JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2000, 51, 4, 777—798

Original articles

U. MACKIEWICZ, K. EMANUEL, B. LEWARTOWSKI

DIHYDROPYRIDINE RECEPTORS FUNCTIONING AS VOLTAGE SENSORS IN CARDIAC MYOCYTES

Department of Clinical Physiology, Medical Center of Postgraduate Education, Warsaw, Poland

Isolated, superfused with $[Ca^{2+}]_0 = 2.5$—3 mM (T = 37°C) and voltage clamped ventricular myocytes of guinea-pig hearts were stimulated by pulses from a holding potential −40 mV to +5 mV (duration 300 ms). They activated L-type Ca^{2+} current and a biphasic contractile response: a phasic component of amplitude ~7% of resting cell length (duration ~150 ms) and a tonic component of amplitude ~3% of resting cell length. The phasic component was inhibited by 10^{-6} M thapsigargin (Tg). Pulses from −40 mV to +5 mV stimulated a similar bi-phasic contractile response in 74% of cells (n = 126) superfused from the beginning of a 30 s period of rest with 5—10 mM Ni^{2+} which blocked the Ca^{2+} current and Na^+–Ca^{2+} exchanger (Ni^{2+}-contractions). Thus, the Ni^{2+}-contractions could be activated only by intracellular Ca^{2+} release. The phasic component of control contractions showed the bell-shaped voltage relation at $[Ca]_0 = 2$ mM and sigmoid relation at $[Ca]_0 = 3$ mM. The phasic component o Ni^{2+}-contractions showed a sigmoid relation at voltages from −40mV to +100 mV and could not be activated at $[Ca]_0 = 2$ mM. It was inhibited by 20 μM nifedipine, a blocker of dihydropyridine receptors, even when activated by the pulses to +70 mV, during which the Ca^{2+} current does not flow. We proved that nifedipine does not affect Na^+–Ca^{2+} exchange. The phasic component of Ni^{2+}-contractions was also inhibited by 2 nM indolizinesulphone SR33557, another dihydropyridine receptor blocker, which halved the phasic component of contractions in sontrol cells without any significant effect on the Ca^{2+} current. Stimulation did not activate contraction in any of 19 cells in which 20 μM nifedipine was superfused from the beginning of 30 s rest instead of 5 mM Ni^{2+}. These cells were depolarized to +5 mV over the rest period in order to prevent intracellular Ca^{2+} loss by Na^+–Ca^{2+} exchange. Residual Ca^{2+} currents were much stronger in cells superfused with nifedipine than residual currents in cells superfused with Ni^{2+} (hardly visible in the records). Our results suggest that a vestigial remnant of a voltage-sensing mechanism similar to that in the skeletal muscle may trigger the Ca^{2+} release from the SR of cardiac myocytes under specific experimental conditions. In normal cells it may be complementary to calcium induced calcium release (CICR).

Key words: *cardiac myocytes, excitation-contraction coupling, dihydropyridine receptors, voltage sensors.*

INTRODUCTION

Contraction of cardiac myocytes is initiated by an increase in $[Ca^{2+}]_i$ the immediate source of which is release from the sarcoplasmic reticulum (SR) (1, 2). Fabiato (3) found that rapid rise in $[Ca^{2+}]_i$ triggers release of Ca^{2+} from the SR. This phenomenon has been called the calcium induced calcium release (CIRC). That an increase in $[Ca^{2+}]_i$ due to activation of L-type Ca^{2+} current is the main trigger for release of Ca^{2+} from the cardiac SR has been generally accepted (1, 2, 4). Recent results obtained by intracellular Ca^{2+} imaging revealed close topographical and functional relations between the L-type Ca^{2+} channels and Ca^{2+} release channels of the SR (5, 6) (and for reviews see 7, 8). In 1987 Cannell *et al.* (9) have suggested that changes in membrane potential in cardiac myocytes may have immediate effects on the Ca^{2+} transient beyond those associated with their effects on Ca^{2+} current, i.e. that the release of Ca^{2+} from the SR may be at least in part regulated by a sarcolemmal charge-coupled release mechanism. This possibility has been recently reinvestigated in several papers the results of which suggest that, a voltage-sensitive mechanism may indeed contribute to release of Ca^{2+} from the SR. Ferrier and Howlett (10), Howlett and Ferrier (11), and Howlett *et al.* (12) were able to activate contractions in voltage clamped ventricular myocytes of guinea pig hearts by voltage steps negative to the threshold of activation of the L-type Ca^{2+} current (I_{CaL}). The following steps to positive potentials activated the Ca^{2+} current and next contraction. The contractions activated by the potentials negative to I_{CaL} threshold but not by those positive to it were inhibited by ryanodine (10) or thapsigargin (12). This suggests that the source of Ca^{2+} activating contractions elicited by pulses negative to the I_{CaL} threshold was the SR. The contractions elicited by potentials negative to I_{CaL} threshold were present despite inhibition of Na^+ current by lidocaine and tetrodotoxin (10, 12), partial or complete withdrawal of Na^+ from the extracellular solution and absence of Na^+ in the solution filling pipettes, which makes the possibility that they were activated by reversed mode Na^+–Ca^{2+} exchange unlikely. They were inhibited by 200 μM Ni^{2+} and 100—300 μM tetracaine but not by verapamil or nifedipine, although these blockers o L-type Ca^{2+} channels inhibited the contractions activated by depolarization to positive potentials (10, 13). The presence of cAMP in the pipette solution was an indispensable condition of stimulation of contractions by voltages negative to the I_{CaL} threshold (14).

Hobai *et al.* (15) were able to stimulate a nearly normal Ca^{2+} transients in cardiac myocytes of rabbits, rats and guinea-pigs superfused with Cd^{2+} or Ni^{2+}, which blocked the calcium currents. These results are at variance with the results of other papers of this group (10, 12) in which contractions activated by putative voltage sensors were blocked by Ni^{2+}. Considering the voltage of depolarizing pulses (−60 mV to +20 mV) used in Hobai *et al.* (15) work we

thought that the calcium transient could be activated in their experiments by dihydropyridine receptors acting like voltage sensors in skeletal muscle (although this hypothesis has not been proposed by the quoted authors). As the divalent cations block conductance but not the charge movement of dihydropyridine receptors (16) it seems likely that Ni^{2+} or Cd^{2+} inhibited Ca^{2+} current but not conformational changes of the L-type Ca^{2+} channels in the Hobai *et al.* (15) experiments. In the present paper we report the results suggesting that contractions stimulated in myocytes of guinea-pig heart superfused with 5–10 mM Ni^{2+}, which inhibits Ca^{2+} currents, may be, in fact, activated by dihydropyridine receptors acting as voltage sensors. This could be a vestigial remnant of a voltage-sensing mechanism similar to that in the skeletal muscle, which may be demonstrated under specific experimental conditions.

MATERIALS AND METHODS

Cell isolation, superfusion and recording of contractions

Guinea pigs of both sexes, weighing 250—300 g, were injected i.p. with 2.500 U heparin followed 30 min later by an overdose of sodium pentobarbital. After the heart was rapidly excised and washed in cold Tyrode solution, the aorta was cannulated and retrogradely perfused for 3 min with a nominally Ca^{2+} free solution, containing 100 μM ethylenebis (oxyethylenenitrilo) tetraacetic acid (EGTA) (for composition of solutions see below). The initial washout period was followed by 10—15 min of perfusion with Ca^{2+}-free Tyrode solution, containing 15 mg collagenase B (Boehringer) and 3 mg protease (Sigma) per 50 ml.

Thereafter the ventricles were cut from the atria and placed in a 50 ml beaker containing the same solution, disrupted with pincettes into small strands, and agitated. The cell suspension was filtered through nylon mesh, and allowed to sediment. The supernatant was discarded and cells were washed twice with Tyrode solution, of which the Ca^{2+} concentration was increased gradually to 1 mM. The cells were stored at room temperature until used.

Cells were placed in the 0.5 ml superfusion chamber mounted on the stage of an inverted microscope (Nikon Diaphot) and allowed to attach to its glass bottom. The chamber was superfused at a rate of ~2 ml/min. Three lines of perfusion solution heated up to the inlet enabled a change in superfusate composition within ~30 s. In experiments with caffeine we used the narrow chamber (4 mm in width and 2 mm in depth) through which a stream of superfusing solution was flowing. The temperature within the chamber was kept at ~37°C.

A TV camera was mounted onto the side port of the microscope and the cell length monitored by video edge-tracking system designed and built by John Parker (Cardiovascular Laboratories, School of Medicine, UCLA).

Ionic currents recordings

Currents were recorded using the whole cell voltage clamp method. Pipettes of 2.3 to 3.4 MΩ resistance were pulled (programmed Flaming/Brown Puller Model −97) from borosilicate glass capillaries. Pulses from holding potential −40 mV to +5 mV (duration 300 ms) were applied at

1 Hz to activate the L-type Ca^{2+} current. Currents were recorded using an Axopatch 1-D amplifier controlled by an IBM-AT 383 computer using the V-clamp software and V-clamp computer interface designed by D. R. Mattesson (Dept. of Biophysics, University of Maryland). Series resistance compensation was used to minimize voltage error due to access resistance. Signals were digitized at 2 kHz, stored on a disc and also directly recorded with a Sefram 8400 chart recorder.

Solutions

During cell isolation and throughout the experiments Tyrode solution of the following composition (in mM) was utilized: 144 NaCl, 5 KCl, 1 $MgCl_2$, 0.43 NaH_2PO_4, 10 N-2-hydroxyethylpiperazine-N'-2-etanesulfonic acid (HEPES), 11 glucose and 5 sodium pyruvate. The pH of the solution was adjusted with NaOH to 7.3 during cell isolation and to 7.4 during experiments. During the experiments the $CaCl_2$ concentration was 2.5—3 mM. The micropipettes were filled with a solution containing (in mM): 135 KCl, 5 NaCl, 1 $MgCl_2$, 10 HEPES, 4 Mg^{2+}-ATP, and 0.05 8-Br-cAMP. pH was adjusted to 7.20 with KOH. In one series of experiments the internal solution contained (in mM): 100 $CsCl_2$, 20 TEA, 10 EGTA 10 HEPES, 4 Mg^{2+}-ATP, and 0.05 8-Br-cAMP.

Statistical evaluation

The results are presented as means $\pm$ SE. Student's t test was used in order to determine the statistical significance of differences between the means. A $p < 0.01$ was taken as significant.

RESULTS

Currents and contractions under control conditions

The pulses from a holding potential of -40 mV to $+5$ mV activated an inward, presumably L-type Ca^{2+} current, and a phasic contraction of an amplitude of $7.0 \pm 1.2\%$ of resting cell length and duration of ~ 150 ms (*Fig. 1* and *2 A*). Relaxation of this contraction was not complete during the voltage clamp as it was followed by a slow tonic contraction of an amplitude of $\sim 3\%$ of resting length and ~ 300 ms duration. Complete relaxation followed repolarization to -40 mV (*Fig. 2 A*).

The effect of 5 mM Ni^{2+} on transmembrane currents and contractions

Hobai *et al.* (15) were able to elicit Ca^{2+} transients by depolarization without apparent activation of Ca^{2+} currents. This was achieved by blocking Ca^{2+} currents with Cd^{2+} or Ni^{2+} and stimulation of cells by the pulses from -60 mV to $+20$ mV. We used a similar approach with an experimental protocol, which permitted investigation of easily reproducible contractions initiated without apparent activation of Ca^{2+} influx. To this end voltage clamped cells were pulsed at the rate of 60/min for 1 min in order to load the

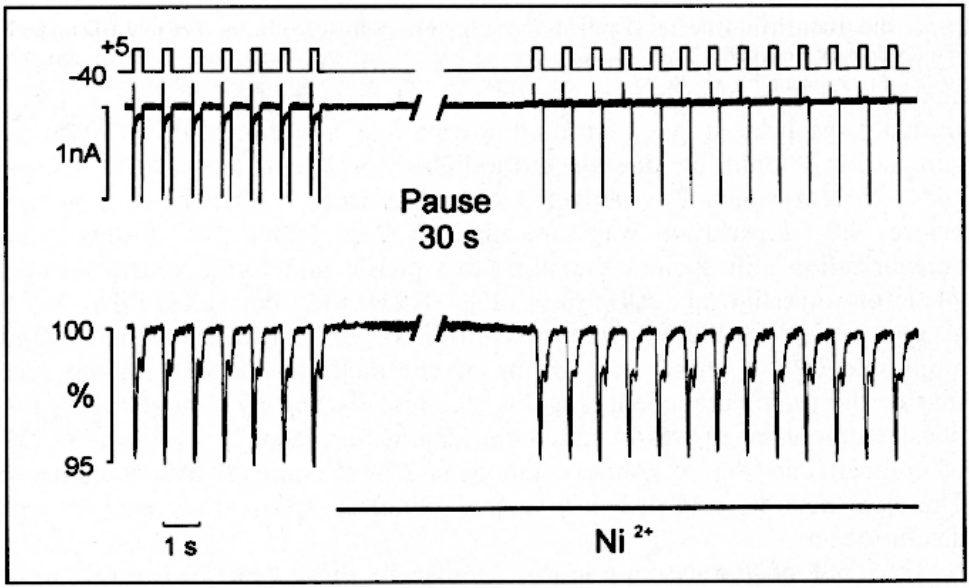

Fig. 1. The protocol of experiments with Ni^{2+} superfusion. Membrane currents (top records) and contractions (shortening downwards) of a representative single ventricular myocyte of guinea pig heart stimulated at the rate of 60/min by the pulses from the holding potential of −40 mV to + 5 mV. 5mM Ni^{2+} superfused from the beginning of the 30 s pause in stimulation. Left scale: cell shortening as % of resting cell length.

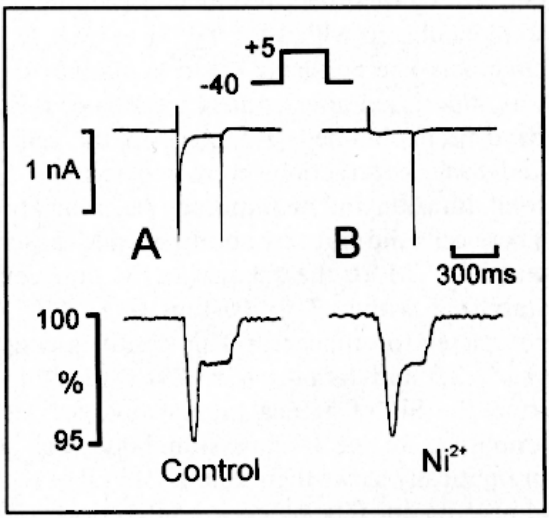

Fig. 2. The effect of 5 mM Ni^{2+} (B) on membrane currents (top records) and contractions (shortening downwards) of a representative single ventricular myocyte of guinea pig heart elicited by changes in membrane voltage shown above the current records. Holding potential −40 mV. Steady state stimulation rate was 60/min. Ni^{2+} was superfused from the beginning of 30 s pause in stimulation as shown in *Fig. 1*. The bottom scale: cell shortening as % of resting cell length.

SR and currents and contractions recorded (*Fig. 1* and *2 A*). Thereafter, stimulation was stopped and superfusing Tyrode solution changed for that containing 5 mM Ni^{2+}. Pulsing at the same rate was resumed after 30 s. At this

time the solution in the superfusing chamber should have been exchanged. 5 mM Ni^{2+} has been shown to block both T-type and L-type Ca^{2+} channels (17) and also the Na^{+}–Ca^{2+} exchanger (18). Therefore Ca^{2+} should have been trapped within the cells and any contractions following stimulation should be due to intracellular Ca^{2+} circulation. The L-type Ca^{2+} current which was activated by pulses from −40 mV to + 5 mV before Ni^{2+} superfusion was now blocked (*Fig. 1* and *2 B*), however, the depolarization still elicited the respective phasic and tonic contractions in 74% of investigated cells (n = 126). They will be called the Ni^{2+} — contractions in the following text. Prior to Ni^{2+} superfusion the average amplitude of the phasic component of contraction was 8.6 ± 1.2 μm and that of the tonic component was 2.4 ± 0.2 μm. During Ni^{2+} superfusion the phasic component of steady state contractions averaged 7.1 ± 0.4 μm (82.5% of control) and that of tonic component 2.1 ± 0.2 μm (87.5% of control). The contractions persisted for several minutes, up to the time of seal deterioration.

In 5 out of 7 cells contractions similar to those described above were obtained when 10 mM Ni^{2+} instead of 5 mM Ni^{2+} was used.

Contractions could not be elicited in cells superfused with 5 mM Ni^{2+} at room temperature stabilized at 24°C.

In order to investigate the source of Ca^{2+} activating control and Ni^{2+} contractions we used thapsigargin (Tg), a blocker of the Ca^{2+}-ATPase of SR (19, 20). Tg treatment results in depletion of Ca^{2+} from the SR (21—24). 8 cells were incubated with 10^{-6} Tg for at least 15 min before plating in the perfusion chamber. The perfusing solution contained 10^{-7} M Tg. In 3 cells 10^{-7} M Tg was superfused after control records of the calcium currents and contractions have been obtained (*Fig. 3 A*). In the cells preincubated with Tg the phasic and tonic contractions were replaced by a slower contractions of increased total duration and of amplitude reaching about 60% of that of the control cells. These cells did not respond to 15 mM caffeine superfusion. In cells superfused with 10^{-7} M Tg the changes in the time course and amplitude of contractions stabilized within 7 to 10 min (*Fig. 3 B*). At this time response to caffeine completely disappeared. This result is consistent with those of Lewartowski *et al.* (23) and Janiak *et al.* (25) who found that complete depletion of Ca^{2+} from the SR of guinea pig cardiomyocytes by Tg had minor effects on the amplitude of electrically stimulated Ca^{2+} transients and contractions but dramatically slows their kinetics. In all (n = 7) cells treated with Tg 5 mM Ni^{2+} almost completely blocked contractions (*Fig. 3 C*). The residual contractions were very slow with delayed onset and peak after the end of repolarization. The experiments with Tg show that the direct source of Ca^{2+} activating the phasic contractions in control cells and in cells superfused with Ni^{2+} is the SR. The source of Ca^{2+} activating the tonic component of contraction, however, is not

clear and solution of this problem deserves a separate study. The following experiments are exclusively directed to the mechanism of release of calcium responsible for the phasic component.

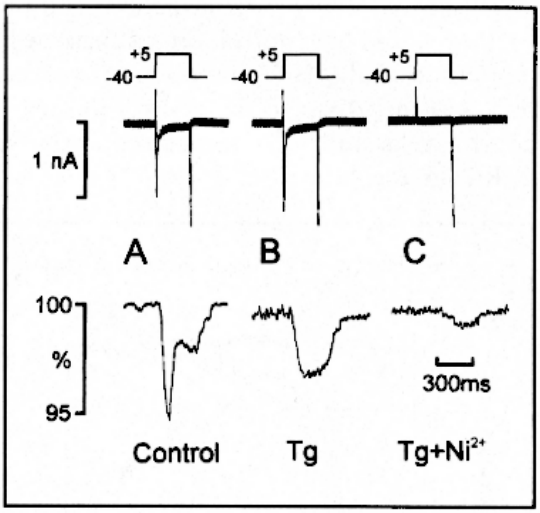

Fig. 3. The effect of 5 mM Ni^{2+} on membrane currents (upper records) and contractions of single ventricular myocyte of guinea pig heart superfused for 20 min with 10^{-7} M thapsigargin (Tg). Stimulation rate was 60/min. Ni^{2+} was superfused from the beginning of 30 s pause in stimulation as shown in *Fig. 1.*

Voltage and calcium dependence of Ni^{2+}-contractions

The results of the above experiments suggest that Ni^{2+}-contractions might be stimulated by Ca^{2+} released from SR due to activation of ryanodine rceptors by the putative voltage sensors. However, it might be argued that, in fact, they were activated by residual Ca^{2+} current hardly seen in our records. One of the methods for differentiation between the activation of ryanodine receptors by Ca^{2+} influx or voltage sensors involves measurement of the voltage dependence of the resulting contractions (12). At highly positive voltages the dihydropyridine receptors are activated, but due to decrease in driving force the Ca^{2+} current is negligible or does not flow. Thus, the sigmoid voltage relation described in skeletal muscle suggests contribution of a voltage sensing mechanism to activation of ryanodine receptors whereas the bell-shaped relation suggests CIRC.

We investigated the contraction-voltage relation at 3 mM Ca^{2+} as used in most of our experiments and at 2 mM Ca^{2+} currently used by others. Cells were stimulated at the rate of 60/min by conditioning pulses from a holding potential of -40 mV to $+5$ mV. Every 10^{th} conditioning pulse was replaced by a test pulse the voltage of which was increased in 10 mV steps from -30 mV to $+100$ mV. *Figure 4* shows the contraction and current voltage relation in control cells superfused with 3 mM Ca^{2+} or 2 mM Ca^{2+} and contraction voltage relation in cells superfused with Ni^{2+} (3 mM Ca^{2+}). Contractions of

the control cells superfused with 2 mM Ca^{2+} showed the typical bell-shaped voltage relation whereas contractions of cells superfused with 3 mM Ca^{2+} showed a sigmoid voltage relation similar to that found in skeletal muscle. The current-voltage relation investigated at two Ca^{2+} concentrations did not differ significantly. The Ni^{2+}-contractions showed a sigmoid voltage relation similar to that found in control cells superfused with 3 mM Ca^{2+}. However, the amplitude of the Ni^{2+}-contraction was significantly lower between −20 mV and +20 mV than in the control (3 mM Ca^{2+}) cells. The difference between control cells and cells superfused with Ni^{2+} may reflect elimination of the CIRC in the latter.

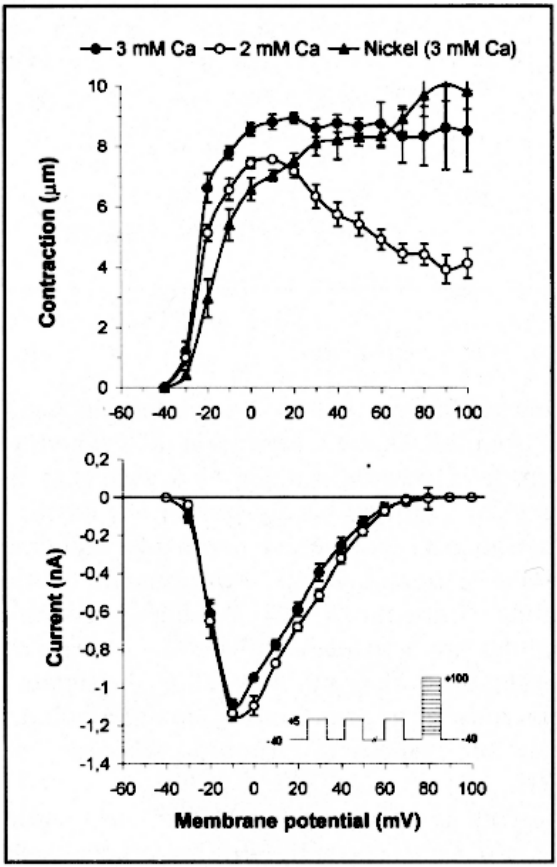

Fig. 4. Contraction — and current — voltage relation of guinea-pig cardiomyocytes stimulated by the pulses from −40 mV to +5 mV at the rate 60/min. Every 10th conditioning pulse was replaced by a test pulse the voltage of which was increased by 10 mV from −30 mV to +100 mV (inset). Each curve represents the mean results from 7 cells. The difference between shortening of cells superfused with Tyrode solution containing 3 mM Ca^{2+} and of cells superfused with 5 mM Ni^{2+} is significant between −20 mM and +20 mV ($p < 0.01$).

The apparent absence of a voltage sensing mechanism in cells superfused with 2 mM Ca^{2+} suggests that Ni^{2}-contractions should not be activated at this Ca^{2+} concentration. The next experiments showed that this is the case. In 12 cells pulses to +5 mV could not elicit Ni^{2+}-contractions when Ca^{2+} concentration in the superfusing solution was lower than 2.5 mM. The series of

several pulses (but not the single pulse) positive to +30 mV were able to activate contraction in 3 out of 5 cells even at 1 mM Ca^{2+}. At lower Ca^{2+} concentrations no Ni^{2+}-contractions could be activated. Substitution of equimolar Ba^{2+} for Ca^{2+} did not change the results.

The effect of lower Ca^{2+} concentration could depend on depletion of SR Ca^{2+}. This possibility was tested in the next series of experiments. 9 cells were superfused with Tyrode solution, Ni^{2+}-contractions were obtained according the protocol shown in *Fig. 1*, and 15 mM caffeine was superfused for 1 s. After subsequent stimulation for 30 s Ca^{2+} concentration was decreased to 1 mM. Next caffeine superfusion was applied after the contractions were inhibited. The amplitude of caffeine contractions obtained at 1 mM Ca^{2+} did not significantly differ from that obtained at 3 mM Ca^{2+} (*Fig. 5*). These experiments show that inhibition of Ni^{2+}-contractions by 1 mM Ca^{2+} did not depend on depletion of SR calcium. Thus, the $[Ca]_0$ dependence of Ni^{2+}-contractions may mean that activation of the putative voltage sensors requires relatively high $[Ca]_0$.

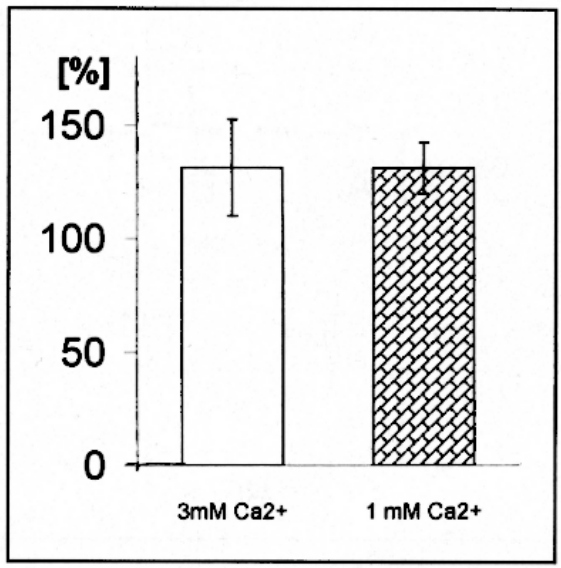

Fig. 5. Contractile response to 15 mM caffeine of single myocytes of guinea-pig heart superfused with Tyrode solution containing 3 mM Ca^{2+} and 5 mM Ni^{2+} (open bar) and of the same cells after the $[Ca]_0$ has been decreased to 1 mM (blackened bar) ($n = 9$, $p > 0.01$). Caffeine contractions calculated in % of shortening of cells electrically stimulated at 3 mM Ca^{2+}.

Molecular nature of the putative voltage sensors

Since contractions in cells superfused with Ni^{2+} were activated by voltage positive to −40 mV, the most likely candidates are sarcolemmal dihydropyridine receptors which could act as the voltage sensors as in the skeletal muscle. Ni^{2+} ions would block their Ca^{2+} conductance, but not their voltage-induced conformational changes. These should be inhibited by organic blockers, which bind to the α_1 subunit of dihydropyridine receptors. Their effect on Ni^{2+}-contractions was tested in the next series of experiments.

Cells superfused with 5 mM Ni^{2+} as shown in *Fig. 1*, were stimulated by pulses from a holding potential of −40 mM to +5 mV (*Fig. 6 A*). The +5 mM pulse was replaced by a pulse to +70 mV at each 10th pulse (*Fig. 6 B*). After the steady state contractions were obtained 20 μM nifedipine was added to the superfusing solution. In 13 cells nifedipine completely blocked the phasic component of contractions stimulated by pulses to +5 mM (*Fig. 6 C*) and also by the pulse to +70 mV (*Fig. 6 D*) during which the Ca^{2+} current does not flow. These results suggest that activation of ryanodine receptors in cells superfused with Ni^{2+} was brought about by conformational changes of dihydropyridine receptors functioning like voltage sensors. Nevertheless, very small residual inward currents were observed in 71 out of 93 cells in which Ni^{2+}-contractions were stimulated (*Fig. 2 B, 6 A, 7 C, 8 C*). So, it may be argued that ryanodine receptors could be activated in cells superfused with Ni^{2+} by residual Ca^{2+} current, which was blocked by nifedipine.

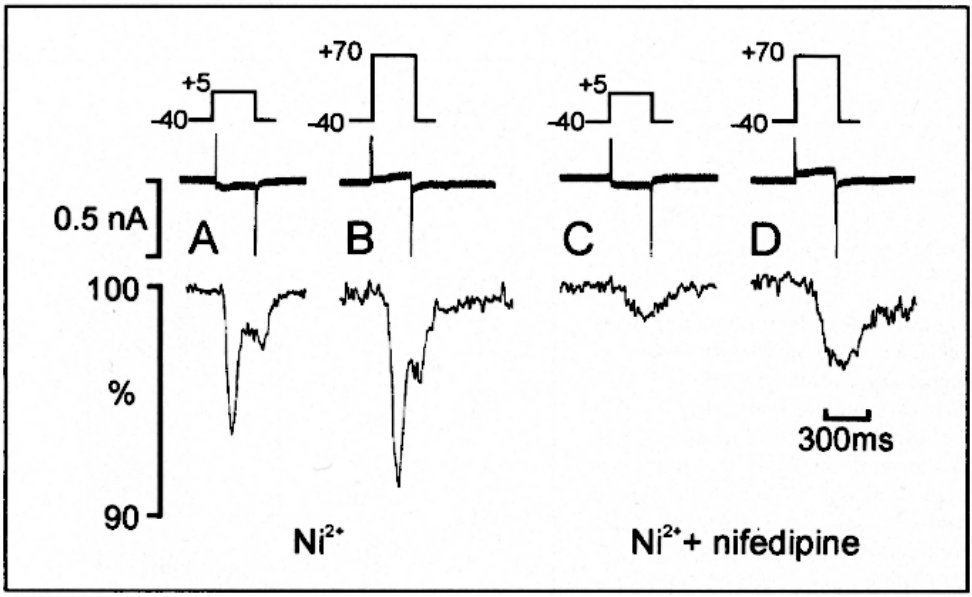

Fig. 6. The effect of 20 μM nifedipine on membrane currents (top records) and contractions of a representative ventricular myocyte of guinea pig heart pretreated with 5.0 mM Ni^{2+}. A, B: steady state contractions stimulated at the rate of 60/min after a pause in stimulation at the beginning of which superfusion with Ni^{2+} was started as shown in *Fig. 1*. Every 10th pulse to +5 mV was replaced by a pulse to +70 mV (B). C, D: two min of superfusion with 20 μM nifedipine. The bottom scale: cell contraction. Amplitude expressed as % of resting length.

We further tested this possibility by using another blocker of dihydropyridine receptors, an indolizinsulphone, SR33557, which has been shown to block the excitation-contraction coupling in skeletal muscle without

substantially affecting the Ca^{2+} current (26). In normal cells 2 nM SR33557 did not affect, or increased, the amplitude of the L-type Ca^{2+} current, while decreasing the amplitude of the phasic component of contraction from 6.4 ± 0.6 μm to 3.3 ± 0.6 μm, i.e. to 51.5% of control ($p < 0.01$, $n = 6$) (*Fig. 7 B*). Next we used 2 nM SR33557 in experiments in which the contractions were stimulated in cells pre-treated with 5 mM Ni^{2+} as shown in *Fig. 1*. After the steady state contractions were obtained (*Fig. 7 C*), 2 nM SR33557 was added to the superfusate. After a few seconds the amplitude of contractions decreased, reaching a new steady state within about 60 s (*Fig. 7 D*). At that time the amplitude of the phasic contraction decreased from 6.8 ± 0.8 μm to 0.8 ± 0.4 μm, i.e. to 11.8% of control ($p < 0.01$, $n = 12$). Since 2 nM SR33557 did not block the Ca^{2+} current in normal cells, its inhibition of the phasic contractions in cells treated with Ni^{2+} could not result from the inhibition of residual Ca^{2+} current.

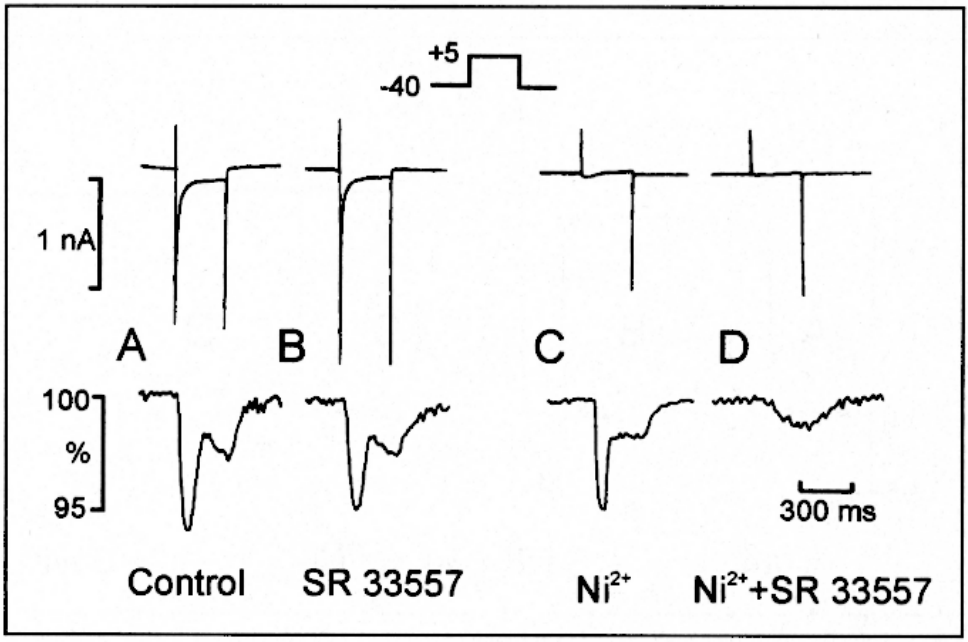

Fig. 7. The effect of 2 nM SR33557 on the membrane currents (top records) and contractions of ventricular myocytes of guinea pig heart. A: a normal myocyte stimulated from the holding potential of −40 mV to +5 mV at a rate of 60/min. B: in the same cell, 2 nM 33557 added to the superfusing solution. C: steady state membrane currents and contractions of another cell recorded after the 30 s pause in stimulation at the beginning of which superfusion with 5 mM Ni^{2+} was commenced. D: 2 nM SR33557 was added to the Ni^{2+} containing solution superfusing the same cell.

Inhibition of contractions stimulated by the pulses to +70 mV by nifedipine and the effect of SR33557 militated against the probability that Ni^{2+}-contractions were activated by residual Ca^{2+} influx. Nevertheless, we

performed additional experiments addressing the problem because opponents of the cardiac voltage sensor hypothesis repeatedly stress the lack of absolute certainty of exclusion of residual Ca^{2+} influx as activator of contractions without apparent Ca^{2+} current.

The nature and significance of the residual currents in cells superfused with Ni^{2+}

In control cells 0.1 μM BayK 8644 greatly increased the Ca^{2+} current and contractions (*Fig. 8 A, B*). In 5 out of 6 cells superfused with 5 mM Ni^{2+}, 1 μM BayK did not stimulate the residual currents (*Fig. 8 C, D*). Only in one cell the current was doubled reaching 8.3% of amplitude of the control, pre-Ni^{2+}, Ca^{2+} current (not shown). Lack of the effect of BayK 8644 in most cells superfused with Na^{2+} suggests that their residual currents were not the Ca^{2+} currents.

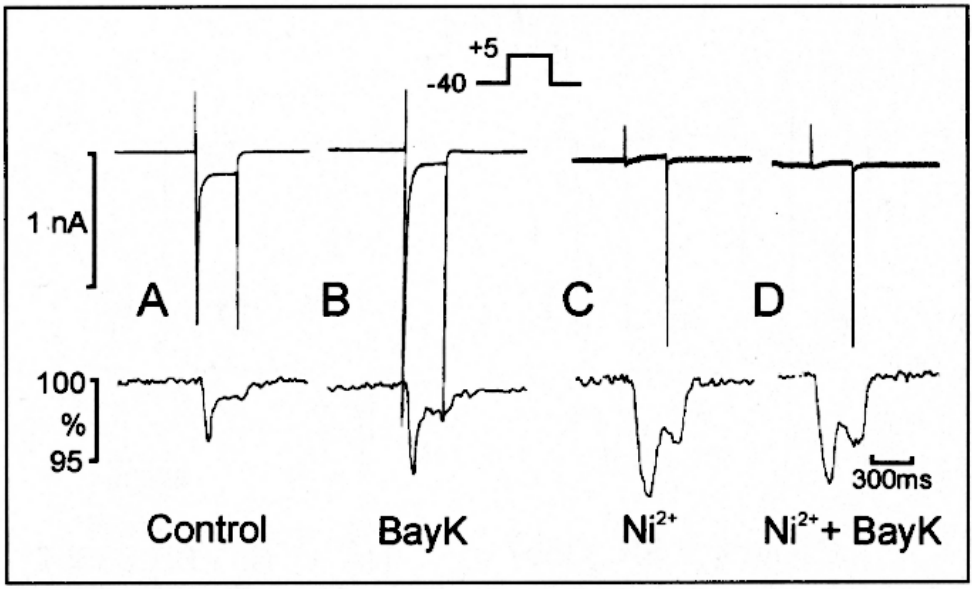

Fig. 8. The effect of BayK 8644 on the membrane currents in a representative control cell and on residual currents in cells superfused with 5 mM Ni^{2+}, A: control; B: same cell superfused for 2 min with 0.1 μM BayK; C- steady state currents and contractions of another cell recorded after the 30 s pause in stimulation at the beginning of which superfusion with 5 Ni^{2+} was started; D: same cell superfused for 2 min with 1 μM BayK.

Nevertheless, in the next series of experiments we determined whether residual inward currents of similar or larger amplitude are able to stimulate contractions when the conformational changes of dihydropyridine receptors are largely inhibited by an organic blocker. We repeated the experiments in

which the Ca^{2+} current was blocked during and after the 30 s break in stimulation, by 20 μM nifedipine instead of Ni^{2+} (*Fig. 9*). In order to prevent Ca^{2+} loss from the cell by Na^{+}–Ca^{2+} exchange, which is not inhibited by nifedipine, the holding potential was switched to +5 mV for the duration of the pause. The holding potential was switched back to −40 mV 5 s before commencing post-rest stimulation. In 19 cells tested this way post rest stimulation either did not elicit contractions, or elicited feeble slow contractions despite residual Ca^{2+} currents much stronger than in the experiments with Ni^{2+} (*Fig. 9 B*). The inward current visible in *Fig. 8 C* was largest among all cells treated with 5 mM Ni^{2+}. Its amplitude attained 4.5% of the control, pre-Ni^{2+}, Ca^{2+} current. The amplitude of residual currents in cells superfused with nifedipine instead of Ni^{2+} ranged from 4.5% to 40% (mean $16.8 \pm 2.3\%$), n = 19) of pre-nifedipine control.

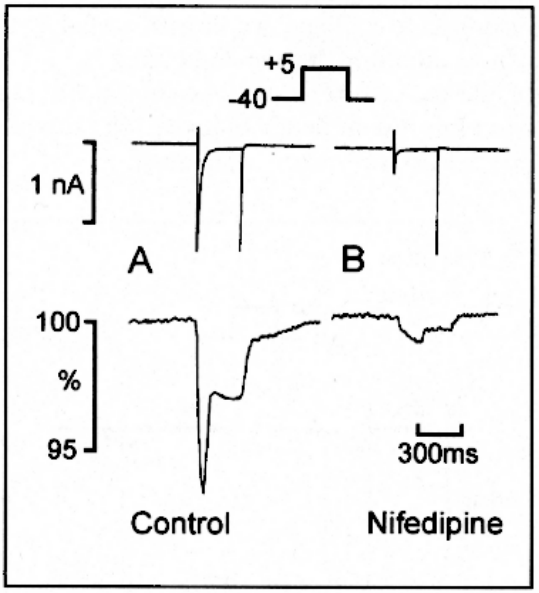

Fig. 9. Membrane currents (top records) and contractions of single myocyte of guinea-pig heart stimulated at the rate of 60/min. B: 20 μM nifedipine superfused from the beginning of 30 s pause in stimulation. Membrane potential was held at +5 mV during the pause in order to inhibit Ca^{2+} loss by Na^{+}–Ca^{2+} exchange.

These results suggest that activation of ryanodine receptors in cells superfused with Ni^{2+} by the residual Ca^{2+} currents is unlikely. Since residual currents of most cells were not stimulated by BayK 8644 we thought that they might be the currents of Na^{+}–Ca^{2+} exchange not completely blocked by 5 mM Ni^{2+} and stimulated by the Ca^{2+} transients in the activated cells. In order to test this possibility 7 cells were dialyzed with an internal solution containing a high concentration of EGTA (see Methods) to chelate intracellular Ca^{2+}. Cells were stimulated by the pulses from a holding potential of −40 mV to

+5 mV (*Fig. 10*). After contractions disappeared stimulation was stopped for 30 s and 5 mM Ni^{2+} superfused as shown in *Fig. 1*. There were no residual inward currents in the records obtained during the stimulation commenced after the pause (*Fig. 10 B*). This suggests that in the main experiments they might result from the residual Na^+–Ca^{2+} exchange not completely blocked by Ni^{2+}. Therefore, it may be argued that ryanodine receptors could be activated in cells superfused with Ni^{2+} by the reversed Na^+–Ca^{2+} exchange, especially when the strongly positive pulses were used. So, it is crucial to know whether nifedipine inhibited the Ni^{2+}-contractions by blocking the Na^+–Ca^{2+} exchange. Since we could not find in the literature the data concerning the effect of nifedipine on Na^+–Ca^{2+} exchange we tested it in our experiments. The voltage clamped cells were superfused with normal Tyrode solution. Stimulation by pulses from −40 mV to +5 mV was stopped for 30 s and the response to 1 s superfusion with 15 mM caffeine tested. Stimulation was resumed for 1 min. Thereafter, stimulation was stopped again and cells superfused with 20 μM nifedipine. After 30 s the response to caffeine was again tested. Nifedipine did not affect the caffeine contraction nor the accompanying Na^+–Ca^{2+} exchange inward current in any of 5 tested cells (*Fig. 11*). The subsequent stimulation showed that Ca^{2+} current was almost completely blocked. These experiments suggest that nifedipine does not affect Na^+–Ca^{2+} exchange.

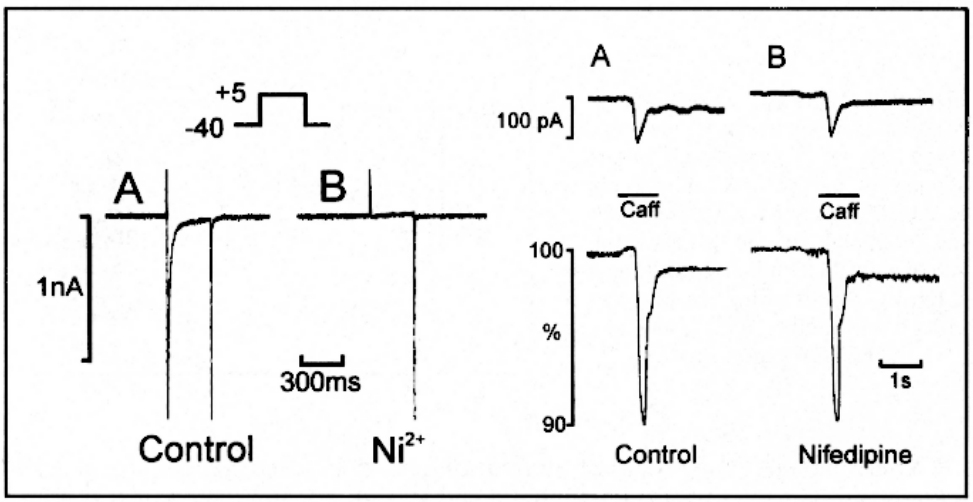

Fig. 10. Membrane currents of ventricular myocyte of guinea pig heart dialyzed with an internal solution containing 34.5 mM EGTA. A: control; B: after 30 s pause in stimulation at the beginning of which superfusion with 5 mM Ni^{2+} was commenced. The cell did not contract (not shown).

Fig. 11. Membrane currents (top records) and contractions elicited by 1 s superfusion of 15 mM caffeine. A: control contraction elicited after 30 s pause in stimulation at 60/min; B: reponse to caffeine at the end of the next 30 s pause in stimulation at the beginning of which 20 μM nifedipine was superfused.

cAMP is not an indispensable condition of stimulation of contractions without activation of Ca^{2+} current

Hobai *et al.* (15) and Ferrier and Howlett (10) have reported that contractions without activation of the Ca^{2+} current cannot be elicited without the presence of 20--100 μM cAMP in the solution filling the micropipette. Therefore in most of our experiments cAMP was added to the internal solution. However, we were able to obtain full size contractions in 5 out of 7 cells superfused with Ni^{2+} in an experiment in which cAMP was omitted (not shown).

DISCUSSION

The important findings of this study are that 1. contractions stimulated in myocytes superfused with 5—10 mM Ni^{2+} (Ni^{2+}-contractions) show a sigmoid voltage relation and are inhibited by organic blocker of dihydropyridine receptors; 2. Ni^{2+}-contractions are activated by Ca^{2+} released from SR; 3. we accumulated substantial evidence that ryanodine receptors in cells superfused with Ni^{2+} were not activated by residual Ca^{2+} influx.

The source of Ca^{2+} activating Ni^{2+}-contractions

Contractions in the control cells and Ni^{2+}-contractions consist of a phasic and tonic component. The phasic component is absent in cells pretreated with 10^{-6} M Tg, the blocker of the Ca^{2+}-ATPase of the SR (19, 20). Contractions remaining in cells pretreated with Tg were almost completely blocked by Ni^{2+}. Thus, the phasic component of control and of Ni^{2+} – contractions is generated by Ca^{2+} released from the SR. The source of Ca^{2+} activating the tonic component of contractions is not clear and deserves a separate study. The following discussion concerns exclusively the phasic component of Ni^{2+}-contractions.

Dihydropyridine receptors as hypothetical voltage sensors in cells superfused with Ni^{2+}

Stimulation of Ni^{2+}-contractions by voltage within the range of activation of dihydropyridine receptors, their sigmoid voltage relation and their inhibition by nifedipine suggest that release of SR Ca^{2+} in cells superfused with Ni^{2+} is brought about by dihydropyridine receptors acting like voltage sensors in skeletal muscle. The mechanism of the effect of Ni^{2+} in our experiments may be explained in the following way. Ni^{2+} blocks the Ca^{2+} current but not the

conformational, voltage dependent changes of the channel proteins, which activate the ryanodine receptors. Divalent cations block the Ca^{2+} current by competing with Ca^{2+} for high-afinity binding to Ca^{2+} channels (27). However, they do not affect the charge movement associated with the channel gating. These are inhibited by organic channel blockers (16, 28). Organic blockers inhibit the charge movement, and in this way uncouple voltage and ryanodine receptors of the SR in our experiments, in a manner similar to their blockade of excitation-contraction coupling in skeletal muscle (29, 30). An alternative hypothesis, already proposed for the skeletal muscle, is that there are two classes of dihydropyridine receptors: one with predominant channel activity and the other with predominant voltage sensor activity (29). The former could be blocked selectively by Ni^{2+}, whereas nifedipine would block both. This hypothesis is supported by the fact that in guinea-pig ventricular myocytes the density of dihydropyridine receptors calculated from charge movement is about 10 fold grater than the density of Ca^{2+} channels calculated from the Ca^{2+} current (16).

However, the hypothesis that ryanodine receptors are activated in cells superfused with Ni^{2+} by dihydropyridine receptors functioning as voltage sensors is challenged by our finding that the Ni^{2+}-contractions depend on $[Ca]_0$ and are inhibited when it is decreased below 2.5 mM. We found that decrease in $[Ca]_0$ from 3 mM to 1 mM does not deplete the SR Ca^{2+}. Thus, the $[Ca]_0$ dependence of Ni^{2+}-contractions may mean either that activation of the putative voltage sensors requires relatively high $[Ca]_0$ or that Ni^{2+}-contractions are activated by residual Ca^{2+} influx blocked by nifedipine.

$[Ca]_0$ dependence of the putative voltage sensors

The first hypothesis is supported by our finding that the bell-shaped contraction-voltage relation of normal cells superfused with 2 mM Ca^{2+} is changed into the sigmoid relation when $[Ca]_0$ is increased to 3 mM Ca^{2+}. At highly positive voltage (up to +100 mV in our experiments) the dihydropyridine receptors are still activated whereas the Ca^{2+} current is negligible or does not flow. Thus, the shift from bell-shaped into a sigmoid contraction-voltage relation upon increase in $[Ca]_0$ from 2 mM to 3 mM suggests that a voltage sensitive mechanism of activation of ryanodine receptors has joined CIRC at higher $[Ca]_0$.

The voltage sensors of skeletal muscle also require the presence of Ca^{2+} in the extracellular space for their function (30) although the critical concentration is much lower than in our experiments (31, 32).

Residual Ca^{2+} influx as hypothetical activator of Ni^{2+}-contractions

The residual Ca^{2+} influx could consist of residual Ca^{2+} current and/or reverse Na^{+}–Ca^{2+} exchange not completely blocked by Ni^{2+}. The first possibility is supported by increase in residual current by BayK 8644 in one of the tested cells. Inhibition of residual inward currents by chelation of intracellular Ca^{2+} suggests that in many cells they could be the currents of Na^{+}–Ca^{2+} exchange not completely blocked by Ni^{2+} and activated by calcium transient in stimulated cells.

The following experimental evidence is not compatible with the possibility that nifedipine inhibited Ni^{2+}-contractions by blocking the residual Ca^{2+} current.

1. Nifedipine blocked the Ni^{2+}-contractions stimulated by the pulses to +70 mV. At this voltage dihydropyridine receptors are activated but Ca^{2+} current does not flow.

2. Another blocker of dihydropyridine receptors, indolizinesulfone SR33557 inhibited the Ni^{2+}-contractions despite the fact that it did not block the Ca^{2+} current in normal cells. This compound is structurally unrelated to other Ca^{2+} channel modulators and has its own binding site on the α_1 subunit of the dihydropyridine receptor binding protein of T tubules of skeletal muscle which is allosterically related to binding sites for other Ca^{2+} channel blockers (33). SR33557 has been shown to discriminate between the two functions of dihydropyridine receptors in skeletal muscle myoballs (26). At low concentrations (between 2 nM and 50 nM) it blocks contractions attributed to the activation of voltage sensors, but decreases the intensity of Ca^{2+} current by only about 30%. At concentrations between 0.1 μM and 2 μM it also completely inhibits the Ca^{2+} current. We found that 2.0 nM SR33557 does not significantly affect the L-type Ca^{2+} current in the control ventricular myocytes of guinea pig heart, but decreases amplitudes of the phasic and tonic components of contractions by 48%. In contrast, in cells superfused with 5 mM Ni^{2+}, in which the Ca^{2+} currents have been already blocked, but a large phasic contraction persists, 2 nM SR33557 inhibited the phasic component of Ni^{2+}-contractions by 88%. Since SR33557 did not affect the Ca^{2+} current in the absence of Ni^{2+}, inhibition of Ni^{2+}-contractions by this compound cannot be attributed to inhibition of some residual Ca^{2+} current. The lack of the effect of 2 nM SR33557 on the Ca^{2+} current in our experiments seems to be at variance with the results of Romey *et al.* (34) who found that SR33557 inhibits the L-type Ca^{2+} current in the cultured myocytes of newborn mice with $EC_{50} = 1.4$ nM. The difference in cell type and temperature might be the reason for this difference.

3. The residual Ca^{2+} currents left after superfusion of nifedipine instead of Ni^{2+} were not able to stimulate contractions despite the fact that their

amplitude was much larger than that of residual currents left during Ni^{2+} superfusion. Conformational changes of dihydropyridine receptors must have been largely inhibited during nifedipine superfusion.

4. As suggested by the experiments with Ca^{2+} sparks and local control theory, recruitment of activated ryanodine receptors or their clusters is graded by the activation of respective dihydropyridine receptors (5, 6, 7, 8, 35, 36) except for Ca^{2+} overloaded cells in which regenerative Ca^{2+} release from SR has been reported. In our experiments control contractions as well as Ni^{2+} — contractions showed graded response to activating voltage between −40 mV and 0 mV, which suggests that activation of ryanodine receptors was graded. Thus, it is very unlikely that residual currents not exceeding 5% of normal Ca^{2+} current activated contractions of almost normal amplitude.

Reverse mode Na^+–Ca^{2+} exchange has been proposed to contribute to activation of ryanodine receptors, particularly under conditions of high cell and SR Ca^{2+} loading (37—43). Thus, it might be argued that residual reverse mode Na^+–Ca^{2+} exchange activates ryanodine receptors to elicit Ca^{2+} release in cells superfused with Ni^{2+}. However, if the Ni^{2+} — contractions were activated by the reverse Na^+–Ca^{2+} exchange they should not have been inhibited by nifedipine. As shown in our experiments (*Fig. 11*) nifedipine does not affect Na^+–Ca^{2+} exchange (see 44). Moreover, in normal cells contribution of reverse mode Na^+–Ca^{2+} exchange to activation of ryanodine receptors does not exceed ~30% (45, 46). As shown by Kimura *et al.* (18) Na^+–Ca^{2+} exchange is strongly blocked by 5 mM Ni^{2+}. I_{Na} was not activated in our cells i.e., one of the factors responsible for reversing of Na^+–Ca^{2+} exchange was not present. So the residual reversed Na^+–Ca^{2+} exchange could be heardly expected to activate nearly normal contraction in cells superfused with Ni^{2+}.

The above evidence suggests that ryanodine receptors in cells superfused with Ni^{2+} were activated by dihydropyridine receptors functioning like voltage sensors rather, than by residual Ca^{2+} influx.

Our conclusion regarding the existence of a voltage sensing Ca^{2+} release mechanism would seem at variance with that of Nabauer *et al.* (47) who concluded that "regulation of calcium release is gated by calcium current, not gating charge, in cardiac myocytes". This notion was based on the results of experiments in which removal of extracellular Ca^{2+} resulted in depolarization-contraction uncoupling in single rat cardiomyocytes, despite an SR replete with Ca^{2+}. A decrease in extracellular Ca^{2+} concentration also inhibited contractions of cells superfused with Ni^{2+} in our experiments. The effect of removal of extracellular Ca^{2+} could not be compensated in Nabauer *et al.* (47) work by Ba^{2+}, which is transported by the Ca^{2+} channel and supports intramembrane charge movement related to excitation-contraction coupling in skeletal muscle. This was also the case in our experiments. So, in

this respect our results do not differ from those of Nabauer *et al.* (47). Other evidence explains why they could not elicit contractions after removal of extracellular Ca^{2+}. First, the experiments of Nabauer *et al.* (47) were performed at room (24°C) temperature at which the voltage sensitive mechanism is not active (11). Even if their experiments were performed at 37°C removal of extracellular Ca^{2+} could inactivate the voltage sensors as we have shown. The failure of Ba^{2+} to substitute for Ca^{2+} may be explained by a difference in cardiac and skeletal muscle voltage sensors.

As referred to in the introduction, the idea of voltage triggered Ca^{2+} release from the cardiac SR has been recently advocated in several papers the results of which, however, differ from ours. Ferrier and Howlett (10), Howlett and Ferrier (11), Howlett *et al.* (12) and Ferrier *et al.* (14) stimulated phasic contractions in cardiac myocytes of rats and guinea pigs by depolarization steps from -65 mV to -40 mV. The L-type Ca^{2+} current is not activated within this voltage range and the contractions were not blocked by verapamil or nifedipine but were blocked by 200 μM Ni^{2+}. Thus, the putative voltage sensing mechanism proposed in the above quoted papers seems to be quite different from that investigated under our experimental conditions.

In conclusion, our results suggest that under some experimental conditions the Ca^{2+} release channels of cardiac SR may be activated by dihydropyridine receptors functioning like voltage sensors as in skeletal muscle. This notion is consistent with the results of Satoh *et al.* (48) who showed that changes of conformation of sarcolemmal dihydropyridine receptors induced by BayK 8644 in resting cardiac myocytes may activate the ryanodine receptors of the SR without activation of Ca^{2+} current. Partial inhibition of contractions by SR33557 in the absence of Ni^{2+} but almost complete inhibition in cells in which the Ca^{2+} current is blocked with Ni^{2+} suggest that the putative voltage sensing mechanism and CICR may work in parallel. Thus, excitation--contraction coupling in cardiac myocytes would not differ qualitatively from that in skeletal muscle in which these two mechanisms cooperate: a positive feedback occurs between Ca^{2+} released from the SR by voltage sensors and secondary activation of ryanodine receptors by the released Ca^{2+} (36, 49). As shown by Yano *et al.* (50) depolarization of the T-tubule membrane induces conformational changes in the junctional foot protein which lead to release of the SR Ca^{2+} and the released Ca^{2+} induces the second phase of conformational changes which result in release of more Ca^{2+}. The double mechanism of activation of the ryanodine receptors of skeletal muscles has been also proposed by Kein *et al.* (51). However, whereas in the skeletal muscle activation of ryanodine receptors by voltage sensors is the main mechanism of excitation-contraction coupling, in cardiac myocytes it is probably only complementary to CICR, "a vestigial remnant of a voltage-dependent

excitation-contraction coupling" (16). This remnant may be demonstrated only under specific experimental conditions.

Acknowledgements:

1. The authors are very grateful to Professor Michel Lazdunski for his suggestion to use SR33557 as a blocker of the putative voltage sensors. The generous supply of SR33557 by Sanofi Recherche is gratefully acknowledged.
2. The authors are greatly indebted to Dr. G.A. Langer and Dr. E. G. Lakatta for reading the manuscript and highly valuable remarks.
3. This work has been supported by a Grant KBN No 4 P05A01915 of the National Research Council.

REFERENCES

1. Bers DM. and Perez-Reyes E. Ca channel in cardiac myocytes: structure and function in Ca influx and intracellular Ca release. *Cardiovasc Res* 1999; 42: 339—360.
2. Langer GA. Excitation-Contraction Coupling and Calcium Compartmentation. In: The Myocardium 1997; pp. 181—227, Ed. GA Langer. Academic Press, San Diego, Cal, USA.
3. Fabiato A. Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. *Am J Physiol* 1983; 245: C1—C14.
4. Eisner DA, Trafford AW, Diaz ME, Overand CL, O'Neil SC. The control of Ca release from the cardiac sarcoplasmic reticulum: regulation versus autoregulation. *Cardiovasc Res* 1998; 38: 589—604.
5. Cannell MB, Cheng H, Lederer WJ. The control of calcium release in heart muscle. *Science* 1995; 268: 1045—1049.
6. López-López JR, Shacklock PS, Balke CW, Wier WG. Local calcium transients triggered by single L-type calcium channel currents in cardiac cells. *Science* 1995; 268: 1042—1045.
7. Cheng H, Lederer MR, Xiao R-P, Gomez AM, Zhou Y-Y, Ziman B, Spurgeon H, Lakatta EG, Lederer WJ. Excitation-contraction coupling in heart: new insights from Ca^{2+} sparks. *Cell Calcium* 1996; 20: 129—140.
8. Wier WG, Balke CW. Ca^{2+} release mechanism, Ca^{2+} sparks, and local control of excitation-contraction coupling in normal heart muscle. *Circ Res* 1999; 85: 770—776.
9. Cannell MB, Berlin JR, Lederer WJ. Effect of membrane potential changes on the calcium transient in single rat cardiac muscle cells. *Science* 1987; 238: 1419—1423.
10. Ferrier GR, Howlett SE. Contractions in guinea-pig ventricullar myocytes triggered by a calcium-release mechanism separate from Na^+ and L-currents. *J Physiol* 1995; 481: 107—122.
11. Howlett SE, Ferrier GR. The voltage-sensitive release mechanism: a new trigger for cardiac contraction. *Canadian J Physiol Pharmacol* 1997; 74: 1044—1057.
12. Howlett SE, Zhu J, Ferrier GR. Contribution of a voltage-sensitive calcium release mechanism to contraction in cardiac ventricular myocytes. *Am J Physiol* 1998; 274: H155—H170.
13. Mason CA, Ferrier GR. Tetracaine can inhibit contractions initiated by a voltage-sensitive release mechanism in guinea-pig ventricular myocytes. *J Physiol* 1999; 519: 851—865.
14. Ferrier GR, Zhu J, Redondo IM, Howlett SE. Role of cAMP-dependent protein kinase A in activation of a voltage-sensitive release mechanism for cardiac contraction in guinea-pig myocytes. *J Physiol* 1998; 513: 185—201.

15. Hobai IA, Howarth FC, Pabbathi VK, Dalton GR, Hanox JC, Zhu JQ, Howlett SE, Ferrier GR, Levi AJ. "Voltage-activated Ca release" in rabbit, rat and guinea-pig cardiac myocytes, and modulation by internal cAMP. *Pflugers Archiv* 1997; 435: 164—173.
16. Hadley RW, Lederer WJ. Properties of L-type calcium channel gating current in isolated guinea pig ventricular myocytes. *J Gen Physiol* 1991; 98: 265—285.
17. Mitra R, Morad M. Two types of calcium channels in guinea pig ventricular myocytes. *Proc Nat Acad Sci USA* 1986; 83: 5340—5344.
18. Kimura J, Miyamae S, Noma A. Identification of sodium-calcium exchange current in single ventricular cells of guinea-pig. *J Physiol* 1987; 384: 199—222.
19. Kirby MSY, Sagara G, Gow S, Inui WJ, Lederer WJ, Rogers TB. Thapsigargin inhibits contraction and Ca^{2+} transients in cardiac cell by specific inhibition of sarcoplasmic reticulum Ca^{2+} pump. *J Biol Chem* 1991; 267: 12545—12551.
20. Thastrup J, Cullen PJ, Drobak B, Hanley MR, Davson AP. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc Nat Acad Sci USA* 1990; 87: 2466—2470.
21. Janczewski AM, Lakatta EG. Thapsigargin inhibits Ca uptake and Ca depletes sarcoplasmic reticulum in intact cardiac myocytes. *Am J Physiol* 1993; 265: H514—H522.
22. Lewartowski B, Wolska BM. The effect of thapsigargin on sarcoplasmic reticulum Ca^{2+} content and contractions in single myocytes of guinea-pig heart. *J Mol Cell Cardiol* 1993; 25: 23—29.
23. Lewartowski B, Różycka M, Janiak R. Effects of thapsigargin in normal and pretreated with ryanodine guinea pig cardiomyocytes. *Am J Physiol* 1994; 266: H1829—H1839.
24. Wrzosek A, Schneider S, Grueninger S, Chiesi M. Effect of thapsigargin on cardiac muscle cells. *Cell Calcium* 1992; 13: 281—292.
25. Janiak R, Lewartowski B. The source of contractile calcium in guinea-pig cardiac myocytes treated with thapsigargin. *J Physiol Pharmacol* 1996; 47: 411—423.
26. Bois P, Romey G, Lazdunski M. Indolizinsulphones. A class of blockers with dual but discriminative effects on L-type Ca^{2+} channel activity and excitation-contraction coupling in skeletal muscle. *Pflugers Archiv* 1991; 419: 651—656.
27. Yang J, Ellinor PT, Sather WA, Zhang J-F, Tsien RW. Molecular determinants of Ca selectivity and ion permeation in L-type Ca channels. *Nature* 1993; 356: 158—161.
28. Bean BP, Rios E. Nonlinear charge movement in mammalian cardiac ventricular cells. Components from Na and Ca channel gating. *J Gen Physiol* 1989; 94: 65—93.
29. Pizarro G, Brum G, Fill M, Fitts R, Rodriguez M, Uribe I, Rios E. The voltage sensor of skeletal muscle excitation-contraction coupling: a comparison with Ca^{2+} channels. In: The Calcium Channel: Structure, Function and Implications 1988; pp. 138—156. Morad, Mayler, Kazda, Schramm Eds. Springer Verlag Berlin Heidelberg.
30. Rios E, Pizzarro G. Voltage sensor of excitation-contraction coupling in skeletal muscle. *Pharmacol Rev* 1991; 71: 849—908.
31. Brum G, Rios E, Stefani E. Effects of extracellular calcium on calcium movements of excitation-contraction coupling in frog skeletal muscle fibres. *J Physiol* 1988; 398: 441—473.
32. Brum G, Fitts R, Pizarro G, Rios G. Voltage sensors of the frog skeletal muscle membrane require calcium to function in excitation-contraction coupling. *J Physiol* 1988; 398: 475—505.
33. Schmid A, Romey G, Barhanin J, Lazdunski M. SR33557, an indolizinesulfone blocker of Ca^{2+} channels: identification of receptor sites and analysis of its mode of action. *Mol Pharmacol* 1989; 35: 766—773.
34. Romey G, Bois P, Lazdunski M. Effects of two chemically related new Ca channel antagonists, SR33557 (fantofarone) and SR33805, on the L-type cardiac channel *Eur J Pharmacol* 1994; 263: 101—105.
35. Rios E, Stern M. Calcium in close quarters: microdomain feedback in excitation-contraction coupling and other cell biological phenomena. *Annu Rev Biomol Struct* 1997; 26: 47—82.

36. Stern MO, Pizzarro G, Rios E. Local control of excitation-contraction coupling in skeletal muscle. *J Gen Physiol* 1997; 110: 415—440.
37. Bouchard RA, Clark RB, Giles WR. Role of sodium-calcium exchange in activation of contraction in rat ventricle. *J Physiol* 1993; 472: 395—413.
38. Kohmoto O, Levi AJ, Bridge JHB. Relation between reverse sodium-calcium exchange and sarcoplasmic reticulum calcium release in guinea pig ventricular cells. *Circ Res* 1994; 74: 550—554.
39. Leblanc N, Hume JR. Sodium current release of calcium from cardiac sarcoplasmic reticulum. *Science* 1990; 248: 372—375.
40. Levesque PC, Leblanc N, Hume JR. Release of calcium from guinea-pig cardiac sarcoplasmic reticulum induced by sodium-calcium exchange. *Cardiovasc Res* 1994; 28: 370—378.
41. Levi AJ, Brooksby P, Hanox JC. A role for depolarization induced calcium entry on the Na^+–Ca^{2+} exchange in triggering intracellular calcium release and contraction in rat ventricular myocytes. *Cardiovasc Res* 1993; 27: 1677—1690.
42. Lipp P, Niggli E. Sodium current-induced signals in isolated guinea-pig ventricular myocytes. *J Physiol* 1994; 474: 439—416.
43. Santi CM, Connor JA, Hernandez-Cruz A. A significant fraction of calcium transients in intact guinea-pig ventricular myocytes is mediated by Na^+–Ca^{2+} exchange. *Cell Signalling* 1995; 7: 803—820.
44. Egger M, Niggli E. Regulatory function of Na–Ca exchange in the heart: milestones and outlook. *J Membrane Biol* 1999; 168: 107—130.
45. Grantham CJ, Cannell MB. Ca influx during the cardiac action potential in guinea pig ventricular myocytes. *Circ Res* 1996; 79: 194—200.
46. Levi AJ, Li J, Spitzer KW, Bridge JHB. Effect on the indo-1 transient of applying Ca channel blocker for single beat in voltage-clamped guinea-pig cardiac myocytes. *J Physiol* 1996; 494: 653—673.
47. Nabauer M, Callewaert G, Cleemann L, Morad M. Regulation of calcium release is gated by calcium current, not gating charge, in cardiac myocytes. *Science* 1989; 244: 800—803.
48. Satoch H, Katoh H, Velez P, Fill M, Bers DM. BayK 8644 increases resting Ca^{2+} spark frequency in ferret ventricular myocytes independent of Ca influx. Contrast with caffeine and ryanodine effects. *Circ Res* 1998; 83: 1192—1204.
49. Jacquemond V, Csernoch L, Klein MG, Schneider MF. Voltage-gated and calcium-gated calcium release during depolarization of skeletal muscle fibres. *Biophys J* 1991; 60: 867—873.
50. Yano M, El-Hayek R, Ikemoto N. Role of calcium feedback in excitation-contraction coupling in isolated triads. *J Biol Chem* 1995; 270: 19936—19942.
51. Klein MG, Cheng H, Santana LF, Jiang YH, Lederer WJ, Schneider MF. Two mechanisms of quantized calcium release in skeletal muscle. *Nature* 1996; 379: 455—458.

Received: April 5, 2000
Accepted: October 18, 2000

Author's address: Professor Bohdan Lewartowski, M.D., Ph. D. Department of Clinical Physiology, Medical Center of Postgraduate Eduction, Marymoncka 99, 01-813 Warsaw, Poland. Tel. 048 22 834-03-67. Fax. 048 22 834-04-70.
E-mail: blew@cmkp.edu.pl