R. JANIAK, B. LEWARTOWSKI

EFFECT OF Na$^+$ CURRENT ON EXCITATION-CONTRACTION COUPLING IN VENTRICULAR MYOCYTES OF GUINEA PIG HEART

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

We investigated the effect of Na$^+$ current on the Ca^{2+} current and Ca^{2+} transients in cardiac myocytes. Myocytes were isolated from the ventricles of guinea-pig hearts by enzymatic dispersion. The membrane currents were recorded by the whole-cell voltage clamping. The Ca^{2+} current was activated by depolarisation from -80 to $+5$ mV preceded by the prepulses to -40 mV. Cellular action potentials (APs) were recorded by current clamping. Intracellular [Ca^{2+}] was assessed by recording of fluorescence of Indo-1 loaded into cells. In current clamped cells (APs recorded) 20 μM tetrodotoxin (TTX) reduced the time to 75% of amplitude of Ca^{2+} transients from 50 ± 6.6 ms to 32 ± 5 ms (n = 7). In voltage clamped cells prepulses from the holding potential of -80 mV to -40 mV 50—100 ms long activated the Na$^+$ current and initiated step increase in [Ca^{2+}] reaching 30—50% of the total amplitude of the transient. Prepulses 10—20 ms long initiated increase in [Ca^{2+}] merging with that elicited by Ca^{2+} current into smooth rising phase. Blocking of Na$^+$ current with TTX or by switching the holding potential from -80 to -40 mV increased the amplitude of the Ca^{2+} current by $38\pm3.2\%$ (n = 8) and $43\pm9\%$ (n = 7), respectively, and eliminated the initial step increase in [Ca^{2+}]. When 10—20 ms prepulses were used, blocking of Na$^+$ current with TTX or switching of the holding potential decreased the time to 75% of amplitude of Ca^{2+} transients from 27 ± 3.7 ms to 12 ± 1.2 ms (n = 5) and from 25 ± 3.1 ms to 14 ± 1.1 ms (n = 9), respectively. 100 μM Cd^{2+} inhibited the initial rise in [Ca^{2+}], however, the inhibition did not correlate with degree of inhibition of Ca^{2+} current.

The Na$^+$ current activated prior to Ca^{2+} current reduces its amplitude and decreases the rate of release of Ca^{2+} from sarcoplasmic reticulum. In voltage clamped cells this could result from Ca^{2+} influx prior to onset of Ca^{2+} current initiated by Na$^+$ current escaping from the voltage control and/or reversal of Na/Ca exchange due to increase in subsarcolemmal [Na$^+$]. In cells in which Ca^{2+} transients were initiated by APs only the second mechanism is conceivable.

K e y   w o r d s: *cardiac myocytes, Na$^+$ current, Ca^{2+} current, Na/Ca exchange, excitation-contraction coupling.*

INTRODUCTION

According to the generally accepted concept of excitation-contraction coupling in the cardiac myocytes sarcoplasmic reticulum (SR) is, under physiological conditions, the main direct source of Ca^{2+} activating

contraction. The Ca^{2+} release channels of the SR are activated in stimulated cell by increase in $[Ca^{2+}]$ in their vicinity i. e. in the SR-sarcolemmal (SR-SL) cleft (1). There are at least two potential routes of diffusion of Ca^{2+} into SR-SL cleft: 1. The L-type sarcolemmal Ca^{2+} channels, activated upon depolarisation of a cell to -40 mV, and 2. Na/Ca exchange reversed due to the shift of the membrane potential positive to the reversal potential of the exchange and/or increase in $[Na^+]_i$. The role of Ca^{2+} influx through the L-type Ca^{2+} channels as the main trigger for the SR Ca^{2+} release is well established. The second route, i. e. reversed Na/Ca exchange was tested by several groups, however, the results are still controversial. Leblanc and Hume (2) found in guinea pig ventricular myocytes that inhibition of Na^+ current by TTX results in decrease of amplitude of the Ca^{2+} transients elicited by APs. Change of the holding potential from -80 to -40 mV, which inactivated the Na^+ channels, also resulted in the decrease of amplitude of the Ca^{2+} transients elicited by depolarisation to 0 mV. Moreover, activation of the Na^+ current in cells pretreated with nisoldipine elicited small Ca^{2+} transients which were blocked by TTX or ryanodine. The authors conclude that Na^+ current may increase the subsarcolemmal Na^+ concentration strongly enough to reverse the Na/Ca exchange. The resulting influx of Ca^{2+} may be sufficient to activate partially the SR Ca^{2+} release channels. However, Bouchard et al. (3) have shown that voltage control may be lost during activation of the Na^+ current in rat ventricular myocytes. Loss of voltage control during activation of the Na^+ current may result in depolarisation activating the Ca^{2+} current unless the series resistance is electronically compensated. The authors feel that this might be the reason why Na^+ current initiated Ca^{2+} transients in the experiments of Leblanc and Hume (2). However, the argument of Bouchard et al. (3) is not valid at least for the experiments in which inihibition of Na^+ current resulted in decrease in amplitude of the transients elicited by APs. The hypothesis of Leblanc and Hume (2) was supported by results of Lipp and Niggli (4) who reported that Na^+ current may initiate the Ca^{2+} transients of the amplitude comparable to that of the transients initiated by the Ca^{2+} current and resistant to the Ca^{2+} channels blocker, Verapamil. Also Levesque et al. (5) were able to elicit the Ca^{2+} transients in guinea pig ventricular myocytes by depolarising steps from -80 to -50 mV in the presence of 5 μM -50 μM nisoldipine or 10 μM D600 applied to both sides of the membrane and also 5 μM nisoldipine plus 50 μM Cd^{2+}. They were blocked by substituting Na^+ with Li^+, by Ni^{2+}, and 10 μM dichlorobenzamil. The authors regard the fact that nisoldipine insensitive and Li^+ sensitive transients may be elicited by APs as the most compelling evidence against the effect of the loss of voltage control. Sipido et al. (6) came to the opposite conclusion, although they performed similar experiments in guinea-pig ventricular myocytes. In their hands Ca^{2+} transients elicited by depolarisations from a holding potential of -90 mV to -50 mM were not blocked by replacement of Na^+ with Li^+, but were strongly reduced by 20 μM

nifedipine or 100 μM Cd^{2+}, although the Ca^{2+} current was not completely blocked. Contrary to the results of Leblanc and Hume (2), TTX superfused during the pause after a series of conditioning APs increased the amplitude of the first Ca^{2+} transient and decreased amplitude of the following transients. This was explained as the result of a slower 0 phase of AP which increased the driving force of the inward Ca^{2+} current. The authors conclude that Ca^{2+} transients or contractions elicited by Na^+ current are most probably initiated by Ca^{2+} current activated due to loss of voltage control.

In experiments in which the depolarisation was imposed from the holding potential of -80 mV or from the resting cell's potential, depolarisation initially activated the Na^+ current which might enhance reversal of Na/Ca exchange (2, 4—7). However, as reported by Levi et al. (8) and Kohmoto et al., (9) depolarisation from a holding potential of -40 mV which inactivates the Na^+ channels may activate the Ca^{2+} influx apparently by reversing the Na/Ca exchange. Recently Grantham and Cannell (10) were able to dissect the inward Ca^{2+} current and outward Na/Ca exchange current at the start of an AP used as a depolarising pulse in voltage clamped myocytes of guinea pig ventricular muscle. They found that although the main route of influx of Ca^{2+} activating the SR Ca^{2+} release channels are the SL Ca^{2+} channels, the reversed Na/Ca exchange may contribute up to 30% of the total influx. Similar conclusion was reached by Levi et al. (11) who found that rapid inhibition of the Ca^{2+} current left intact 24—34% of the rapid rising phase of the Ca^{2+} transient attributable to release of Ca^{2+} from the SR.

In this paper we report a novel effect of Na^+ current on the Ca^{2+} transients and Ca^{2+} current. Inhibition of Na^+ current by TTX increased the rate of the rising phase of Ca^{2+} transients elicited by APs. In voltage clamped cells the Na^+ current initiated the Ca^{2+} influx, but decreased the amplitude of the Ca^{2+} current and the rate of rising phase of Ca^{2+} transients. We propose that increase in the subsarcolemmal $[Ca^{2+}]$ initiated by the Na^+ current may activate a fraction of the Ca^{2+} release channels of SR prior to the activation of the Ca^{2+} current also in physiologically stimulated cells. This would have two effects: 1. Total release of Ca^{2+} from the SR would be more gradual i. e. slower 2. Rise in the subsarcolemmal $[Ca^{2+}]$ would limit subsequent activation of the Ca^{2+} current.

METHODS

Cell isolation and superfusion

Guinea pigs of both sexes weighing 250—300 g were injected intraperitoneally with 2.500 U heparin followed by an overdose of pentobarbital sodium. After the heart was rapidly excised amd washed in cold Tyrode solution, the aorta was cannulated and retrogradely perfused for 5 min with nominally Ca^{2+} free Tyrode solution containing 10 μM EGTA (ethylene-bis

oxyethylenenitrilotetraacetic acid) (for compositions of the solutions see below). The initial perfusion period was followed by 20 min perfusion with Ca^{2+} free solution (without EGTA) containing 20 mg collagenase B (Boehringer) and 3 mg protease (Sigma) per 50 ml. Thereafter the ventricles were minced with scissors in the same, enzyme containing solution to which 0.2 mM Ca^{2+} was added. The cell suspension was filtered and cells allowed to sediment. Thereafter they were washed twice with Tyrode solution containing 1.0 mM Ca^{2+}. Cells were stored at room temperature until used.

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 85—23, revised 1985).

Cells were placed in the 0.5 ml superfusion chamber mounted on the stage of an inverted microscope (Nikop Diaphot) and superfused at the rate of 2 ml/min. They were illuminated with the red (650—750 nm) light through the bright-field illumination optics of microscope.

Measurement of Indo 1 fluorescence

The 500 μl of cell suspension were incubated for 20 min at room temperature with 7 μl of 1.0 mM Indo 1-AM dissolved in dry dimethyl sulfoxide (DMSO) and washed with Tyrode solution. A drop of suspension of cells loaded with the dye was added to the Tyrode solution filling the superfusion chamber. A Nikon mercury lamp was used as a source of UV light for epifluorescence. A concentric diaphragm enabled illumination of a fregment of cell. The fluorescent light was split by the dichronic mirror into 410 and 495 nm beams which were passed to two photomultipliers mounted in the side port of the microscope. The ratio of 410 to 495 nm fluorescence was obtained from the output of Dual Channel Ratio Fluorometer (Biomedical Instrumentation Group — University of Pennsylvania). No attempts to calibrate the signals in terms of Ca^{2+} concentration were made.

Electrophysiological investigation

The ionic currents were recorded by the whole cell clamp method. Pipettes of 2.5 to 3.6 M resistance were pulled from borosilicate glass capillaries (World Precision Instruments, USA). The cell membrane under electrode was disrupted by suction. Prepulses from a holding potential of −80 mV to −40 mV of the duration of 10 to 100 ms were used in order to activate and inactivate the Na^+ current. They were followed by the step depolarisation to +5 mV for 200 ms. In some experiments cells were stimulated by one-step depolarisation from −80 mV to +5 mV. The holding potential could be switched from −80 mV to −40 mV during ∼15 s pause in pulsing. Currents were measured with an Axopatch 1-D amplifier controlled by an IBM AT 386 computer using the V-clamp software and V-clamp computer interface generously offered by D. R. Matteson (Dept. of Biophysics, University of Maryland). Series resistance compensation was used to reduce voltage error due to the access resistance. The cellular action potentials (APs) were recorded with the same amplifier set to the current clamp mode. Cells were stimulated by the current pulses injected through the recording electrode.

The signals were passed through the computer interface, digitized at 2 kHz and stored on a disc for off line analysis. They were also directly recorded with the Gould TA 240 chart recorder.

Solutions

For cells isolation and throughout the experiments we used the Tyrode solution of the following composition (in mM): 144.0 NaCl, 5.0 KCl, 1.0 $MgCl_2$, 0.43 NaH_2PO_4, 10.0 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 11.0 glucose, 5.0 sodium pyruvate

and 20.0 tetraethylammonium (TEA). The pH of the solution was adjusted with NaOH to 7.30 for isolation of cells or to 7.40 for experiments. In the experiments $CaCl_2$ was added to concentration of 3.0 mM. The patch pipettes were filled with a solution containing (in mM): 100.0 potassium aspartate, 35.0 KCl, 10.0 NaCl, 20.0 TEA and 10.0 HEPES. In some experiments the internal solution contained also 1.0 mM $MgCl_2$. The pH was adjusted with KOH to 7.20.

DMSO, Indo 1-AM and thapsigargin were purchased from Sigma, USA. Tetrodotoxin was from Serva, Germany.

Statistical evaluation

In all experiments the control measurements and measurements after interventions were performed in the same cell. Therefore the Student's t test for paired samples was used for the evaluation of significance of differences between the means. $P < 0.02$ was accepted as the level of cofidence.

RESULTS

1. The effect of tetrodotoxin (TTX) on Ca^{2+} transients elicited by APs

Cells were stimulated at the rate of 0.5 Hz. Under our experimental conditions the full effect of TTX developed during ~ 45 s of its superfusion. If cells were stimulated at that time the sarcoplasmic $[Na^+]$ could decrease as compared with the normal cell due to the progressing inhibition of the Na^+ current. In order to avoid this difference we stopped stimulation for 60—90 s at the onset of superfusion of 20 µM TTX. Therefore stimulation of cells was stopped for 60—90 s also in control runs. This way we could compare the first postrest and following APs and Ca^{2+} transients recorded in the same cell before and during the TTX superfusion (*Fig. 1*). In the control runs the rate of rise and amplitude of the rapid rising phase of the initial post rest Ca^{2+} transient were increased or slightly decreased as compared with the steady state transients, apparently depending on the rate of rest decay of the individual cells. The rate of rise and amplitude of the second postrest Ca^{2+} transient were decreased. The transients reached their pre-rest steady state during ~ 15 seconds of the following pacing (*Fig 1 A—C, top*). In all experiments TTX increased the rate of rapid rising phase of the first postrest Ca^{2+} transient and of the following transients (*Fig. 1 A—C, bottom*). In order to evaluate this difference quantitatively we measured the time from the onset of each Ca^{2+} transient to 75% of its total amplitude. This solved the problem of choice of the moment of the transition between the rapid phase and the following phase of the transient. The difference between the control values and those measured after TTX superfusion was highly significant for the first, the second and steady state postrest transients (*Fig. 2*). Since the amplitude of the transients did not change significantly, it may be inferred that TTX increased
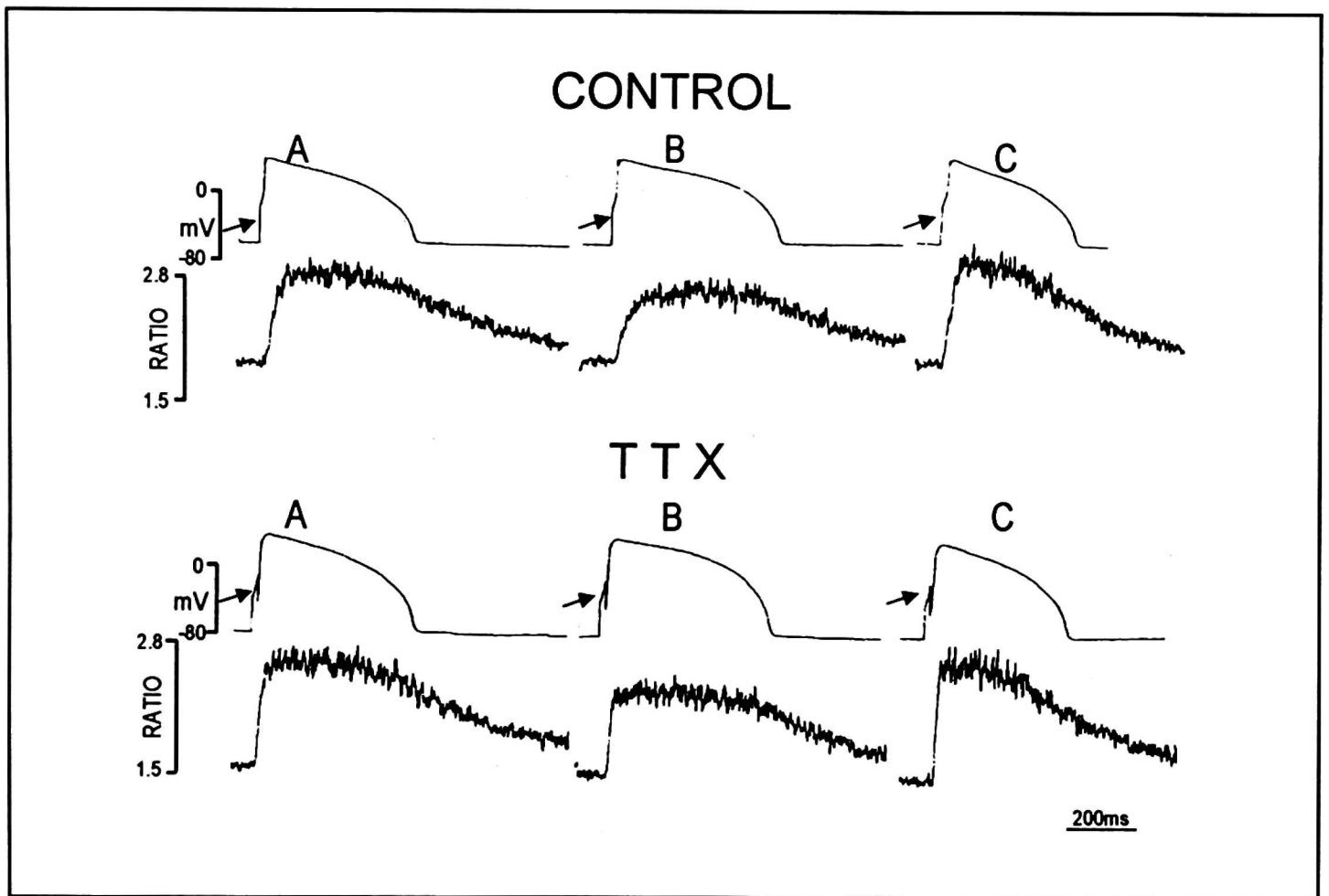
Fig. 1. Effect of terodotoxin (TTX) on APs (top records) and Indo 1 fluorescence of single ventricular myocyte of guinea-pig heart stimulated at the basic rate of 30/min. Control: A — the first cycle after 60 s pause in stimulation; B — the second cycle after the pause; C — steady state stimulation. TTX: 20 μM terodotoxin was superfused from the beginning of the second pause in stimulation. A, B, C — .the first, the second cycle after the pause, and steady state cycle, respectively. Low excitability of cells treated with TTX required strong stimuli resulting in large artifacts (pointed by arrows).
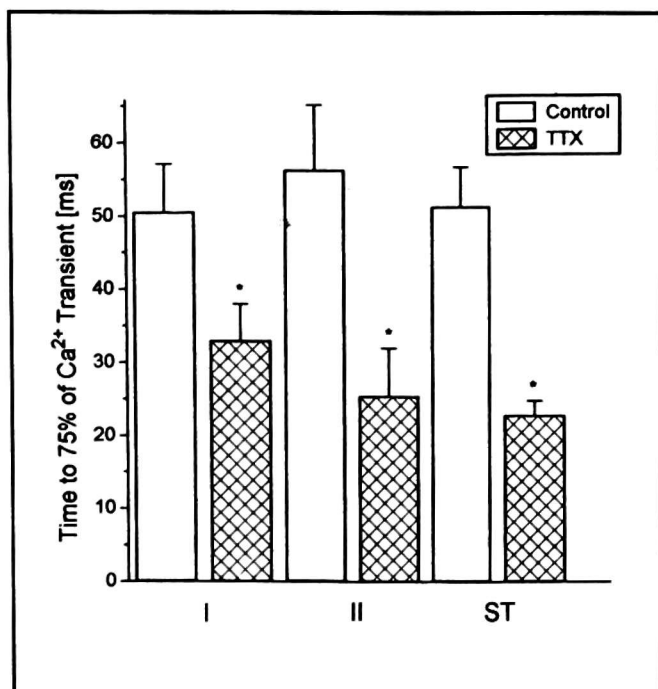
Fig. 2. The effects of 20 μM terodotoxin (TTX) on the time from the onset of Ca^{2+} transients to 75% of their total amplitude. The transients initiated by APs. I-the first transient after 60—90 s pause in stimulation; II-the second transient after the pause; ST — steady state transients. Mean ±SE, n = 7, P < 0.02 for all differences.

the rate of release of Ca^{2+} from the SR. The compound also decreased the amplitude of APs as well as their total duration. These changes were least visible in the first postrest AP and gradually increased during postrest stimulation.

In order to analyze the mechanism of the effects of TTX we performed the experiments in voltage clamped cells.

2. The effect of inhibition of Na^+ current on Ca^{2+} current and Ca^{2+} transients in voltage clamped cells

Cells were depolarised for 200 ms to +5 mV from the holding potential of −80 mV either in one step or after a prepulse to −40 mV. Duration of the prepulse was set at 10, 20, 50 or 100 ms. Prepulse activated a large inward current which inactivated within ∼10 ms and was completely blocked by TTX. So we infer that it was the Na^+ current (*Fig. 3*). The following step to +5 mV activated an inward current which slowly inactivated and was blocked by 100 μM Cd^{2+}. So we infer that it was the Ca^{2+} current (*Fig. 7*). One-step depolarisation to +5 mV activated the large inward current which was blocked by TTX. The compound dissected from the total current the Ca^{2+} current which was blocked by Cd^{2+}. In the untreated cells it was visible as apparent slowing of inactivation of the large inward current (not shown).

The prepulses from −80 to −40 mV of the duration of 50 or 100 ms initiated a step increase in Ca^{2+} concentration, the amplitude of which ranged from ∼20% to 60% of the total amplitude of the transient. The beginning of rise in $[Ca^{2+}]$ coincided with the inactivation phase of the Na^+ current (*Fig. 3 C*). The following step to +5 mV initiated a further increase in $[Ca^{2+}]$, the beginning of which coincided with the peak of the Ca^{2+} current. The rate of rise of the second phase of the Ca^{2+} transient was usually lower then that of the first phase (*Fig. 3 C*). The increase in $[Ca^{2+}]$ initiated by prepulses of the duration of 10 or 20 ms merged with the increase initiated by depolarisation to +5 mV into one smooth rapid rising phase of the transient. The beginning of this phase coincided with the inactivation phase of the Na^+ current and clearly preceded the apparent activation of the Ca^{2+} current (*Fig. 3 A and E*).

When the prepulses of various durations were applied in the same cell it became clear that amplitude of the Ca^{2+} current is directly related to their duration i.e. to the delay between the activation of Na^+ current and Ca^{2+} current (*Fig. 4*).

The prepulses did not initiate increase in $[Ca^{2+}]$ in 5 cells pretreated for 15 min with 10^{-6} M thapsigargin (TG), a selective inhibitor of the SR Ca^{2+} — ATPase (12—15). In 2 out of 5 cells only a slight, hardly visible change was noticed (not shown).
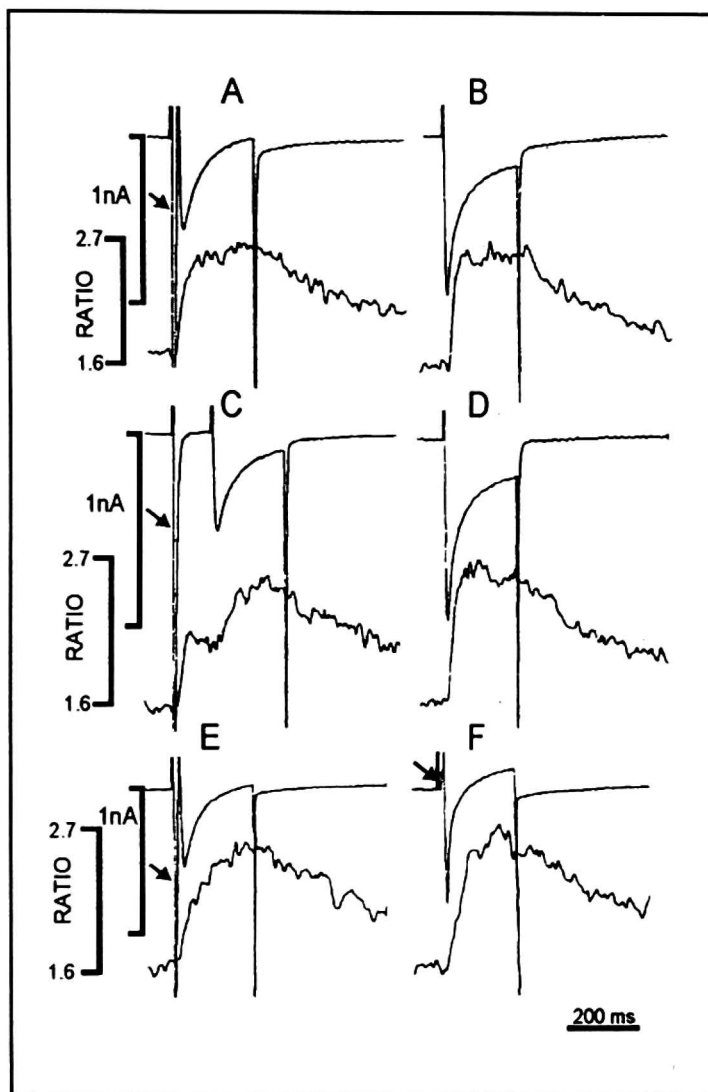
Fig. 3. The effect of blocking of Na$^+$ current on Ca^{2+} current and Ca^{2+} transients of single myocytes stimulated at a rate of 30/min by prepulses from the holding potential of -80 mV to -40 mV followed by step depolarisation to $+5$ mV for 200 ms. Duration of the prepulse 20 ms (A, E, F) or 100 ms (C). A, C, E: control records. Na$^+$ current (pointed by arrows) off scale. B, D: holding potential switched to -40 mV. F: 20 μM TTX superfused during 60 s pause in stimulation.

Fig. 4. The effect of duration of prepulse from -80 mV to -40 mV on Ca^{2+} current activated by depolarisation to 5 mV. The Na$^+$ current pointed by arrows, off scale. Numbers above the records indicate duration of prepulse.
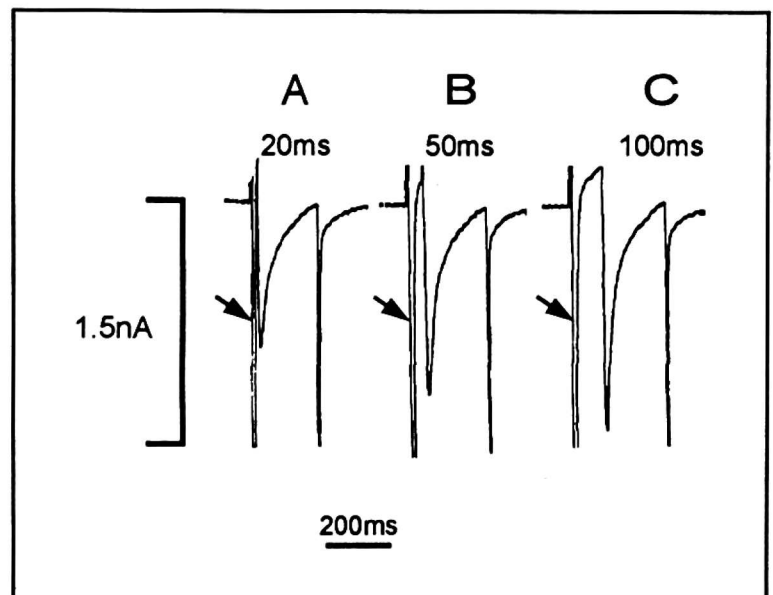
These results showed that prepulses from -80 to -40 mV initiated release of Ca^{2+} from the SR and hindered activation of the Ca^{2+} current. The following experiments showed that the primary factor responsible for these effects was the Na$^+$ current.

Inhibition of the Na$^+$ current by switching the holding potential to -40 mV had the following effects. When 20 ms prepulse was used in the control

runs, switching of the holding potential resulted in delay of beginning of the rapid rising phase of the transient to the nadir of the Ca^{2+} current. The time from the onset to 75% of the total amplitude of the transients decreased (*Fig. 3A, B and Fig. 5*). The change was highly significant (P < 0.02). In all 7 tested cells the amplitude of the Ca^{2+} current increased by $43 \pm 9\%$ (*Fig. 3A, B*). When 50 ms or 100 ms pulses were used, switching of the holding potential to -40 mV resulted in delay of the rapid rising phase of the Ca^{2+} transients to the nadir of the Ca^{2+} current and in increase in its rate and amplitude *Fig. 3C, D*). The amplitude of the Ca^{2+} current greatly increased (*Fig. 3C, D*).

Fig. 5. The effect of inhibition of Na^+ current by switching of the holding potential from -80 mV to -40 mV or by 20 μM tetrodotoxin (TTX) on the time from the onset to 75% of the total amplitude of the Ca^{2+} transients. The transients initiated by the prepulses from -80 mV to -40 mV of the duration of 20 ms followed by depolarisation to $+5$ mV for 200 ms. Mean $\pm$ SE, n = 9 (switching potential) or 5 (TTX). P < 0.02 for all differences.
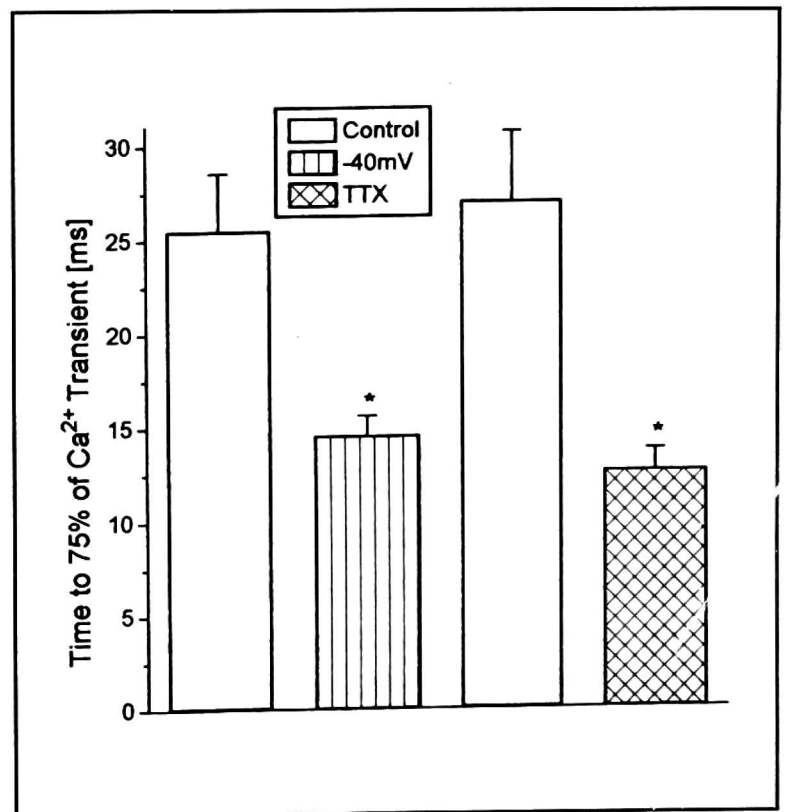
Inhibition of the Na^+ current by 20 μM TTX had the effects similar to those of switching the holding potential to -40 mV. The initial steps of the Ca^{2+} transients elicited in the control runs by the 100 or 50 ms prepulses were inhibited. They were replaced by the smooth rising phase of the transients beginning about the time of the nadir of the Ca^{2+} current. The rate of the rapid rising phase was greater than that of initial and final steps of the control transient. In the experiments in which 10 or 20 ms prepulses were used, the beginning of the rising phase of the transients was also delayed to the moment of the peak Ca^{2+} current. The time from the onset to 75% of the total amplitude of the transients decreased. The change was highly significant statistically (P < 0.02). The amplitude of the transients also tended to increase, however, the change was not significant (*Fig. 3E, F and Fig. 5*). In all cells the amplitude of the Ca^{2+} current increased by $38 \pm 3.2\%$ (P < 0.02, n = 8) (*Fig. 3E, F and Fig. 6*).

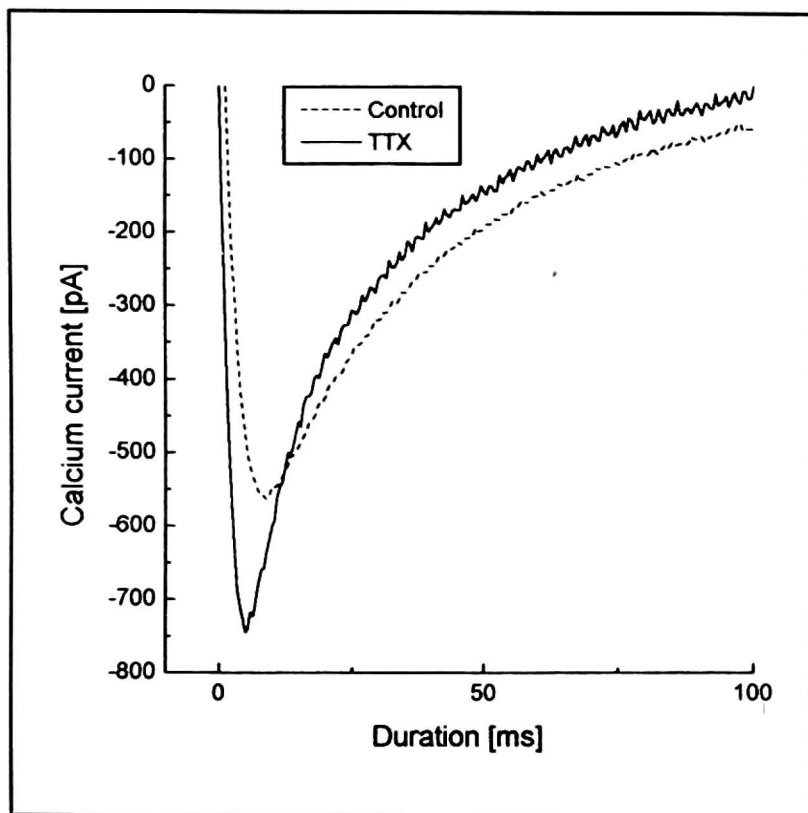*Fig. 6.* The effect of inhibition of the Na⁺ current on the mean of Ca²⁺ currents recorded in 6 cells during the first cycles after 45—60 s break in stimulation. Step depolarisation to +5 mV activating the Ca²⁺ current was preceded by a prepulse from −80 to −40 mV of the duration of 20 ms activating and inactivating the Na⁺ current (not shown). Broken line: control records during the first cycle after break in stimulation. Continues line — 20 µM terodotoxin superfused for 45—60 s during the next break in stimulation. Controls and superfusion of TTX in the same cells.

TTX or switching the holding potential to −40 mV did not have a consistent effect on the rate and amplitude of the rapid rising phase of the Ca²⁺ transients elicited by depolarisation from −80 mV to +5 mV. It increased in 4 cells, did not change in 6 cells and slighty decreased in 3 cells (not shown). These results might seem contradictory to those of experiments in which prepulses were used. We will address this discrepancy in the Discussion section.

Results of the above experiments suggest that the Na⁺ current initiates a Ca²⁺ influx into the diadic space which partially activates the SR Ca²⁺ release channels. The increase of Ca²⁺concentration before activation of the SL Ca²⁺ current may result in its partial inhibition. There are at least two possible mechanisms of Ca²⁺ influx due to activation of the Na⁺ channels: 1. Increase in the [Na⁺] concentration in the subsarcolemmal "fuzzy space" i. e. a diadic cleft may reverse the Na/Ca exchange with resulting Ca²⁺ influx, as proposed by Leblanc and Hume (2). 2. Activation of the SL Ca²⁺ channels due to the loss of voltage control during activation of Na⁺ current (3). We tried to test these possibilities in the following series of experiments.

3. Activation of the SL Ca²⁺ channels by the Na⁺ current vs. the reversed Na/Ca exchange

Bouchard *et al.* (3) found that loss of voltage control during activation of Na⁺ current may be prevented by proper electronic compensation of the series resistance. Therefore we checked the effect of the series resistance compensation

on the Ca^{2+} transients initiated by the voltage steps from -80 to -40 mV. We did not see any difference between the Ca^{2+} transients recorded without and with the compensation (not shown).

Then we used the SL Ca^{2+} channel blocker in order to eliminate the Ca^{2+} current. Since exchange of the solutions in our perfusion chamber required ~ 30 s to be completed, we had to stop stimulation of cells for this time in order to preserve the SR Ca^{2+} content despite beginning of action of th Ca^{2+} channel blocker. Therefore we chose a non use dependent blocker, 100 µM Cd^{2+} believed not to inhibit the Na/Ca exchange in this concentration. Cd^{2+} has been reported to inhibit more or less also the Na^+ current (6, 16). However, we could not see any consistent effect of Cd^{2+} on the I_{Na} in our experiments. It is true, that it is not possible to record I_{Na} with the electrodes of the resistance of 2—4 MΩ used in this work without a fraction of it escaping from voltage control. However, if Cd^{2+} had significant effect it should have been seen also under our experimental conditions. We applied the 100 ms prepulses from -80 to -40 mV followed by depolarisation to $+5$ mV for 200 ms and compared the control post rest and steady state records with the records taken after 45 s—60 s pause in stimulation at the beginning of which superfusion of Cd^{2+} was started.

As reported also by Sipido et al., (6) we were never able to block the Ca^{2+} current completely. After 45 s—60 s superfusion of Cd^{2+} the amplitude of the first post rest Ca^{2+} current was reduced by 62%—94% with respect to control and it did not further change during the steady state post rest stimulation. The increase in $[Ca^{2+}]$ elicited by the first post rest prepulse was inhibited by Cd^{2+} in a variable degree. It completely disappeared in 2 cells, in 5 cells its amplitude was reduced by 30% 87% and in one cell it did not change. In all cells in which the initial step of $[Ca^{2+}]$ was not completely inihibited by Cd^{2+}, the rate of its rise was decreased. In 3 cells the rapid step-wise increase was still visible (Fig. 7), whereas in 3 others it was replaced by monotonous rise in $[Ca^{2+}]$ starting during the inactivation phase of the Na^+ current and continuing till the activation of the remnants of Ca^{2+} current (Fig. 8). The second phase of the Ca^{2+} transient elicited by Ca^{2+} current was also inhibited, however, to a less degree than the first one. Surprisingly enough, the degree of inhibition of the first and second phase of the first post rest Ca^{2+} transient was not related to the degree of inhibition of the Ca^{2+} current (the table). The following post rest Ca^{2+} transients were strongly inhibited by Cd^{2+}. They took form of a monotonous rise in $[Ca^{2+}]$ with a maximal amplitude ranging from 15% to 85% of control. The rise in $[Ca^{2+}]$ began at the inactivation phase of the Na^+ current in 3 cells and was delayed to the activation of the remants of the Ca^{2+} current in 3 other cells.

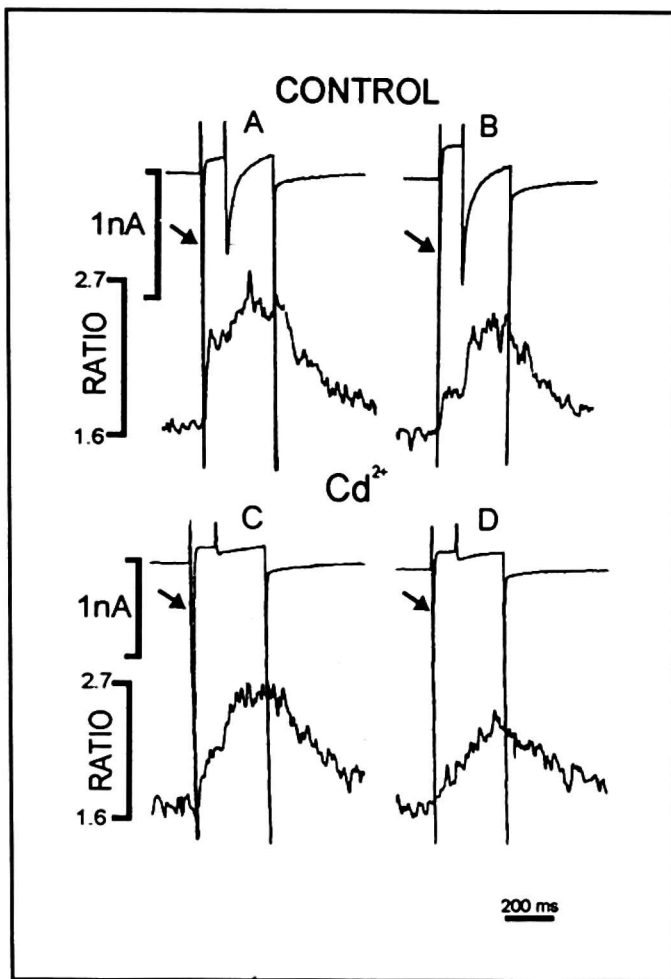Fig. 7. The effect of 100 μM Cd^{2+} on Ca^{2+} transients elicited by prepulse from −80 mV to −40 mV for 100 ms followed by step depolarisation to +5 mV for 200 ms and on the respective Ca^{2+} currents. Upper panel. A: the first cycle recorded after 45 s break in stimulation at the rate of 30/min. B: a cycle recorded during steady state stimulation. Bottom panel. A: the first cycle recorded after the next 45 s break in stimulation of the same cell. Cd^{2+} superfused from the beginning of the break. B: second cycle after the break. Na^{+} current off scale.

Fig. 8. The effect of the SR Ca^{2+} load on the inhibition of Ca^{2+} transient by 100 μM Cd^{2+} Upper panel. A, B: the first cycles after 45 s breaks in stimulation. 100 μM Cd^{2+} superfused from the beginning of the second break (before cycle B). Stimulation rate before the breaks 30/min. Bottom panel. Cd^{2+} washed out and experiment illustrated in upper panel repeated in the same cll, but the steady state stimulation rate increased to 60/min. 100 μM Cd^{2+} superfused from the beginning of 45 s break preceding cycle D.
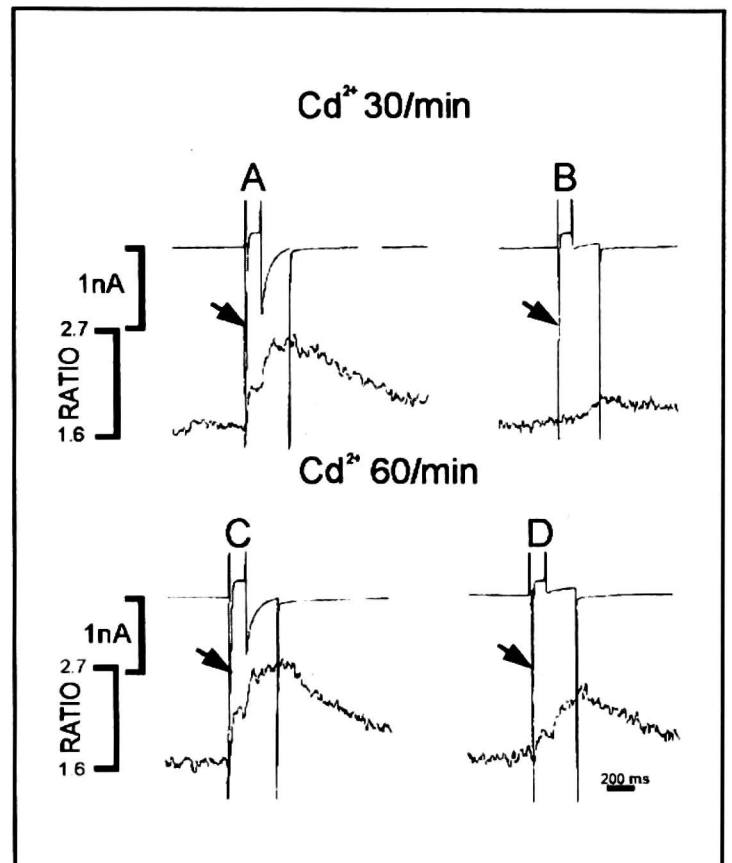
In one cell in which the initial rise in [Ca^{2+}] was inhibited by Cd^{2+} we washed out the blocker and repeated the experiment after the rate of stimulation has been increased from 30/min to 60/min. In the second part of

experiment inhibition was much less than in the first part (*Fig. 8*). This result suggests that degree of inhibition could be related to the SR Ca^{2+} load.

The results of these experiments certainly suggest that some activation of the Ca^{2+} current by the Na^+ current could be involved in the stimulation of the SR Ca^{2+} release by the later. However, lack of the consistent relation between degree of inhibition of the Ca^{2+} current and step-wise rise in $[Ca^{2+}]$ elicited by Na^+ current suggest that other factor was also involved.

DISCUSSION

In this study we found that inhibition of the Na^+ current by TTX resulted in an increase of the rate of the rapid rising phase of post rest and steady state Ca^{2+} transients initiated by APs (*Fig. 1, 2*). This result is consistent with that of Sipido *et al.* (6) who found that TTX initially increased the rate of rise and total amplitude of the Ca^{2+} transients activated by APs. The amplitude of the following transients decreased in their experiments. We also observed the decrease, however, during long enough steady state stimulation in most cells the amplitude regained the pre- TTX level. The results of Sipido *et al.* (6) and ours seem to be at variance with those of Leblanc and Hume (2), who observed decrease in amplitude of the Ca^{2+} transients elicited by APs under the effect of TTX. The reason of this discrepancy is not quite clear. The only difference between their and our protocol was that they stimulated cells every 30 s during superfusion of TTX whereas we applied the 1—1.5 min pause in stimulation and recorded post rest and following APs and Ca^{2+} transients of cells stimulated at the rate of 30/min. As the authors inform that the Ca^{2+} transient shown in their *Fig. 1A2* was recorded during steady state stimulation it is possible that they saw only the phase of decrease of amplitude seen also in our and in Sipido *et al.* (6) experiments. It is difficult to tell from the records of Ca^{2+} transients whether there was a change in the rate of their rise.

The results of experiments in which activation of Na^+ and Ca^{2+} currents were separated may help to explain the mechanism of the effect of TTX in our experiments. We found that prepulses from -80 mV to -40 mV reduced the amplitude of the Ca^{2+} current, inhibition being inversely related to the duration of the prepulse (*Fig. 4*). Blocking of the Na^+ current by switching the holding potential from -80 to -40 mV or by TTX removed the inhibition and increased the rate of the rising phase of the Ca^{2+} current (*Fig. 3 and 6*). This result suggests that some factor appeared within the cell due to activation of the Na^+ current which limited the activation of the Ca^{2+} current. Apparently the factor disappeared with time.

The $[Na^+]$ may increase in the subsarcolemmal "fuzzy space" to the level much higher than that in the bulk sarcoplasm (17—19). This had been recently proved by Wendt-Gallitelli *et al.* (20) by the electron probe microanalysis.

These authors found steep heterogeneous subsarcolemmal gradients of $[Na^+]$ which locally reached up to 80 mM. Although in their experiments the gradients were apparently generated by Na^+ influx for exchange with Ca^{2+}, even higher gradients may be expected to develop due to activation of the Na^+ channels. High $[Na^+]$ could inhibit the Ca^{2+} current as proposed by Balke and Wier (21). Moreover, high subsarcolemmal $[Na^+]$ could reverse the Na/Ca exchange which would result in Ca^{2+} influx. Indeed, in our experiments the Na^+ current initiated release of Ca^{2+} from the SR, apparently due to stimulation of the Ca^{2+} influx. The influx could also result from activation of the SL Ca^{2+} channels due to the loss of voltage control during activation of the Na^+ current as suggested by Bouchard et al. (3). Whatever was the route, the influx could increase the subsarcolemmal $[Ca^{2+}]$. As predicted by the recently published model of Langer and Peskoff (22), $[Ca^{2+}]$ in the SR–SL cleft may increase within the initial 20 ms of cell stimulation to the level of 600 μM and decay to 20 μM within 200 ms. Indeed, Isenberg et al. (23) using the high speed digital imaging microscopy have shown recently the existence of sharp intrasarcomere $[Ca^{2+}]$ gradients which develop during initial 15 ms after start of depolarisation. The increase in subsarcolemmal $[Ca^{2+}]$ initiated in our experiments by Na^+ current might account for inhibition of Ca^{2+} current sen in our experiments since it has been shown to be regulated by $[Ca^{2+}]_i$ (24, 25). According to Hirano and Hiraoka (26) the SL Ca^{2+} channel activity is suppressed by the bulk $[Ca^{2+}]_i > 600$ nM. The Ca^{2+} influx prior to activation of Ca^{2+} current could also activate a fraction of the SR Ca^{2+} release channels rendering the total release more gradual.

The apparent decrease in the rate of Ca^{2+} release from the SR in our experiments seems to be at variance with the results of Lipp and Niggli (4). They reported that the rate of rise of the Ca^{2+} signals initiated by Na^+ current was greater than the rate of signals initiated by the Ca^{2+} current. We propose the following explanation of this apparent discrepancy. In our experiments the amplitude of the Ca^{2+} signals initiated by Na^+ current as compared with the signals initiated by the Ca^{2+} current was much lower than in the experiments of Lipp and Niggli (4). The reason of this difference might be the more negative holding potential prior to activation of the Na^+ current than in our experiments (-90 mV vs. -80 mV). Moreover, these authors recorded the Ca^{2+} signals initiated by the Na^+ current or the Ca^{2+} current separately or in one depolarising step. We observed slowing of the Ca^{2+} signal by the Na^+ current when the currents were activated in succession. Small Ca^{2+} transient activated in our experiments by the Na^+ current when fused with the signal initiated by the Ca^{2+} current slowed the apparent rate of the rising phase.

We are not able to tell for sure what was the route of the Ca^{2+} influx initiated by the Na^+ current. The rise in Ca^{2+} concentration initiated by prepulses from -80 to -40 mV was not affected by series resistance compensation as suggested by Bouchard et al. (3). Superfusion of Cd^{2+} during

45 s — 60 s pause in stimulation resulted in a variable inhibition of Ca^{2+} current and of rise in Ca^{2+} concentration under the effect of prepulse during the first post rest cycle (*Fig. 7*). The Ca^{2+} current activation due to escape of the Na^+ current from the voltage control (if present) should have been inhibited to the same degree as Ca^{2+} current activated by the following voltage step to $+5$ mV. Surprisingly enough degree of inhibition of rise in $[Ca^{2+}]$ was not related to the degree of inhibition of the Ca^{2+} current (the *table*). These results suggest that although the Ca^{2+} current, which might be activated due to escape of the Na^+ current from voltage control at the beginning of the prepulse, could contribute to the triggering of Ca^{2+} release from the SR, another factors were also important. Certainly it was not the voltage as proposed by Ferrier and Howlett (27) since the rise in $[Ca^{2+}]$ was completely inhibited by TTX. The most likely candidate is the reversed Na/Ca exchange, which might be effective in variable degree due to variable Ca^{2+} loading of the SR. As shown recently by Janczewski *et al.* (28) the ratio of amount of Ca^{2+} released from the SR by the given Ca^{2+} influx (the gain index) is directly releated to the SR Ca^{2+} load. In our experiments the loat at the first post rest cycle could differ from cell to cell due to various rate of their rest decay. The result of experiment illustrated in *Fig. 8,* in which increase in the rate of prerest stimulation increased the initial rise in $[Ca^{2+}]$ in a cell pretreated with Cd^{2+}, lends support to this supposition.

Table 1. The effect of 100 μM Cd^{2+} on Ca^{2+} current and Ca^{2+} transients elicited by prepulse from -80 to -40 mV for 100 ms followed by depolarisation to $+5$ mV for 200 ms.

% inhibition of Ca^{2+} current	% inhibition of the phase of Ca^{2+} transient initiated by prepulse	% inhibition of the phase of Ca^{2+} transient initiated by Ca^{2+} current
71	100	25
89	100	90
78	87	85
75	75	50
92	64	30
92	64	30
82	35	0
94	30	20
62	0	85

In summary, we found that prepulses to -40 mV which activate the Na^+ current reduce the activation of the following Ca^{2+} current and decrease the

rate of the rapid rising phase of the Ca^{2+} transients. It is likely that these effects are at least in part related to the Ca^{2+} influx initiated by the Na^+ current. Inhibition of the activation of Ca^{2+} channels by the Na^+ ions by modulation of their c-AMP dependent phosphorylation is also conceivable (21). Increase by TTX of the rate of rise of the rapid phase of the Ca^{2+} transients elicited by the APs suggests that similar events may take place also in physiologically stimulated cells. Exact relative time course of activation of the Na^+ and Ca^{2+} currents and of the SR Ca^{2+} release channels during the upstroke of an AP is, to our knowledge, not known. However, an attempt to its reconstruction based on the kinetics of these currents known from the voltage clamp experiments and local control theories for excitation-contraction coupling (29—32) seems to be justified.

The majority of Na^+ channels are already activated at -40 mV with the first latency not exceeding 1.5 ms (32, 33), macroscopic current reaching its peak within 2 ms at most (33—35). The L-type Ca^{2+} channels are activated by depolarisation to -40 mV with the first latency of -25% of the channels ranging from 1 ms to 10 ms (36). So, most likely, a considerable proportion of the Ca^{2+} channels are activated with the few ms delay with respect to activation of the Na^+ channels. Thus it is conceivable that the elementary Na^+ currents activated prior to elementary Ca^{2+} currents may reduce activation of the later by a number of potential mechanisms. Shift of the membrane potential to the positive values decrease the Ca^{2+} current by decrease of its driving force. Local increase in subsarcolemmal $[Na^+]$ may reverse the Na/Ca exchange which would result in the local increase in subsarcolemmal Ca^{2+} concentration. This could reduce activation of the Ca^{2+} channels and activate the SR Ca^{2+} release channels which would further increase the subsarcolemmal $[Ca^{2+}]$ prior to activation of some proportion of the Ca^{2+} channels. Activation of a fraction of the SR Ca^{2+} release channels before activation of significant percentage of the SL Ca^{2+} channels would decrease the overall rate of the SR Ca^{2+} release rendering it more gradual because a fraction of SR channels activated by the early reversal of the Na/Ca exchange would be refractory at the moment of activation of the SL Ca^{2+} channels (30). On the other hand the limited activation of the SL Ca^{2+} channels would limit activation of the remaining SR Ca^{2+} channels (32). The importance of action of the Na^+ current prior to activation of the SL Ca^{2+} channels for the discussed phenomena seems to be confirmed by lack of consistent effect of Na^+ current inihition in cells depolarized in one step from -80 mV to $+5$ mV. In these cells the Na^+ current and Ca^{2+} current should be activated almost simultaneously. The proposed hypothesis, if valid, would predict that conditions enhancing the reversed Na/Ca exchange would tend to limit activation of the SL Ca^{2+} channels. Thus the relative contribution of Na/Ca exchange to the trigger of Ca^{2+} release would increase, as already proposed by Kohmoto et al. (9).

The physiological meaning of our results is not clear. One of the possible effects of the proposed mechanism may be the limitation of the rate and amplitude of the increase in subsarcolemmal $[Ca^{2+}]$ to which the Na/Ca exchangers are eventually exposed. This would decrease the proportion of the released Ca^{2+} which is extruded by the Na/Ca exchange working later in the cycle in the "Ca^{2+} out mode", rendering the Ca^{2+} exchange cycle more economical.

REFERENCES

1. Fabiato A. Stimulated calcium current can both cause calcium loading and trigger calcium release from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. *J Gen Physiol* 1985; 85: 291—320.
2. Leblanc N, Hume JR. Sodium current release of calcium from cardiac sarcoplasmic reticulum. *Science* 1990; 248: 372—375.
3. Bouchard RA, Clark RB, Giles WR. Role of sodium-calcium exchange in activation of contraction in rat ventricle. *J Physiol (Lond)* 1993; 472: 395—413.
4. Lipp P, Niggli E. Sodium current-induced signals in isolated guinea-pig ventricular myocytes. *J Physiol (Lond)* 1994; 474: 439—416.
5. 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.
6. Sipido KR, Carmeliet E, Pappano A. Na^+ current and Ca^{2+} release from the sarcoplasmic reticulum during action potentials in guinea pig ventricular myocytes. *J Physiol (Lond)* 1995; 489: 1—17.
7. 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.
8. Levi AJ, Brooksby P, Hancox JC. A role for depolarisation induced calcium entry on the Na–Ca exchange in triggering intracellular calcium release and contraction in rat ventricular myocytes. *Cardiovasc Res* 1993; 27: 1677—1690.
9. 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.
10. Grantham CJ, Cannell MB. Ca^{2+} influx during the cardiac action potential in guinea pig ventricular myocytes. *Circ Res* 1996; 79: 194—200.
11. Levi AJ, Li J, Spitzer KW, Bridge JHB. Effect on the indo-1 transient of applying Ca^{2+} channel blocker for single beat in voltage-clamped guinea-pig cardiac myocytes. *J Physiol (Lond),* 1996; 494: 653—673.
12. Kirby MS, Sagara YG, Gow S, Inui G, Lederer WG, Rogers TB. Thapsigargin inhibits contraction and Ca^{2+} transient in cardiac cells by specific inhibition of the sarcoplasmic reticulum Ca^{2+} pump. *J Biol Chem* 1992: 267: 12545—12551.
13. 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.

14. Thastrup JP, Cullen J, 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 Natl Acad Sci USA* 1990; 87: 2466—2470.

15. Wrzosek A, Schneider H, Grueninger S, Chiesi M. Effect of thapsigargin on cardiac muscle cells. *Cell Calcium* 1992; 13: 281—292.

16. Di Francesco D, Ferroni A, Viscentin S, Zaza, A. Cadmium-induced blockade of the cardiac fast Na channels in calf Purkinje fibres. *Proc Roy Soc R* 1985; 223: 475—484.

17. Carneliet E. A fuzzy subsarcolemmal space for intracellular Na^+ in cardiac cells? *Cardiovasc Res* 1992; 26: 433—442.

18. Lederer WJ, Niggli E, Hadley RW. Sodium-calcium exchange in excitable cells: fuzzy space. *Science* 1990; 248: 283.

19. Niggli E, Lipp P. Subcellular restricted spaces: significance for cell signalling and excitation-contraction coupling. *J Muscle Res Cell Motility* 1993; 14: 288—291.

20. Wendt-Gallitelli MF, Voigt T, Isenberg G. Microheterogeneity of subsarcolemmal sodium gradients. Electron probe microanalysis in guinea-pig ventricular myocytes. *J Physiol (Lond)* 1993; 472: 33—44.

21. Balke CW, Wier WG. Modulation of L-type calcium channels by sodium ions. *Proc Natl Acad Sci USA* 1992; 89: 4417—1121.

22. Langer GA, Peskoff A. Calcium concentration and movement in the diadic cleft space of the cardiac cells. *Biophys J* 1996; 70: 1169—1182.

23. Isenberg G, Etter EF, Wendt-Gallitelli MF *et al.* Intrasarcomere $[Ca^{2+}]$ gradients in ventricular myocytes revealed by high speed digital imaging microscopy. *Proc Natl Acad Sci USA* 1996; 93: 5413—5418.

24. Bates SE, Gurney AM. Ca^{2+} — dependent block and potentiation of L-type calcium current in guinea-pig ventricular myocytes. *J Physiol (Lond)* 1993; 466: 345—363.

25. Boyett MR, Kirby MS, Orchard CH. Rapid regulation of the "second inward current" by intracellular calcium in isolated rat and ferret ventricular myocytes. *J Physiol (Lond)* 1988; 407: 77—102.

26. Hirano Y, Hiraoka M. Dual modulation of unitary L-type Ca^{2+} channel currents by $[Ca^{2+}]$ in fura-2-loaded guinea-pig ventricular myocytes. *J Physiol (Lond)* 1994; 480: 449—463.

27. Ferrier GR, Howlett SE. Contractions in guinea-pig ventricular myocytes triggered by calcium-release mechanism separate from Na^+ and Ca^{2+} currents. *J Physiol (Lond)* 1995; 484: 107—122.

28. Janczewski AM, Spurgeon HA, Stern MD, Lakatta EG. Effects of sarcoplasmic reticulum Ca^{2+} load on the gain function of Ca^{2+} release by Ca^{2+} current in rat cells. *Am J Physiol* 1995; 268: H916—H920.

29. Cannell MB, Cheng H, Lederer WJ. The control of calcium release in heart muscle. *Science* 1995; 268: 1045—1049.

30. Cheng H, Lederer MR, Lederer WJ, Cannell MB. Calcium sparks and $[Ca^{2+}]$ waves in cardiac myocytes. *Am J Physiol* 1996; 270: C148–C159.

31. Lopez-Lopez 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.

32. Santana LF, Cheng H, Gomez AM, Canell MB, Lederer WJ. Relation between the sarcolemmal Ca^{2+} current and Ca^{2+} sparks and local control theories for cardiac excitation-contraction coupling. *Circ Res* 1996; 78: 166—171.

33. Berman MF, Camardo JS, Robinson RB, Siegelbaum SA. Single sodium channels from canine ventricular myocytes: voltage dependence and relative rates of activation and inactivation. *J Physiol (Lond)* 1989; 415: 503—531.

34. Scanley BE, Hanck DA, Hay T, Fozzard HA. Kinetic analysis of single sodium channels from canine cardiac Purkinje cells. *J Gen Physiol* 1990; 95: 411—437.

35. Follmer CH, Ten Eick RE, Yeh JZ. Sodium current kinetics in cat atrial myocytes. *J Physiol (Lond)* 1987; 384: 169—197.
36. Pelzer D, Cavalie A, Trautwein W. Activation and inactivation of single calcium channels in cardiac cells. *Experimental Brain Res Series* 1986; 14: 17—34.

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