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B. LEWARTOWSKI, K. ZDANOWSKI, B. M. WOLSKA.

THE EFFECT OF MENADIONE ON SARCOPLASMIC RETICULUM Ca^{2+} AND CONTRACTIONS OF SINGLE GUINEA-PIG CARDIOMYOCYTES

Department of Clinical Physiology, [Medical Center of Postgraduate Education, Marymoncka 99, 01-813 Warsaw, Poland.

We investigated the effect of 2-methyl-1,4-naphtoquinone (Menadione) on sarcoplasmic reticulum (SR) Ca^{2+} content and electrically stimulated contractions (ESCs) of single isolated myocytes of guinea-pig ventricular myocardium. The contractures initiated by means of microinjections of caffeine into the close vicinity of the cell were used as an indirect index of the SR Ca^{2+} content. Superfusion of the cells for 45 min with Menadione resulted in gradual disappearance of contractile respones to caffeine, prolongation of time to peak amplitude of ESCs by $48 \pm 15\%$ and complete inhibition of postrest and postextrasystolic potentiation. These results are consistent with those of Floreani and Carpenedo (7) who found that Menadione strongly inhibits the SR Ca^{2+} ATPase. Despite depletion of the SR Ca^{2+} the amplitude of ESCs did not change which suggests that contractions were initiated in the cells treated with Menadione by Ca^{2+} derived from the sources other than the SR.

Key words: Sarcoplasmic reticulum, Ca^{2+} ATPase, Menadione, cardiomyocytes, excitation-contraction coupling.

INTRODUCTION

According to general consensus the main source of Ca^{2+} activating contractile system of the cardiac myocytes is sarcoplasmic reticulum (SR) (for review see 1). The Ca^{2+} is released from the SR through the release channel activated by small but rapid increase in the sarcoplasmic Ca^{2+} concentration brought about by activation of the sarcolemmal Ca^{2+} channels (2). Results obtained in the last decade in multicellular preparations and in single, isolated myocytes seem to confirm the important role of the SR in excitation-contraction coupling as a major source of activator

Ca^{2+}: 1. SR Ca^{2+} content decreases during prolonged rest in parallel to decrease in contractility (3, 4, 5, 6). 2. Rapid depletion of Ca^{2+} from the SR of single myocytes by means of caffeine „spritz" or rapid caffeine superfusion results in dramatic inhibition of the post-caffeine contractions (6).

However, we (6) also found that it was possible to activate contractions of single myocytes of guinea-pig heart the SR of which has been deprived of Ca^{2+} by means of prolonged superfusion with 0.1 μM ryanodine. The amplitude of the contractions was nearly normal, albeit their time to peak amplitude was prolonged. Ryanodine delayed the rest decay of contractions and their postrest recovery. In this paper we extended the investigation on the role of the SR in excitation-contraction coupling in cardiac myocytes to inhibition of the SR Ca^{2+} uptake by means of 2-methyl-1,4-naphtoquinone (Menadione). This compound has been shown by Floreani and Carpenedo (7) and by Floreani et al. (8) to block relatively selectively the SR Ca^{2+} ATPase.

Superfusion of single isolated myocytes of guinea-pig ventricular myocardium with Menadione resulted in gradual depletion of the SR Ca^{2+} assessed by means of caffeine contractures (5, 6, 9). Despite the depletion of the SR Ca^{2+} electrical stimulation initiated contractions of normal amplitude and increased time to peak amplitude. These results and our experiments with ryanodine (6) suggest that a major function of the SR in the cardic excitation- contraction coupling in the guinea-pig myocardium is the control of the time-course of contraction.

METHODS

The hearts of quinea-pigs were rapidly isolated under Nembutal anesthesia and perfused for 3 min with nominally Ca^{2+} free solution (see below), then for 20—30 min with the same solution containing 2 mg of protease and 50 mg of collagenase per 50 ml and for the next 5 min with the same solution containing no enzymes but 0.2 mM Ca^{2+}. The ventricles were then placed in the beaker containing 50 ml of the solution with 0.2 mM Ca^{2+} and gently agitated. The dispersed cells were filtered through the nylon mesh and the Ca^{2+} concentration was adjusted to 1.0 mM. The cells could then be stored for several hours at room temperature.

The cells were placed in the superfusion chamber mounted on the stage of an inverted microscope and became attached to its bottom. They were field stimulated by means of platinum electrodes placed in the superfusion solution. The temperature of the solution was kept at 24°C. Changes in the length of the cells were monitored by means of the edge video-tracking system (10). For the sake of clarity the protocols of experiments will be described in the Results section.

The SR Ca^{2+} was released by means of short (100 ms) exposures to caffeine (15.0 mM) injected into the close vicinity of the cell from a micropipette placed few μM from the cell downstream in respect to the flow of the superfusate. The micropipette was connected to a pneumatic ejection system activated by a pulse from a stimulator.

Superfusion solution of the following composition was used throughout this work (in mM): NaCl 144; KCl 5.0; $CaCl_2$ 2.0; $MgCl_2$ 1.0; NaH_2PO_4 0.43; HEPES 10.0; glucose 11.0. The pH of the solution was adjusted with NaOH to 7.38.

Chemicals. Protease and collagenase were purchased from Sigma. Two forms of Menadione were used: 1. Anhydrous No M-5625, Lot 66C-0341 was purchased from Sigma. It was dissolved in alcohol (10 mg/1 ml) and added to the large volume of the superfusate. 2. The water soluble form (No 55701 B) was purchased from Fluka. It was directly dissolved in the superfusate. The effects of both forms of Menadione were identical, although the anhydrous form was about 20 times more potent than the water soluble one. Therefore the results have been pooled.

Statistical analysis. The quantitative results are given as means $\pm$SD. The significance of differences between the means was evaluated by means of the Student's t test for paired samples.

RESULTS

Estimation of the SR Ca^{2+} content and of the SR Ca^{2+} uptake.

Application of caffeine instead of a steady state electrically stimulated contraction (ESC) resulted in a transient contracture (*Fig. 1A*, *Fig. 4*). Its amplitude averaged 129$\pm$23% (SD, n = 56) of that of steady state ESCs. The amplitude was stable in the given cell under the steady experimental conditions. The second injection of caffeine did not evoke any contractile response if the cell remained quiescent after the first injection (Fig. 1A). The prolongation of the delay between the applications of caffeine did not result in the reappearance of the second contractile response (*Fig. 4*). This result is consistent with our previous observations (11) and suggests that Ca^{2+} released from the SR by caffeine is extruded from the cell i. e. that neither recirculation of Ca^{2+} nor the net Ca^{2+} uptake occured in the resting cell under these experimental conditions. Such a cell selectively deprived of the SR Ca^{2+} provides a very convenient model for investigating the ability of SR to take up Ca^{2+} under various experimental conditions.

The electrical stimulus applied after the caffeine contracture evoked a very weak contractile response, the amplitude of which averaged 18.6$\pm$10% (n = 56) of that of the steady state ESCs (Fig. 1A). The amplitude of this contraction did not increase irrespective of the duration of the delay after the preceding caffeine injection (not shown). Since the SR Ca^{2+} had been depleted, the post-caffeine ESC apparently resulted from the influx of Ca^{2+} from the extracellular space. The top panel of Fig 1A shows the following sequence of interventions. Caffeine applied instead of the last steady state ESC initiated a contractile response the amplitude of which was a little lower than that of the control ESCs. The next injection of caffeine applied 4 s later was without any contractile effect. A single electrical stimulus applied 4 s after the second injection

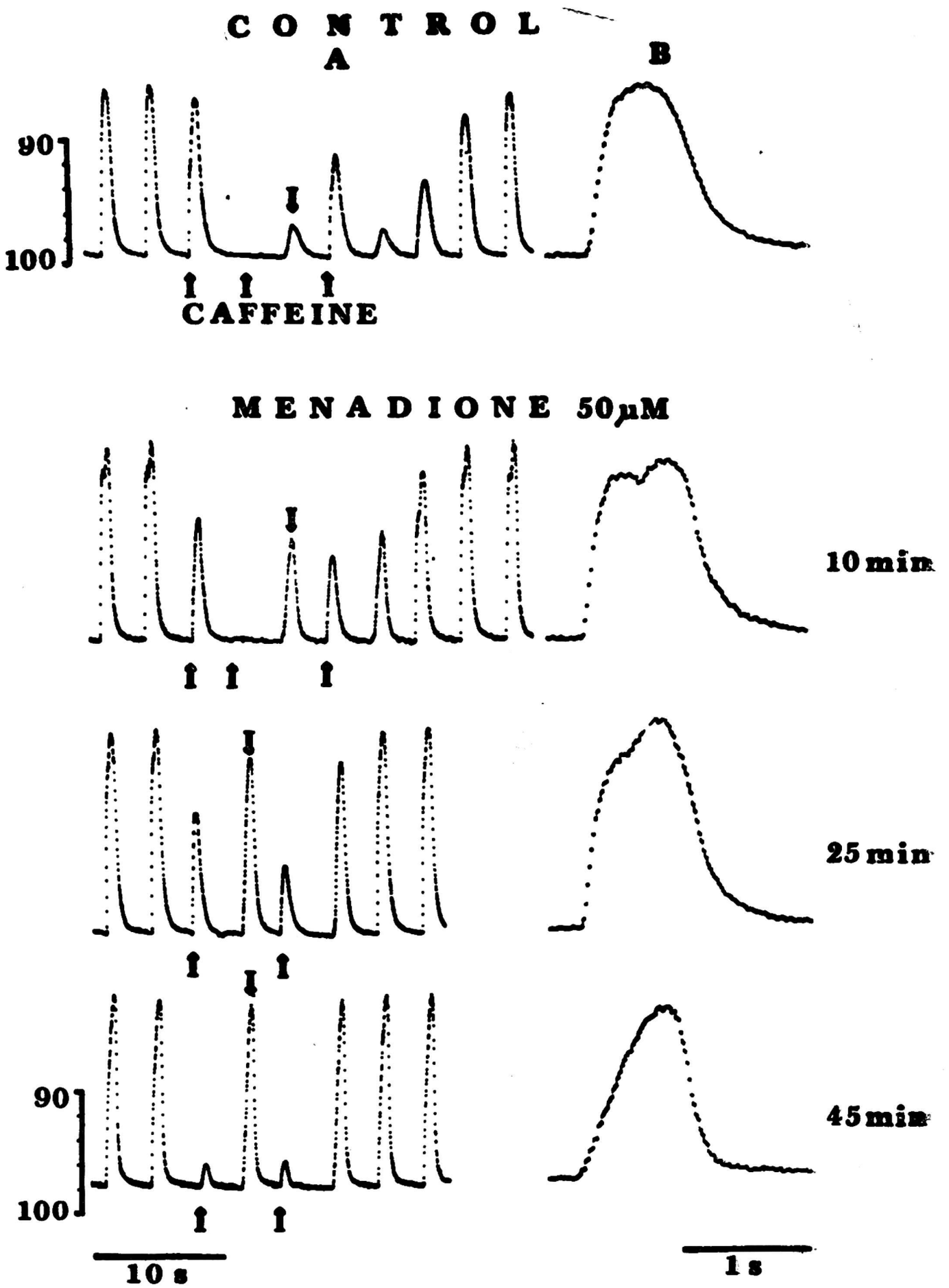

Fig. 1. A. The effect of Menadione (anhydrous) on the caffeine contractures (arrows pointing upwards), on the first post-caffeine contractions (the arrows pointing downwards) and on steady state electrically stimulated contractions. B — Steady state contractions registered at the higher paper speed. The scales to the left — % of the resting cell length. The experiment has been performed in one isolated myocyte of guinea-pig ventricular myocardium. Note a decrease in the diastolic cell length clearly visible in the bottom panel A.

of caffeine initiated the small contraction. The next application of caffeine, initiated contracture the amplitude of which attained 71% of that of the first caffeine contracture. Thus a single post-caffeine excitation immediately restored the contractile response to caffeine. The average amplitude of the restored contracture was 54±10% (n = 32) of that of the steady state ESCs. The amplitude of the caffeine contracture restored by a single excitation may be used as a relative index of the ability of the SR to take up Ca^{2+} diffusing into the sarcoplasm during this excitation. The steady state stimulation restored the pre-caffeine amplitude of ESCs over several beats (Fig 1A).

The effect of Menadione on ability of the SR to take up Ca^{2+} and on the steady state ESC.

Addition of Menadione (1.0 mM water soluble or 0.05 mM anhydrous) to the superfusing solution resulted in an immediate and short lasting slight positive inotropic response which subsided over few contractions. In most of the cells a slight negative inotropic effect followed. Also this effect dissapeared over several min. Within about 10 min the amplitude of the contractile response to the first injection of caffeine and to caffeine injected after a single excitation began to decrease (Fig 1A). At the same time the amplitude of the first post-caffeine ESC increased. In parallel to these changes a second slow component appeared in the steady state ESC (*Fig. 1B*). Its amplitude was initially lower than that of the first fast component, but it slowly increased.

Over the subsequent 30—45 min all these changes progressed. The contractile responses to caffeine have decreased until they disappeared completely or nearly completely (the bottom panel of Fig 1A). The amplitude of the first post-caffeine ESC increased until it did not differ from that of the steady state ESCs. The amplitude of the first fast component

Table 1. The effect of Menadione (1.0 mM water soluble or 0.05 mM anhydrous) on the time course and amplitude of steady state contractions (stimulation rate 20—30 min) of the isolated single myocytes of guinea-pig ventricular myocardium.

	Control	Menadione
duration of contraction * (ms)	808±104	923±171**
	$p > 0.2$	
time to peak amplitude (ms)	402±120	595±127
	$p < 0.001$	
relaxation phase* (ms)	406±99	328±87
	$p < 0.05$	
amplitude (% of control)		96±18

* measured at the level of 80% of relaxation. ** Mean±SD, n = 15.

of the steady state ESCs have decreased until it disappeared completely whereas the second slow component increased. The eventual changes in the steady state ESCs consisted of statistically significant prolongation of the time to peak amplitude and of shortening of the relaxation phase. Total duration of contraction increased but this change was not significant statistically. The total amplitude of contractions averaged 96±18% of the pre-Menadione control (the bottom panel of *Fig. 1B* and the *Table 1*).

In 8 out of 15 cells studied the small spontaneous contractile waves appeared between about the 10-th and 20-th min of superfusion with Menadione (not shown). They dissapeared completely during the later phases of experiment.

Within about 10—15 min after disappearance of contractile responses to caffeine the diastolic length of the cell began to decrease. This was preceded by the appearance of the low tail of relaxation. Within subsequent 10—15 min many cells died.

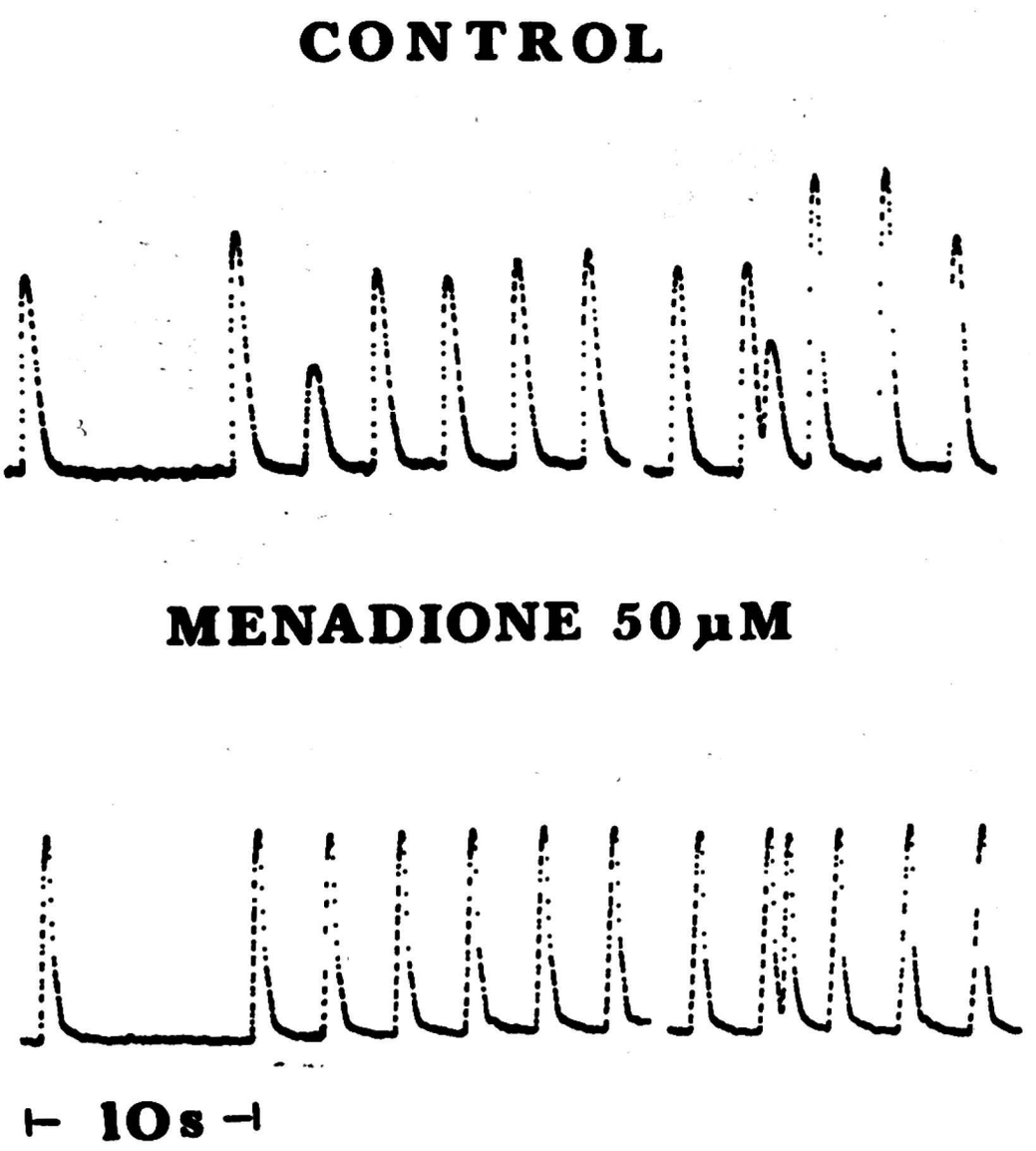

Fig. 2. The effect of Menadione on postrest potentiation (left panels) and on postextrasystolic potentiation of an isolated myocyte of guinea-pig ventricular myocardium.

The effect of Menadione on postrest and postextrasystolic potentiation.

Fig. 2 (the left panel) shows the postrest ESCs. The first contraction is potentiated, the second one is decreased. The pre-rest amplitude is recovered over the few subsequent beats. Fig. 2 shows also the potentiation of ESCs after the single intrapolated contraction. After the effects of Menadione described in the preceding section had fully developed, a short rest did not affect the amplitude of the ESCs (*Fig. 2*). Also the postextrasystolic potentiation was completely inhibited by Menadione (Fig 2).

The effect of Menadione on the rest-decay of ESCs.

A longer rest (5 min) resulted in the decrease of the amplitude of the postrest ESCs to 25±11% of control (Fig. 3). Rest decay of ESC was greatly retarded by Menadione. After 5 min of rest amplitude of ESC did not change appreciably (not shown) and at least 10 min was required

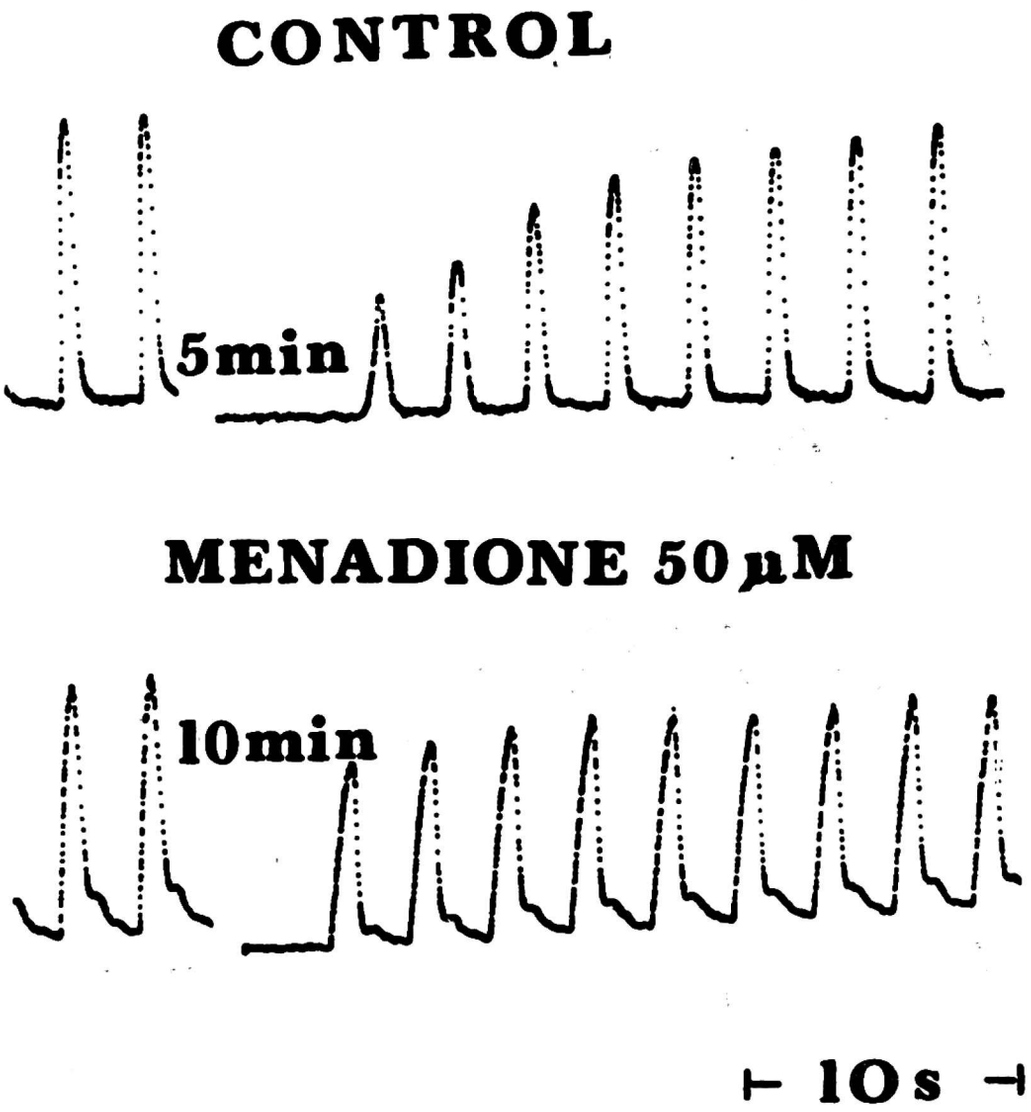

Fig. 3. The effect of Menadione on rest decay of electrically stimulated contractions of the isolated myocyte of guinea-pig ventricular myocardium.

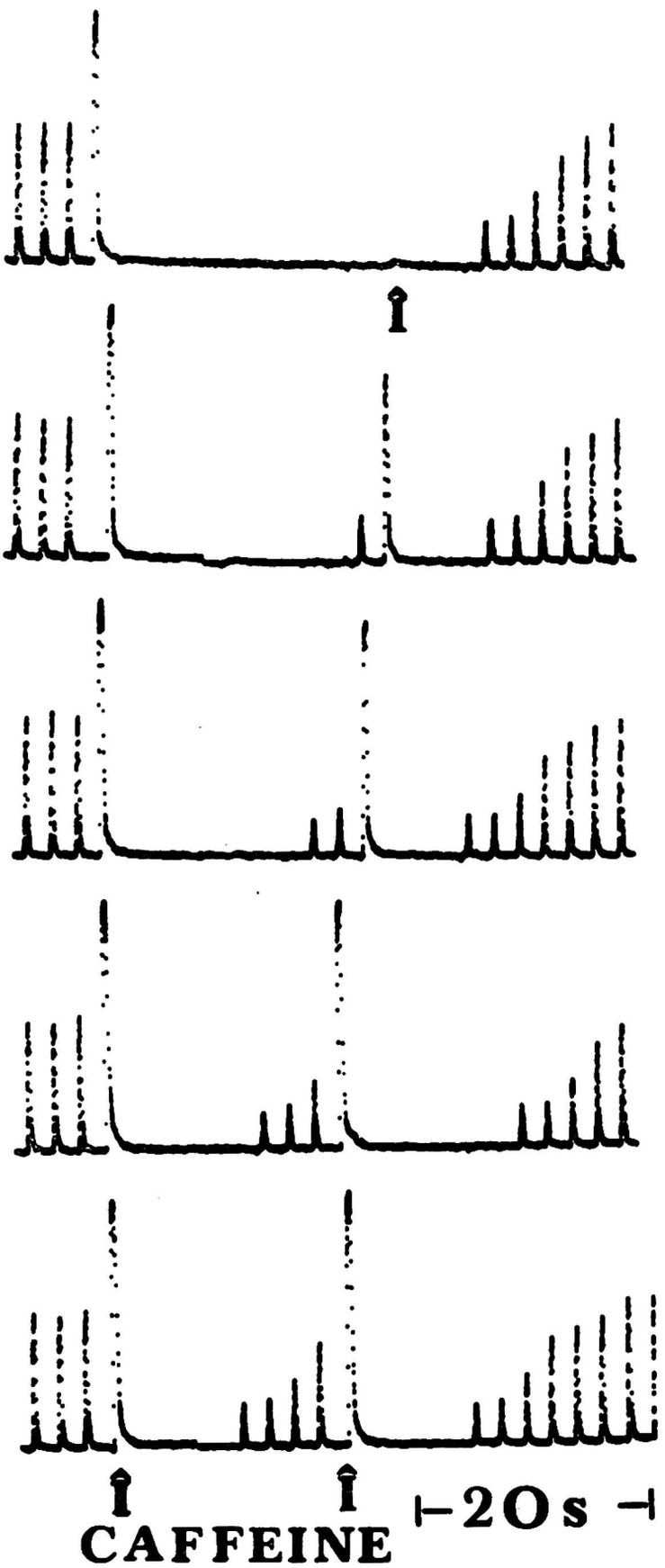

Fig. 4. Restoration of the contractile response to the second injection of caffeine (arrows) by the increasing numbers of post-caffeine electrically stimulated contractions in an isolated myocyte of guinea-pig ventricular myocardium.

in order to obtain its decrease by 50 %. Postrest recovery of the contractile amplitude was slow and it was accompanied by a decrease in the diastolic cell length (*Fig. 3*).

In the experiments performed in October and November, 1990 the water-soluble from of Menadione was without any effect or evoked only the spontaneous contractions. The water-insoluble form remained active. It seems that the possible reason of this discrepancy might be the seasonal variations in the properties of myocardium resulting in changes in permeability of sarcolemma of the isolated cells. Permeability of the water--soluble form is less than that of the insoluble form. The seasonal variations in the quality of the isolated cardiomyocytes are well known to the researcherers, although they are very rarely aknowledged in their papers.

DISCUSSION

The important finding of this study is that superfusion of isolated single myocytes of guinea-pig heart with Menadione resulted in a set of changes suggesting that SR was not able to take up and store Ca^{2+}: 1. The caffeine contractures initiated instead of a steady state ESC were inhibited. These contractures are regarded as a relative index of the SR Ca^{2+} content (for review see 6). 2. Single post-caffeine ESC became ineffective in restoring the contractile response to the next application of caffeine. 3. Postrest potentiation and postextrasystolic potentiation were completely inhibited. Since these events are associated with the normal SR function (1, 12, 13, 14), their disappearance has proved that SR was inactivated. These effects of Menadione are consistent with the results of Floreani and Carpenedo (7) and Floreani et al (8) who found that this compound strongly inhibits Ca^{2+} ATPase of the SR but not the Na/Ca exchanger or the sarcolemmal Na^+, K^+ATPase.

Despite the apparent inability of the SR to take up and store Ca^{2+}, electrical stimulation elicited contractions of normal amplitude, albeit with significantly prolonged time to peak amplitude. These results are consistent with those of Lewartowski et al. (6) who found that deprivation of SR of the ability to store Ca^{2+} by means of long superfusion with low concentrations of ryanodine does not preclude ESC of normal amplitude though with prolonged time to peak amplitude. The contractions in the cells superfused with the solution containing Menadione must have been initiated by Ca^{2+} diffusing into sarcoplasm from the sources other than SR. The most likely candidate is sarcolemma. Three routes of sarcolemmal Ca^{2+} influx into the sarcoplasm may be considered: 1. Transsarcolemmal diffusion through the activated Ca^{2+} channels; However, recent calculations of the influx of extracellular Ca^{2+} based on measurements of the

Ca^{2+} current have confirmed that it is too small for direct activation of significant contraction (15). 2. The Ca^{2+} may be transported into the cells by means of Na/Ca exchange working in the „in mode" during the initial phase of the plateau of the action potential (16). 3. The Ca^{2+} may be derived from the rapidly exchanging compartment defined by Langer et al (17) which is apparently localised at the sarcolemma or at a cellular site in very rapid equilibration with sarcolemmal sites. This compartment is believed to contain most of cellular exchangeable Ca^{2+} (17). Increase in its capacity results in a positive inotropic effect (10, 18).

Since the relative contribution of these sources of Ca^{2+} to activation of contraction is not known we will refer to them collectively as the sarcolemmal derived Ca^{2+} (SD-Ca^{2+}). The amount of SD-Ca^{2+} is apparently sufficient to activate contraction of the full amplitude in the cells treated with Menadione. One may wonder whether this is also the case in normal cells. In the cells treated with Menadione, Ca^{2+} current may be enhanced for two reasons. First, this compound was shown to inhibit slightly the phosphodiesterase (8) which might increase the cAMP concentration in the cell. Second, it has been shown by DuBell and Houser (5), Wier et al (19) and Yue (20) that upstroke of the Ca^{2+} transient is slowed down, its aplitude is decreased and its duration is increased in the cardiomyocytes treated with ryanodine. It is very likely that similar changes in the Ca^{2+} transient occur also in the cells treated with Menadione as both compounds prolong the time to peak tension or peak amplitude of contraction. Slow rise of the intracellular Ca^{2+} concentration and decrease in the maximal concentration would enhance the Ca^{2+} influx through the Ca^{2+} channels (22, 23). Also the Ca^{2+} influx by means of Na/Ca exchange would be enhanced (22). Thus the total amount of the SD-Ca^{2+} entering the sarcoplasm may be increased. Moreover, slower rise and increased duration of the Ca^{2+} transient may make it more effective in activation of the contractile system despite lower maximal sarcoplasmic Ca^{2+} concentration (20).

Nevertheless our results show that the Ca^{2+} influx during a single excitation may be quite large in the quinea-pig cardiac myocytes. As shown in Fig. 1 and 4 the SR Ca^{2+} is completely depleted after application of caffeine which is manifest in the lack of contractile response to the next application of caffeine and a very weak post caffeine ESC. A single excitation restores the caffeine contracture of the amplitude averaging 54% of that of steady state caffeine contracture. As shown in Fig. 4 the restored contracture may be much larger and reach 75% of the control. Intracellular redistribution of Ca^{2+} is unlikely as the recovery of caffeine contracture does not occur in the resting cell (the top panel of Fig. 4). Thus the source of the Ca^{2+} trapped by the SR during a single excitation must be the sarcolemma. The amount of Ca^{2+} trapped by the SR during

a single post-caffeine excitation is difficult to asses. However, as illustrated in Fig. 4 the contractile response to caffeine may be completely recovered over the three post-caffeine ESCs. Since the total SR Ca^{2+} content is larger than the amount of Ca^{2+} released to activate ESC (*Fig. 4*) it may be concluded that amount of Ca^{2+} trapped by the SR during single excitation is not much less than that necessary to activate a steady state ESC.

The results of this work suggest that the major role of the SR in activation of contraction of the guinea-pig ventricular myocytes is to take up SD-Ca^{2+}, the amount of which would be sufficient to activate directly contraction of considerable strength, and to release it rapidly at the very beginning of the next excitation as shown by the very early and rapid onset and upstroke of the intracellular Ca^{2+} transients when the SR function is normal (5, 19, 20, 21). When the ability of SR to store and release Ca^{2+} is inhibited by ryanodine, the upstroke of Ca^{2+} transient is slowed down, its amplitude is decreased and its duration is prolonged (5, 19, 20). Probably similar changes in the Ca^{2+} transient appear in the cells treated with Menadione. This role of the SR is clearly visible when one watches the transition from the normal contraction of a myocyte to that under the eventual effect of Menadione as illustrated in *Fig. 1.* The initial change consists of appearance of a second, late component of contraction. This appeares when the SR begins to lose its ability to take up Ca^{2+} which is manifested in a decrease in the amplitude of caffeine contractures. The initial rapid component disappeares and the second component rises and stays steady when the SR function is completely blocked. Thus the initial rapid component depends on Ca^{2+} release from the SR, whereas the late component apparently depends on SD-Ca^{2+}. The concept of such two components of contraction and their mechanism has been already offered by others (15, 24). When the SR function is normal the SD-Ca^{2+} is trapped and released at the next excitation. Thus only the rapid component appeares. When the SR function is impaired, the SR may be bypassed by SD-Ca^{2+} which may reach the contractile system and directly activate contraction. In this case only the late component appeares. The trapping of Ca^{2+} by the SR and the effect of its impairment is clearly illustrated in Fig. 1A. In the normal cell the amplitude of the first post-caffeine ESC averages 18.6% of that of the control steady state ESCs. In some cells it is so small that it is hardly visible in the record or by direct inspection on the screen of a TV monitor. However, this small ESC restores contractile response to the next application of ceffeine which shows that at least a considerable fraction of the SD-Ca^{2+} which entered sarcoplasm during a single post-caffeine ESC must have been trapped by the SR. As the caffeine contractures decrease under the effect of Menadione, the amplitude of the first post-caffeine ESC increases until it eventually reaches

the level of that of the steady state ESCs. At all phases of these changes its shape is typical for a contraction initiated when the SR Ca^{2+} is depleted: it has a prolonged time to peak amplitude and a sharp transition to the relaxation phase. The small amplitude of the post caffeine ESC was regarded as the proof of the SR being a major source of Ca^{2+} activating contraction (6). However, the present results suggest an alternative interpretation: the amplitude is so small because most of the SD-Ca^{2+} released during the first post-caffeine ESC is trapped by the SR before it is able to reach the contractile proteins. This interpretration is consistent with the results of Fabiato (2, 25) who was not able to activate contraction of the skinned cardiomyocytes by an increase in extracellular Ca^{2+} concentration if the SR was deprived of Ca^{2+}. When the ability of the SR to trap Ca^{2+} is blocked by means of Menadione, the SD-Ca^{2+} may bypass the SR and reach the contractile proteins to activate a contraction of full amplitude. We observed similar changes in the first post-caffeine ESC when the SR was deprived of the ability to store Ca^{2+} by means of a long superfusion with the low concentrations of ryanodine (6).

The relaxation phase was significantly shortened by Menadione while the amplitude of ESCs did not change. Thus the relaxation rate, although not measured directly in these experiments evidently increased. Apparently the SR either does not play any important role as a relaxing factor in the normal cells or it has been replaced by the other transport systems in the cells treated with Menadione.

The diastolic cell length decreased and eventually the cells became rounded 20—35 min after the SR function had been completely blocked by Menadione. This observation could mean that the normal SR function is necessary for maintaining the normal Ca^{2+} content in the cells. However, Menadione is not quite specific and may show a number of effects other then blocking of the SR Ca^{2+}ATPase (for review see 8). Among others Menadione may contribute to increase in the concentration of free radicals. This effect might be responsible for the eventual injury of the cell membrane and for increased non specific permeability to Ca^{2+} ions resulting in the cellular Ca^{2+} overload. Increased production of free radicals may be also responsible for the transient occurence of the spontaneous contractile waves. We observed such waves under the effect of H_2O_2 (unpublished).

In conclusion we propose that the amount of the SD-Ca^{2+} is large enough to activate directly ESC in the guinea-pig cardiomyocytes treated with Menadione. In the normal cells this SD-Ca^{2+} is taken up by the SR before it is able to reach the contractile proteins and released at the very beginning of the next excitation. Thus the major role of the SR in the quinea-pig cardiomyocytes would be the control of the time-course of contraction.

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Authors' address: Department of Clinical Physiology, Medical Center of Postgraduate Education, Marymoncka 99, 01-813 Warsaw, Poland.