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Actions of Calcium Channel Blockers on the Beating Rhythm and Membrane Potential of Cultured Chick Embryonic Heart Cells

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ABSTRACT. Actions of Ca channel blockers such as verapamil and nifedipine on the electrical and mechanical activities of cultured cells from chick embryonic ventricles were studied. The action potentials were divided into three types based on their shape and amplitude; a sino-atrial cell type, a Purkinje fiber type and a ventricular cell type. Some cells without spontaneous beating responded to intracellular stimulation with ventricular type action potential. Both verapamil and nifedipine decreased the rate and strength of spontaneous contractions depending on their concentration. These drugs also reduced the height of plateau of ventricular type action potential much more than that of Purkinje fiber type. Nifedipine, at high concentrations, blocked initiation of the upstroke of the action potential from pacemaker potential. The occasional omission of action potentials resulted in a slow beat rate and irregular rhythm. During the recovery phase from nifedipine treatment, the rate and contractions transiently increased. Positive chronotropic and inotropic interventions during recovery could be explained by the increased Ca influx resulting from the increased concentration gradient between inside and outside of the cells.

Key words : cultured heart cell — membrane potential — spontaneous beating — verapamil — nifedipine

Cultured heart cells from the chick embryos continue to beat spontaneously, and their membrane potentials as well as their mechanical activities have been extensively investigated.[1-5] These cells are free from nerve innervation and are easily exposed to extracellularly applied chemicals and drugs because of the small size and thinness of the cell aggregates. Therefore, they are a good preparation for investigating the nature of spontaneous activity and the mode of actions of some drugs on the electrical and mechanical events in each beat.

It has been postulated that the Ca current plays an important role not only in plateau potential but also in sinus pacemaker potential,[6-10] and it is selectively inhibited by "Ca-antagonists" or "Ca-channel blockers".[11-13] The present study was carried out to describe the various types of action potentials of cultured heart cells. The study was extended to the actions of verapamil and nifedipine on the rhythm and shape of spontaneous and driven action potentials as well as contractions.

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METHODS

The heart was removed from 7 to 11-day-old chick embryos. The ventricle was separated from the atrium and was cut into small cubes of about 0.5 mm^3. These tissue pieces were suspended in 0.2% trypsin phosphate buffer solution (NaCl 137, KCl 2.7, Na_2HPO_4 8.1 and KH_2PO_4 1.5 (mM)) for 20 min, and then were divided into single cells. The cells thus separated were washed out with a phosphate buffer solution without trypsin, and were scattered into culture dishes in the proportion of 10^6 cells per dish. The culture medium was MEM solution and contained (mM) NaCl 116, KCl 5.4, NaH_2PO_4 0.9, $MgSO_4$ 0.8, $CaCl_2$ 1.8, $NaHCO_3$ 12, glucose 5.5, vitamins, amino acids, antibiotics and 10% fetal bovine serum. The cells were incubated for 3 to 9 days, being gassed with a mixture of 95% air and 5% CO_2. The cells attached to the bottom of the culture dish and fused into aggregates which repeated spontaneous beating.

The beating was recorded by a photoelectrical device and the membrane potential was recorded with an intracellular glass microelectrode filled with 3 M KCl. Verapamil (provided by Eisai Pharm. Co., Tokyo) was dissolved in distilled water at a concentration of 60 μg/ml or 1.32×10^{-4} M before each of the experiments. Nifedipine (Sigma) was dissolved in dimethyl sulfoxide (Wako Pure Chemicals Ltd.) at a concentration of 10^{-5} M.

RESULTS

1. Changes in beat rate and in contraction strength caused by verapamil and nifedipine

The actions of verapamil and nifedipine on the spontaneously beating rhythm and contraction strength were investigated by recording the edge movement of the cell aggregate. Fig. 1A shows the record of the edge movement before and after application of verapamil at concentrations of 1.32×10^{-6} M (0.6 μg/ml), 2.64×10^{-6} M and 3.96×10^{-6} M. At a concentration of 1.32×10^{-6} M, the actions of verapamil on both rhythm and contraction were not detected. The beating rate, however, decreased from 100/min in the control to 65/min

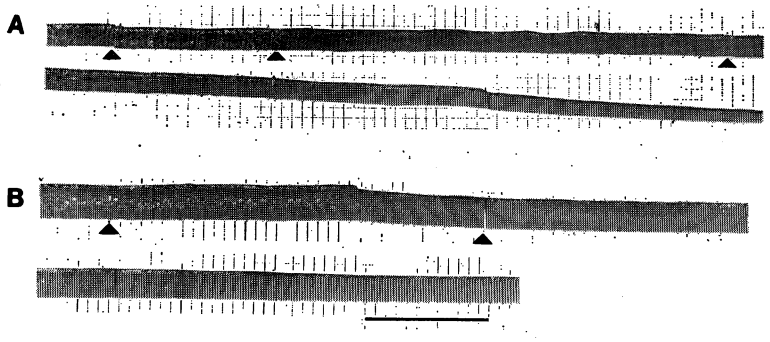

Fig. 1. Edge movement at one position of the cell aggregate. Two experimental records are shown. 0.02 ml of verapamil solution of 1.32×10^{-4} M (60 μg/ml) was applied to a culture medium of 2 ml at each arrow mark. The downward deflection indicates contraction. Time scale: 1 min. 9-day-old embryo.

at 2.64×10^{-6} M and to 40/min at 3.96×10^{-6} M. The amplitude of the edge movement also decreased to half of the control. Fig. 1B is the record of edge movement for another preparation after application of verapamil at a final concentration of 2.64×10^{-6} M, showing a decrease in beat rate from 124/min to 78/min and a decrease in contraction amplitude to 65% of the control.

The negative chronotropic action of verapamil examined with 5 preparations from different embryos is summarized in Fig. 2. The threshold concentration of verapamil for the negative chronotropic action seemed to be around 1.32×10^{-6} M, and the concentration for blockade of the spontaneous activity was ten times as high as that for the threshold.

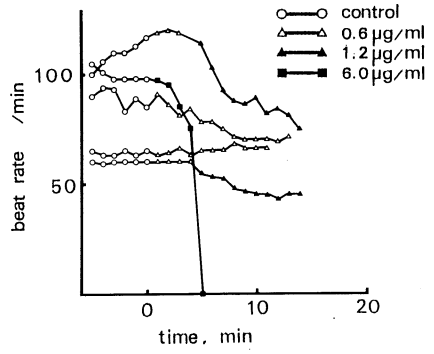

Fig. 2. Changes in beat rates under verapamil of different concentrations. At time 0, verapamil was applied at the final concentrations indicated. 9 to 11-day-old embryos.

Fig. 3 shows the inhibitory effect of nifedipine on the rhythm and strength of the contractions. Here, the edge movement was measured at two positions opposite each other along the long axis of the cell aggregate. If the preparation, after having stopped beating in nifedipine solution, was illuminated by a xenon lamp, it started to beat again within 1 or 2 minutes. The rise in temperature of the medium solution was 0.5°C, which was too small to change the beating rate described below. The peculiar feature during the recovery phase was that

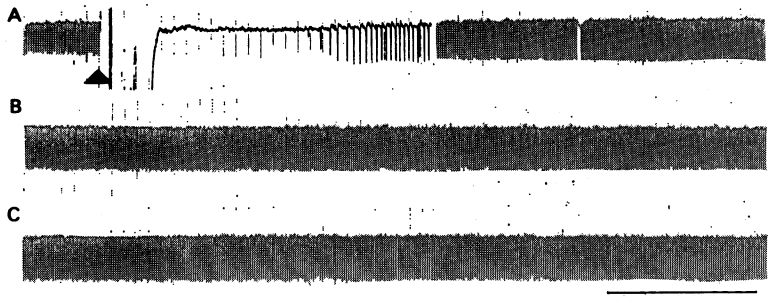

Fig. 3. Edge movement as an index of change in the diameter of the cell aggregate in the presence of 800 nM nifedipine. The three rows are continuous recordings; at the time indicated by the arrow, nifedipine was applied and then, 40 sec later, it was dissociated by xenon illumination. The downward deflection indicates contraction. Time scale: 1 min. 8-day-old embryo.

both the beating rate and contractions were transiently accelerated. At present, it has not been decided whether this positive inotropic intervention is caused by the direct effect of nifedipine removal or by the secondary effect of the increase in the beating rate.

2. Different types of action potential in cultured cells

Usually, the diastolic potential was low immediately after the electrode was inserted into the cell, but it increased in amplitude gradually in 1 min, probably because of sealing over of the membrane once injured during electrode impalement. Most of the cells of the 7 to 10-day-old embryos examined here showed action potentials of 70–80 mV with an overshoot potential of about +15 mV. They were accompanied by pacemaker potentials with a low amplitude (Fig. 4B,C), and their shape resembled those observed in adult Purkinje fibers. The second type of action potential showed a distinct plateau (Fig. 4E,F), quite similar to the one observed in adult ventricle cells. The cells, which stopped spontaneous beating several hours after the start of the experiment, could generate action potential when intracellularly stimulated (Fig. 4G). The driven action potential always showed a plateau of ventricular type. The third type of action potential showed a distinct pacemaker potential accompanied by diastolic potential of as low as −40 mV and a peak action potential of −10 mV (Fig. 4 A). There was only one pacemaker in each aggregate. In some cells, especially later in the experiments, the pacemaker potential failed to lead to the action potential and tended to decay before attaining the threshold potential (Fig. 4 D,F).

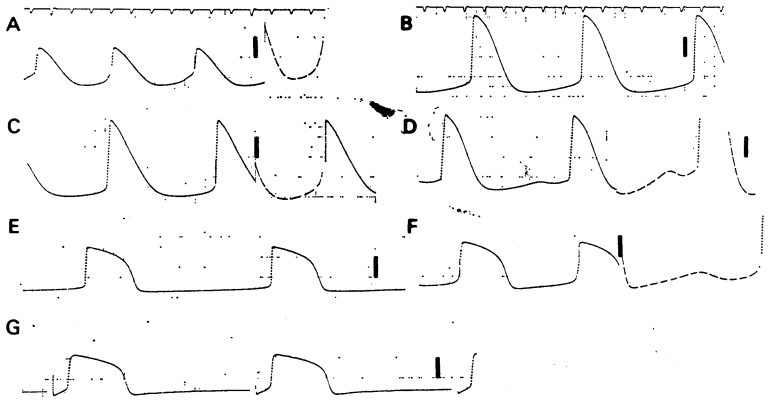

Fig. 4. Different types of spontaneous action potentials recorded from the cells of different embryos. A: sino-atrial node type, B, C and D: Purkinje fiber type, E and F: ventricular cell type, G: driven action potential. Time scale: 0.1 sec. Vertical bars are calibrations of 20 mV in A–D and 40 mV in E–G. Broken traces in A, C, D and F show the high gain recordings of subthreshold potential for the calibration bar of 10 mV. 7 to 10-day-old embryos.

3. Actions of verapamil and of nifedipine on the spontaneous action potentials

It was not difficult to leave the electrode inside the cell and record the membrane potential continuously for 10–20 min. Fig. 5 is a record illustrating

the effect of verapamil on the spontaneous action potentials of the Purkinje type. There were prolonged intervals between each action potential, a decreased rate for the rise of the pacemaker potential and a slight decrease of the peak amplitude. The threshold potential did not appear to be altered.

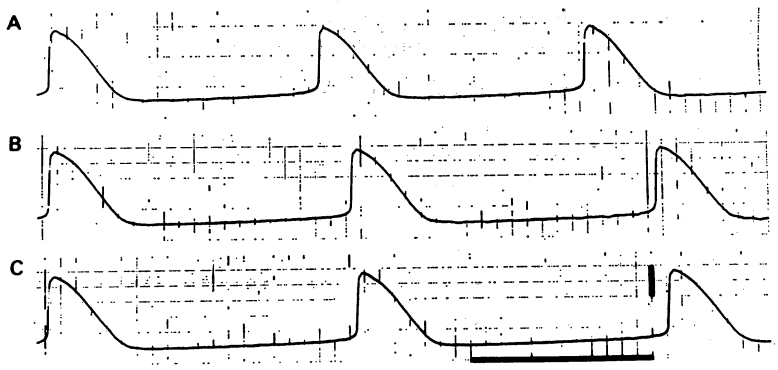

Fig. 5. Effect of verapamil (2.64×10^{-6} M) on the spontaneous action potentials. A: control, B: 3 min after and C: 6 min after application of verapamil. Calibrations were 40 mV and 1 sec. 7-day-old embryo.

In a nifedipine solution, the same changes as seen in verapamil were observed in the shape and size of Purkinje type action potential (Fig. 6A). In contrast, the plateau potential of ventricular type was markedly reduced and shortened (Fig. 6B).

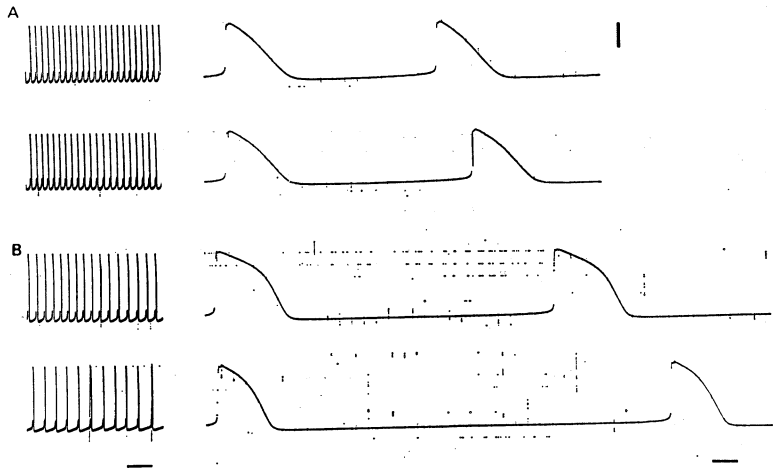

Fig. 6. Effects of nifedipine on the spontaneous action potentials. A: Purkinje fiber type action potentials of the control (upper trace) and under 300 nM nifedipine (lower trace), showing the prolongation of intervals. 11-day-old embryo. B: ventricular cell type action potentials of the control (upper trace) and under 600 nM nifedipine (lower trace), showing the shortening or fall of the plateau. The vertical calibration was 40 mV and the horizontal ones were 4 sec (left row) and 0.1 sec (right row). 10-day-old embryo.

4. Actions of verapamil and of nifedipine on the driven action potentials

The question remains whether the changes in the shape and size of the action potentials took place in cells whose membrane potential was being recorded or they were caused by slowing conduction or by a decrease in electrotonic spread from the neighboring cells.

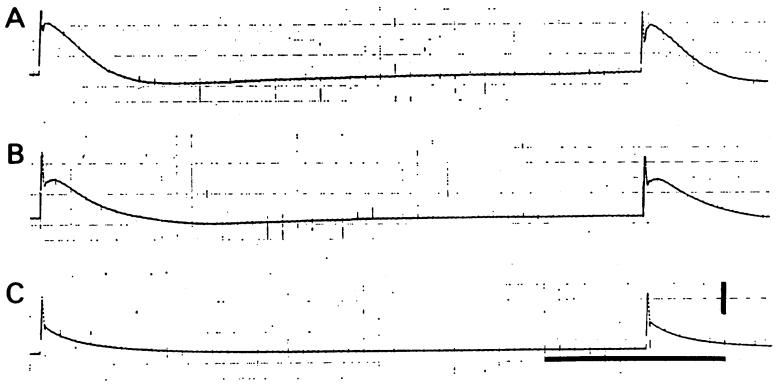

Fig. 7. Effects of verapamil (1.32×10^{-5} M) on the driven action potentials. A: control, B and C: 5 min and 7 min after application. Calibrations were 40 mV and 1 sec. 7-day-old embryo.

Some cultured cells ceased to beat spontaneously in the course of the experiment but generated action potential when they were stimulated. Fig. 7 shows one example of the action potentials stimulated intracellularly through the same electrode as the recording one at a rate of 1/sec. The upstroke of the action potential was not recorded because of the large stimulating artefact, but it was not preceded by the pacemaker potential. The driven action

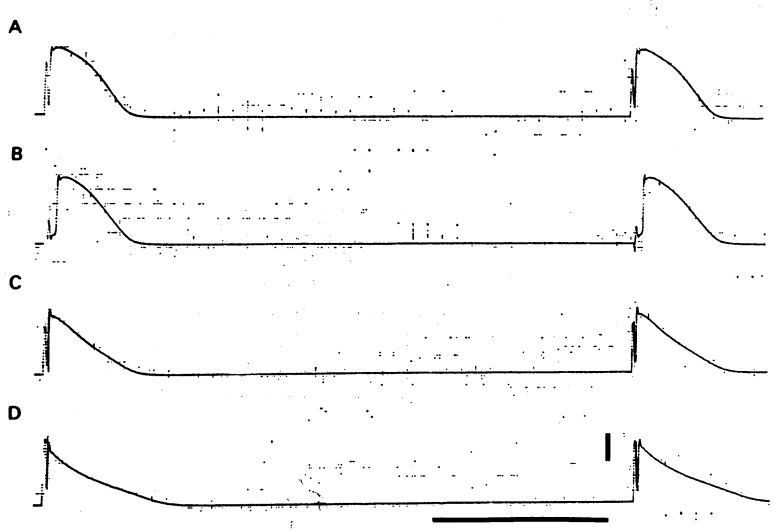

Fig. 8. Effects of nifedipine (600 nM) on the driven action potential. A: control, B, C and D: 3 min, 5.5 min and 10.5 min after application. Calibrations were 40 mV and 1 sec. 11-day-old embryo.

potential was usually accompanied by a plateau, and it was decreased or shortened by both verapamil (Fig. 7) and nifedipine (Fig. 8). The peak amplitude of the upstroke was more or less reduced. The results suggest that the changes in action potential take place in the cell from which the membrane potential is recorded. From Figs. 5-8, another conclusion can be deduced; that ventricular type plateau potentials are more sensitive to nifedipine than those of Purkinje type.

5. Actions of verapamil and nifedipine on the pacemaker potential

It was often observed microscopically that a few cell aggregates beat at an irregular rhythm. The membrane potentials of these cells are shown in Fig. 9. The pacemaker potentials developed regularly, but some of them failed to lead to full-sized action potentials and decayed to the diastolic potential level before attaining threshold potentials. Abortive potentials took place one after another and looked like oscillatory potentials. No contraction was observed under the microscope following abortive action potentials. Occasional blockade

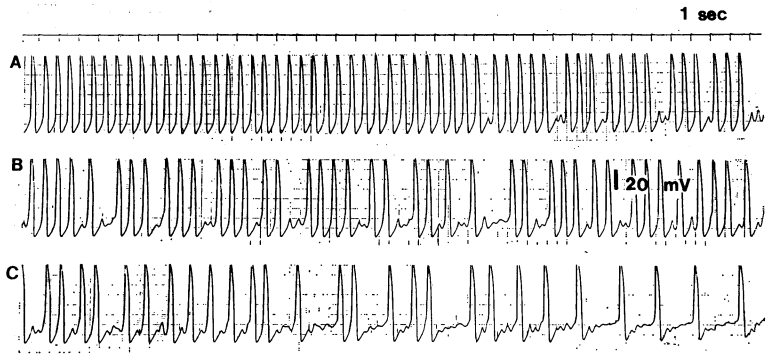

Fig. 9. A, B and C are continuous recordings showing that some of the pacemaker potentials failed to produce action potentials. 7-day-old embryo.

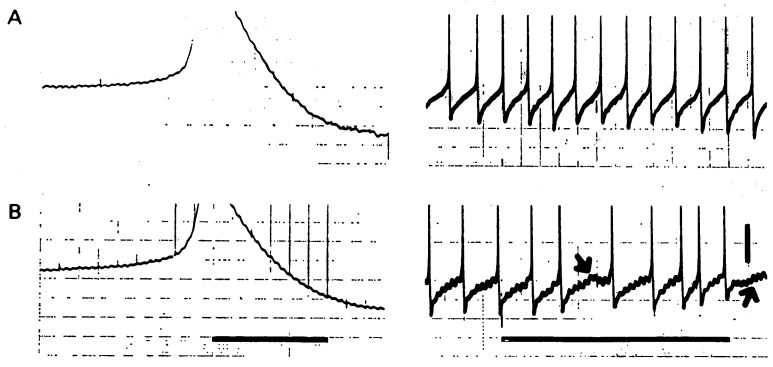

Fig. 10. Failure of initiation of action potential following pacemaker potential (marked by arrows) and small fluctuation in pacemaker potential in the presence of verapamil. A: control, B: 8 min after application of 2.2×10^{-6} M verapamil. The vertical calibration was 20 mV and the horizontal calibrations were 0.1 sec and 10 sec for fast (left row) and slow (right row) recordings, respectively. 9-day-old embryo.

of the action potential was one of the causes of the irregular beating.

Verapamil tended to turn full-sized action potentials into abortive ones. Fig. 10 shows that the pacemaker potential did not always lead to an action potential but fluctuated below the threshold level, with a superimposed small oscillation potential. The slope of the pacemaker potential itself was also slowed. Nifedipine also produced irregular beating, as is shown in Fig. 11. Some of the pacemaker potentials repolarized to the diastolic potential level instead of generating action potentials. Here also the slope of the pacemaker potential was decreased. Blockade of the action potential and slowing of the pacemaker potential by verapamil and nifedipine were the causes of negative chronotropic intervention and irregular beating.

The membrane potential changes during recovery from the treatment with nifedipine was characteristic and is shown in Fig. 12. The spontaneous excitation

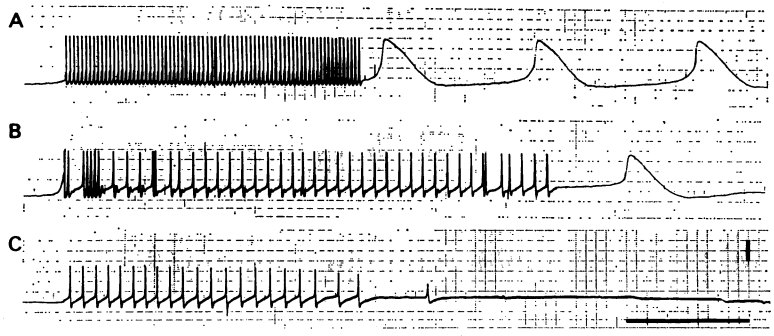

Fig. 11. Failure of initiation of action potential in the presence of nifedipine. A: control, B: 2 min after application of 300 nM nifedipine, C: continuous recording after B. Note that the delayed after-depolarization follows repolarization in B and C. The vertical calibration was 40 mV, and the horizontal ones were 0.1 sec and 4 sec, respectively. 10-day-old embryo. 38.5°C.

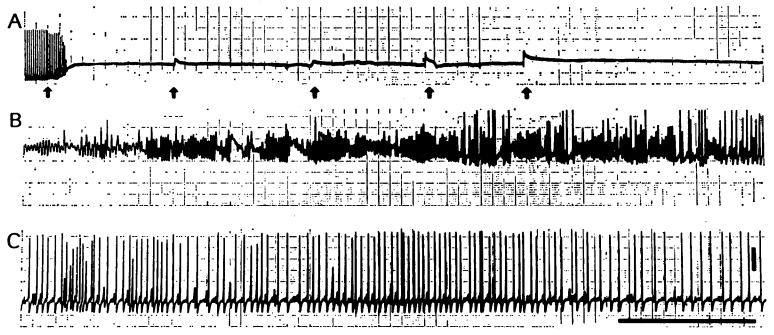

Fig. 12. Recovery of spontaneous electrical activity by a stroboscope flash light after treatment with nifedipine. The first arrow from the left in the top recording indicated the time of 900 nM nifedipine application. The 2nd through 5th arrows indicated the timing of the flash. The middle recording followed 1 min after the end of the top recording, and the bottom recording came about 20 min after the middle one. The vertical calibrations were 40 mV for the top recording and 20 mV for the middle and bottom recordings. The horizontal one was 10 sec. 10-day-old embryo.

was abolished a few minutes after application of nifedipine at high concentrations. The membrane potential was maintained at a less negative level (-60 mV) than the maximum diastolic potential. After the spontaneous activity ceased, the cells were illuminated by light from a flash stroboscope several times. Within 6 to 10 minutes the activities were gradually restored, and the membrane potential changes that appeared at the early phase of recovery were oscillation potentials with a small amplitude as low as 10 mV. They were progressively augmented in a peak and finally attained an amplitude sufficient to produce the action potential. It is also noteworthy that the rhythm of the action potential at the beginning of the recovery was faster than the control rhythm. As has been shown in Fig. 3, the strength of the contraction was also enhanced during the recovery from nifedipine treatment. Positive chronotropic and inotropic interventions during the recovery phase of nifedipine treatment were also observed for frog atrial muscle (unpublished results).

DISCUSSION

1. The properties of the cultured cells

Cultured heart cells are a good preparation for investigating the nature of spontaneous activity and the mode of action of some drugs on the electrical and mechanical events in each beat, because they have no neural innervation and are thin and small enough to allow the drugs to act rapidly. Embryonic cells are different from adult cells in the sense that the Ca current contributes not only to the plateau potential but also to the upstroke of the spike potential.[4] The pacemaker potential is also partly carried by Ca current.[8,14]

The shape of the action potential recorded from most of the cells resembled to that of adult Purkinje fiber, which has pacemaker potential and only a short plateau. Some cells showed a distinct plateau potential like that of adult ventricle cells. Only a few cells showed action potential similar to that of the sino-atrial cells. Various types of action potentials have already been described by Lehmkuhl and Sperelakis.[1] In the present study, the potentials could be divided into three types; a sino-atrial type, a Purkinje type and a ventricular type. Within one aggregate, all the cells showed the same size and the same shape of action potentials, indicating that the cells had an electrically isopotential syncytium. In addition, the cell aggregates continued to beat synchronously and only one pacemaker played a role in determining the beating rhythm. This property of the cultured heart cells is also favorable for investigating the pacemaker mechanism.

2. Recording of the strength of contractions

Clusin[5] measured the edge movement at 3 portions of a cell aggregate suspended in culture medium and succeeded in evaluating the strength and temporal characteristics of contractions. In the present work, the cell aggregates attached themselves to the bottom of culture dishes and their edge movement was measured at only one or two positions. Therefore, our measurement probably underestimated the strength of contractions. Nevertheless, our recording indicated shortening in the radial direction and expressed qualitatively the

strength of contractions. And it is quite certain that verapamil and nifedipine inhibit the contraction of cultured cells.

3. Upstroke and plateau of the action potential

Both verapamil and nifedipine depressed the height and shortened the duration of the plateau potential of ventricular type cells, and less markedly of Purkinje type cells. Since these drugs predominantly blocked Ca current, it was supposed that the plateau of Purkinje type cells is determined mainly by K current through the x channel, while the plateau of ventricular type is built up mainly by Ca current. The action of verapamil suppressed the plateau potential of driven activity at high stimulation frequencies much more than that at low stimulation frequencies, while the action of nifedipine was less dependent on frequency. The details of frequency dependence of drug action will be described in another paper. Verapamil, in addition to its depressing action on the plateau, reduced the peak and the maximum rate of upstroke of the action potential, whereas nifedipine reduced the peak only. McDonald and Sachs[15] reported that D600, another Ca-channel blocker, reduced and shortened action potential without affecting the maximum rate in chick embryonic cells. One reason for the difference between McDonald and Sachs with D600 and ours with verapamil may be due to the ages of the embryos. It has been proved that the upstroke of action potential in young embryonic cells is carried not only by Na^+ but also by Ca^{2+} and, therefore, the peak is dependent on Ca^{2+} current.[15,16] According to Nayler and Horowitz,[12] L-verapamil inhibits fast Na current much more than it does Ca current, and commercially available verapamil contains both L-and D-isomers. This is the reason why verapamil but not nifedipine reduces the maximum rate of action potential.

4. Pacemaker potential

Clay and Shrier[17] showed that the current in pacemaker potential was carried mainly by time-dependent and hyperpolarization-activated inward going K current, i_{K_2}. This current is also called either i_h or i_f. If nifedipine or verapamil inhibits i_{K_2}, then the slope of the pacemaker potential will be decreased. This is one probable explanation for the slowing of the beat rate. In rabbit sino-atrial node, however, i_{Ca} as well as i_{K_2} contributes to the pacemaker potential.[8,18,19] Therefore, it is easy to consider that the inhibitory action of these drugs on Ca conductance in the pacemaker cells reduces the rate of the pacemaker potential. Recent experiments of Brown *et al.*[9] and Hagiwara *et al.*[20] have revealed that $i_{Ca\cdot T}$ plays an important role in the later phase of pacemaker potential and nifedipine blocks $i_{Ca\cdot L}$ selectively. The blockade of the Ca-channel must thus be the main reason for the inhibition of pacemaker potential.

5. Initiation of irregular beating

Nifedipine and verapamil block the generation of action potential that follows pacemaker potential. The first possible explanation for abortion of action potential is that verapamil and nifedipine inhibit the later part of the pacemaker potential, which is carried by Ca^{2+},[9,20] and consequently fail to generate the action potential. Another possibility is that during prolonged pacemaker potential the K current through the s channel may increase and the membrane

may be repolarized. Lastly, the change in $[Na^+]_i$ also participates in the blockade of action potential generation. Under the influence of nifedipine and verapamil, $[Ca^{2+}]_i$ is lowered because of the inhibition of slow inward current, and low $[Ca^{2+}]_i$ in turn decreases Ca efflux through the electrogenic Na-Ca exchange mechanism, and consequently results in the fall of $[Na^+]_i$. This explanation is supported by the results of Noma and Irisawa[21] who observed abortive action potentials when the Na-K pump was inhibited by lowering $[Na^+]_o$ or loading K^+_i. These changes in the intracellular ionic environment may be the causes of abortive action potential.

During the recovery phase from treatment with high concentrations of nifedipine, oscillation potential was observed before the regular excitation resumed. Nathan and Bhattacharyya[22] reported that small potential fluctuations below 1.5 mV peak amplitude took place when $[Ca^{2+}]_i$ was expected to be moderately raised. It is expected that $[Ca^{2+}]_i$ will rise if Ca-channels recover from blockade after removal of drug action, but the Na-Ca exchange mechanism will still remain in the inhibited state. Further investigation on the electrical activity during recovery will be described elsewhere.

Acknowledgments

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