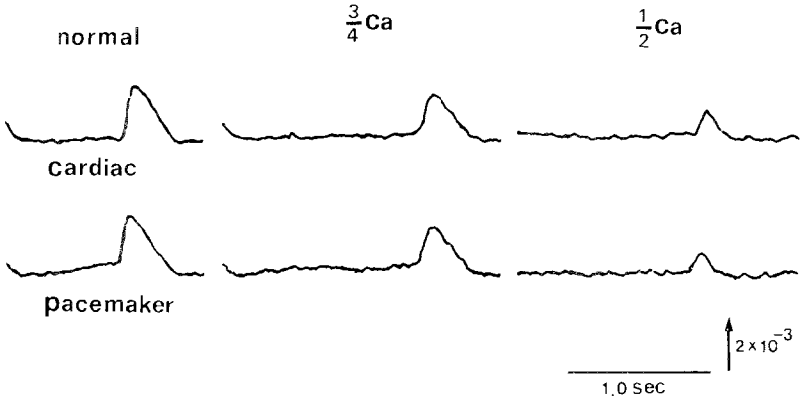

Fig. 5. Effects of low external Ca^{2+} concentration on the configuration of optical action signals in a 9-somite embryonic precontractile heart. Upper and lower signals were recorded simultaneously from the left cono-ventricle (upper) and the left pre-atrium region (lower) corresponding to pacemaking area. In the standard solution, the upper traces resembled a typical cardiac type action potential, and the lower traces resembled a pacemaker type action potential. Note that in the case of the lower signals, a slow diastolic depolarization is unclear at low Ca^{2+} concentrations. Using an AC coupled system (time constant 1.5 s), it is possible to compare the rate of slow diastolic depolarization in the two recordings under normal and 3/4 Ca^{2+}. Note that the base line (corresponding to the resting level) of cardiac type action signal is constant. Such a nature also is seen in Fig. 2*A*. Ca^{2+} concentration was changed by replacing some of the $CaCl_2$ with $MgCl_2$. Temperature: 36.8 °C

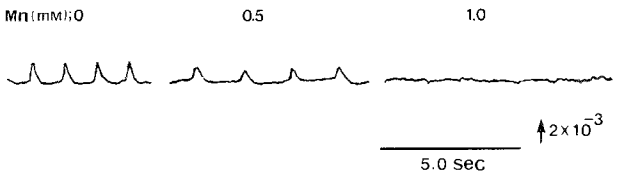

Fig. 6. Effect of Mn^{2+} on the spontaneous action potential in a 9-somite embryonic precontractile heart. Temperature: 36.9 °C. The action potential was completely abolished in a 1.0 mM Mn^{2+}-containing solution. $MnCl_2$ of 0.5 or 1.0 mM was added to the standard bathing solution

the amplitude, and the rate of rise were reduced in both the cardiac and pacemaker type action potentials. Therefore, it is reasonable to assume that these action potentials in the embryonic precontractile heart consist of the plateau phase, corresponding to that labeled "phase 2" in the adult heart, resulting from the slow Ca^{2+} current.

Furthermore, the lower traces in Fig. 5 indicate that the rate of diastolic depolarization decreases with a reduction in the external Ca^{2+} concentration. Therefore it seems likely that the low Ca^{2+}-induced decrease in the frequency of recurrence of the spontaneous action potential in the embryonic precontractile heart is closely related to the decrease in the rate of diastolic depolarization.

Inhibitory Effects of Some Transition Metal Ions

Some transition metal ions such as Co^{2+}, Mn^{2+}, Ni^{2+} and La^{3+} are known to block Ca^{2+}-dependent action potentials in various cells (for reviews *see* Hagiwara, 1973; Hagiwara & Byerly, 1981). Thus we studied the effects of these ions on the generation of spontaneous action potentials in the early embryonic hearts at the 7- to 9-somite stage of development.

Figure 6 shows the effect of adding Mn^{2+} to the bathing solution on the spontaneous optical action potentials in an 8-somite embryonic heart. Mn^{2+} produced a decrease not only in the amplitude, but also in the frequency of spontaneous action potentials in the 7- to 9-somite embryonic hearts. Similar findings were obtained after the addition of Ni^{2+}, Co^{2+} or La^{3+} to the bathing solution and all such responses were dose dependent.

Figure 7 shows examples of the relationships between the dose and response in the inhibitory effects of the transition metal ions on the amplitude (*A*), the frequency (*B*), and the rate of rise (*C*) of the spontaneous optical action potentials obtained from an 8- or a 9-somite embryonic heart. The amplitude, the frequency, and the rate of rise were normalized to that in the normal bathing solution. Values obtained in an 8-somite embryonic heart are given by filled symbols, those in a 9-somite embryonic heart by open ones. It is clear from this Figure that the inhibitory effects of the cations on the amplitude, the frequency and the rate of rise of the spontaneous action potentials are parallel, and that the order of effectiveness is

$$La^{3+} \gg Co^{2+} > Mn^{2+} \simeq Ni^{2+}.$$

Furthermore, both the La^{3+}- and the Co^{2+}-resistance in the 8-somite embryonic heart was less than that in the 9-somite preparation.

Spontaneous action potentials in the 7- to 9-somite embryonic heart were abolished by adding D-600 (1 μM) or ruthenium red (10 μM).

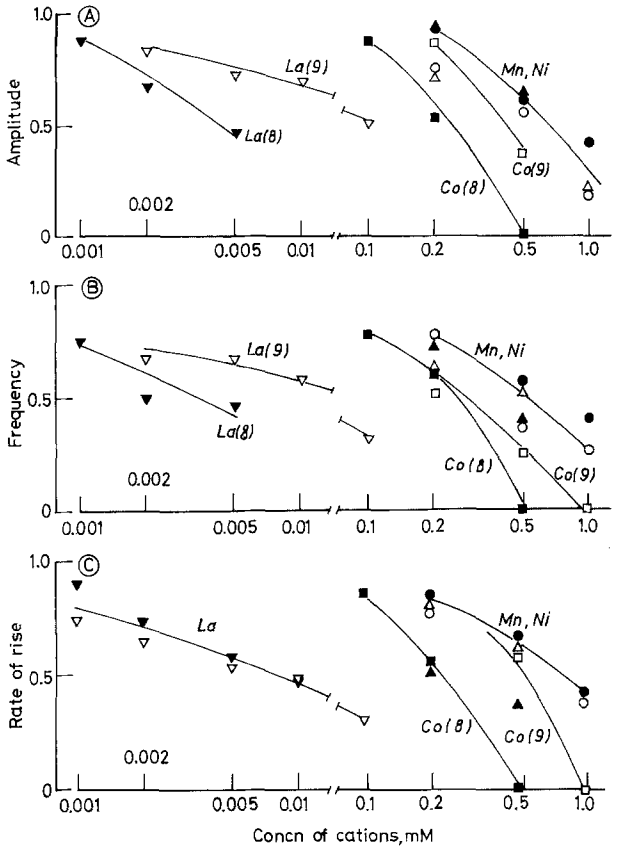

Fig. 7. Inhibitory effects of some transition metal ions on amplitude (*A*), frequency (*B*) or the rate of rise (*C*) of spontaneous optical action signals in the 8- or the 9-somite embryonic heart. Co^{2+}, squares; Mn^{2+}, circles; Ni^{2+}, triangles; La^{3+}, inverse triangles. Values obtained in an 8-somite embryonic heart are given by filled symbols, those in a 9-somite embryonic heart by open ones. The ordinate is amplitude, frequency or the rate of rise of spontaneous action potentials, and these are normalized to the values observed in the absence of the inhibitors. The abscissae indicate the concentration of inhibitors added to the bathing solution. The measurements were carried out at 37.0±0.1 °C. Data in *A*, *B* and *C* were obtained from the same preparation for each inhibitory ion species

Effect of Sr^{2+}

Sr^{2+} has been found to carry inward current in all Ca-channels studied, so that the ability of Sr^{2+} to substitute for Ca^{2+} in maintaining regenerative responses is one of the criteria used in identifying a Ca-action potential (Hagiwara & Byerly, 1981).

Accordingly, we applied Sr^{2+} to the early embryonic hearts. When Ca^{2+} in the bathing solution was completely replaced with Sr^{2+}, rhythmical spontaneous action potentials continued, but the duration was markedly prolonged. On the other hand, the effects of Sr^{2+} on the frequency and on the amplitude could not be uniquely established (Fig. 8).

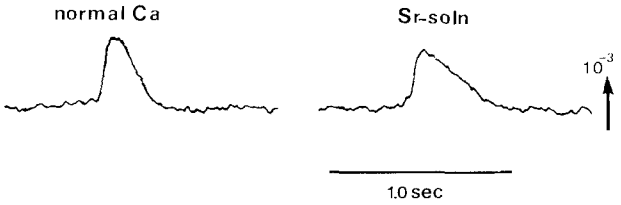

Fig. 8. Effects of Sr^{2+} on the spontaneous action potential. The traces were obtained from the left cono-ventricular region in a 9-somite embryonic heart. Note the prolongation of the plateau phase in Sr^{2+} solution. Temperature: 37.2 °C. The Sr solution was a complete replacement of Ca^{2+} in the standard bathing solution by Sr^{2+}

Discussion

This paper is concerned with the ionic properties of spontaneous action potentials in 7- to 9-somite embryonic precontractile chick hearts.

The nature of these action potentials was determined to be: (i) insensitive to TTX, (ii) independent of the external Na^+, (iii) blocked by transition metal ions such as Co^{2+}, Mn^{2+}, Ni^{2+} or La^{3+}, (iv) dependent on the external Ca^{2+}, and (v) obtained in solution containing Sr^{2+} substituted for Ca^{2+}. Such characteristics have been generally used to identify Ca-dependent action potentials (Hagiwara & Byerly, 1981). In addition, the absence of both "phase 0" and "phase 1" which are labeled in the adult heart (Hoffman & Cranefield, 1976), characterized configurationally the spontaneous potentials in the 7- to 9-somite embryonic hearts.

On the basis of these observations, we tentatively conclude that the spontaneous action potentials in the early embryonic hearts do not depend upon a component due to a fast Na^+ current, and that they are best characterized as Ca^{2+}-action potentials, generated by the slow inward Ca^{2+} current which is responsible for the plateau phase (phase 2).

Pappano (1972) observed that action potential overshoot in 6-day-old atria was dependent on external Ca^{2+}-concentration. Ishima (1968) also reported that in 2- to 5-day embryonic chick hearts, the action potential measured by microelectrode is insensitive to TTX, suppressed by Mn^{2+} and occurred in Na^+-free solution in which Na^+ is replaced with choline ions. On the other hand, in similar experiments, Shigenobu and Sperelakis (1971), and Shigenobu (1978) reported that both La^{3+} and Mn^{2+} had no effect on the TTX-insensitive action potential and that excitability was lost in Na^+-free solution. They concluded that the TTX-insensitive action potential of the young

heart is a Na^+ spike. Furthermore, they stated that cell membranes of the embryonic noninnervated muscles do not possess receptor sites for tetrodotoxin even though there is Na^+ channel activation. Although it is difficult to compare directly the results of Shigenobu and Sperelakis (1971) with the present work because there are differences in the stages of development, we have never obtained evidence, in the 7- to 9-somite embryonic hearts, which is consistent with their data. We feel that the following characteristics of the genesis of channel activation are reasonably well established by this paper; in the early phases of cardiogenesis, the Ca^{2+} channels first appear in the myocardial cell membrane, and the Na^+ channels are formed progressively at a later stage of development. It is not reasonable to generally assume that the differentiation in channel activity in the early embryonic heart is related unequivocally to the noninnervation or innervation process. In various developing nerve cells, there is a change in the ionic dependence of the action potential from a long duration Ca^{2+}-dependent spike to a brief Na^+-dependent spike (Baccaglini & Spitzer, 1977; Matsuda, Yoshida & Yonezawa, 1978; Spitzer, 1979, 1981). Generation of Ca^{2+} channels in the membrane of early developing skeletal muscle has been identified (Kano & Shimada, 1973; Kidokoro, 1973; Kano, 1975; Kidokoro, 1975).

It is worth mentioning that the spontaneous action potentials recorded from the pacemaking area are strikingly dependent on the external Ca^{2+} concentration. Slowing of the rate of diastolic depolarization with decrease in the external Ca^{2+} concentration provides some important information on the genesis of the pacemaker potential. We demonstrated previously a developmental increase in the rate of diastolic depolarization during the progression from 7- to 9-somite stages (Fujii et al., 1981*b*). This process may explain a possible mechanism of the genesis of the pacemaker potential and its development; the increase in the rate of diastolic depolarization throughout the early phases of cardiogenesis may be the result of an increase in the Ca^{2+}-inward current through the Ca^{2+} channels participating in the diastolic depolarization. This idea suggests that the developmental increase in the rate of diastolic depolarization may also be due to an increase in the number of Ca^{2+} channels. Inhibitory effects of Mn^{2+}, Co^{2+}, Ni^{2+} or La^{3+} strongly support this assumption. Similarly, in the adult rabbit sinoatrial node cell, it is proposed that the contribution of the slow inward current is more important for the generation of the pacemaker potential than is the gradual decay of the potassium conductance (Yanagihara & Irisawa, 1980).

We have also demonstrated (Fujii et al., 1981*b*) that the spontaneous rhythmicity of heart cells is already generated at the 7-somite developmental stage, and that the organization of the rhythm is complete at approximately the time of the initiation of the heartbeat. From finding that disorder in the rhythmicity is produced by low external Ca^{2+} concentration, it is suggested that Ca^{2+} play an important role in the cellular and/or subcellular processes related to the genesis of cardiac rhythmicity and its development.

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