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# THE SOURCE OF CONTRACTICLE CALCIUM IN GUINEA-PIG CARDIAC MYOCYTES TREATED WITH THAPSIGARGIN

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We investigated the source of activator $Ca^{2+}$ in the cells deprived of sarcoplasmic reticulum (SR) $Ca^{2+}$ by pretreatment with $10^{-6}$ mM thapsigargin (TG). These cells show $Ca^{2+}$ transients of nearly normal amplitude, albeit with the slowed kinetics. We found in the voltage clamped and loaded with Indo 1-AM cells that at depolarizing potentials from −30 to +70mV, blocking of sarcolemmal $Ca^{2+}$ channels with 20 μM nifedipine or 20 μM $Cd^{2+}$ reduced $Ca^{2+}$ transients and contractions as much in the cells treated with TG as in the normal cells. The residual $Ca^{2+}$ transients were mostly subthreshold for the contractile system. The result suggests that in the cells treated with TG, $Ca^{2+}$ influx by the reversed Na/Ca exchange is not more important for activation of contraction than in the normal cells. In the normal cells shortening of one of the depolarizing pulses (+5 mV) applied at a steady rate of 30/min from 200 ms to 5, 10, 20, 30, 50, or 100 ms little affected amplitude of the respective $Ca^{2+}$ transients, although their duration was decreased proportionally to the decrease of the duration of the pulse. In the cells pretreated with TG, 20 ms pulses initiated $Ca^{2+}$ transients which were hardly visible in the records of fluorescence. Their amplitude increased with increase in the duration of the pulses linearly correlating with the charge transfered with the $Ca^{2+}$ current. We propose that the direct source of $Ca^{2+}$ activating contraction in the guinea-pig ventricular myocytes is sarcolemmal $Ca^{2+}$ influx mostly through the sarcolemmal $Ca^{2+}$ channels. The alternative hypothesis is that there is some yet unidentified cellular source of activator $Ca^{2+}$ (internal leaflet of sarcolemma?) from which it may be released by sarcolemmal $Ca^{2+}$ influx.

Key words: *cardiac myocytes, sarcoplasmic reticulum $Ca^{2+}$, thapsigargin, $Ca^{2+}$ channels, Na/Ca exchange, excitation-contraction coupling.*

## INTRODUCTION

It is generally accepted, that sarcoplasmic reticulum (SR) is the main source of $Ca^{2+}$ activating contraction in mammalian cardiac myocytes (1). $Ca^{2+}$ is released from SR due to activation of SR release channels by $Ca^{2+}$ diffusing through the sarcolemmal channels ($Ca^{2+}$-induced $Ca^{2+}$ release, 2). However,

recently several groups have reported that in the single myocytes of guinea-pig and rabbit heart thapsigargin (TG) or cyclopiazonic acid reduce the amplitude of shortening of the electrically stimulated cells only by 30% and 50%, respectively, despite complete depletion of $Ca^{2+}$ from the sarcoplasmic reticulum (SR) (3—5). In guinea-pig cardiomyocytes loaded with Indo 1-AM, TG did not affect significantly amplitude of $Ca^{2+}$ transients or contractions (6) despite complete inhibition of their contractile response to caffeine. These results suggest that contraction largely depends in these species on $Ca^{2+}$ derived from the sources other than SR.

The most apparent source of contractile $Ca^{2+}$ other than SR is $Ca^{2+}$ influx through the activated sarcolemmal $Ca^{2+}$ channels. However, it is generally believed that due to buffering of $Ca^{2+}$ by intracellular sites (troponin, calmodulin, other $Ca^{2+}$ binding proteins, negatively charged phospholipids of internal leaflet of sarcolemma) $Ca^{2+}$ influx by sarcolemmal channels is too small to rise sarcoplasmic $Ca^{2+}$ concentration high enough to activate significant contraction (1, 2). The $Ca^{2+}$ current is not significantly changed in guinea-pig cardiac myocytes treated with TG (6).

The other source might be the reversed Na/Ca exchange. The exchange works in a "$Ca^{2+}$ out mode" when the membrane potential is negative to the exchange reversal potential, i.e., at the diastolic potential. However, during the 0 phase and the initial part of the plateau of the action potential $V_m$ becomes strongly positive to the Na/Ca exchange reversal potential until the later rises due to rapid increase in $[Ca^{2+}]_i$. Therefore it has been proposed, that $Ca^{2+}$influx by the reversed Na/Ca exchange over the initial portion of plateau might significantly add to $Ca^{2+}$ influx through the sarcolemmal $Ca^{2+}$ channels (7—9). $Ca^{2+}$ influx at the time might be promoted by the local, subsarcolemmal $Na^+$ concentration rised at the initial parts of AP due to $Na^+$ influx through the sarcolemmal $Na^+$ channels (10).

However, it has been proved more recently that in cells in which $Ca^{2+}$ current has been blocked, the reversed Na/Ca exchange is able to initiate significant $Ca^{2+}$ transient and contraction only when SR contains sufficient amount of $Ca^{2+}$. When SR is deprived of $Ca^{2+}$ by ryanodine and/or TG, the reversed Na/Ca exchange may initiate only a slight elevation of $[Ca^{2+}]_i$ and feeble contraction (11—14) even when $Ca^{2+}$ influx is promoted by dialising the cell with high $Na^{2+}$ concentration in the internal (patch pipette) solution (11). Therefore it is now proposed that the reversed Na/Ca exchange may contribute together with $Ca^{2+}$ current to the trigger for $Ca^{2+}$-induced $Ca^{2+}$ release from SR but its role as a source of contractile $Ca^{2+}$ is negligible (11—14).

In this paper we reinvestigated the effects of blocking of sarcolemmal $Ca^{2+}$ channels in normal, voltage clamped cells, and in the cells treated with TG. We found that blocking of $Ca^{2+}$ current in voltage clamped myocytes reduces $Ca^{2+}$ transients and contractions to insignificant levels at all depolarizing

potentials (up to +70 mV). Moreover, in the cells treated with TG the amplitude of the $Ca^{2+}$ transient was linearly related to the charge transfered with $Ca^{2+}$ current. Hence we propose that the source of contractile $Ca^{2+}$ in the cells treated with TG is $Ca^{2+}$ influx through the activated sarcolemmal $Ca^{2+}$ channels or, that there is some source other than SR in the myocytes treated with TG from which the $Ca^{2+}$ current may release more $Ca^{2+}$ (some additional $Ca^{2+}$-induced $Ca^{2+}$ release).

## METHODS

### *Cell isolation, superfusion and recording of cotractions*

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 and washed in cold Tyrode solution, the aorta was cannulated and retrogradely perfused for 5 min with nominally $Ca^{2+}$-free Tyrode solution containing 10μM EGTA (Ethylene-bis oxyethylenenitrilo) tetraacetic acid) (for composition of solutions see below). Initial washout period was followed by 20 min of perfusion with $Ca^{2+}$-free solution 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 $Ca^{2+}$ has been 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 temparature until used.

Cells were placed in the 1 ml superfusion chamber and became attached to its glass bottom. The chamber was mounted on the stage of an inverted microscope (Nikon Diaphot) and perfused at the rate of 2 ml/min. A TV camera was mounted in the place of one of the eye pieces of the microscope and the image of cells displayed on the screen of a TV monitor. The length of cell was monitored by a TV edge-tracking system. Cells were illuminated with the red (650—750 nm) light through the bright-field illumination optics of microscope.

### *Measurement of Indo 1-AM fluorescence*

500 μl of cell suspension were incubated for 20 min at room temperature with 5 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 fragment of the cell. The fluorescent light was split by the dichroic mirror into 410 and 495 nm beams which were passed to two multipliers mounted in the side port of the microscope. The ratio of 410 to 495 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 in cells were recorded using whole cell clamp method. Pipettes of 2.5 to 3.6 MΩ resistance were pulled from borosilicate glass capillaries. The cell membrane under the

electrode was disrupted by suction or the perforated patch method was applied. In the latter case 400 to 600 μM nystatin was added to the solution filling pipette. Pulses from a holding potential of -80 to -45 mV (duration 50 ms) followed by pulses to +5 mV (duration 20 to 300 ms) were applied at 0.5 Hz to inactivate the fast $Na^+$ current and activate the $Ca^{2+}$ current, respectively. Currents were measured using an Axopatch 1-D amplifier controlled by an IBM AT 386 computer using the V-clamp software and V-clamp computer interface designed by D.R. Matteson (Dept. of Biophysics, University of Maryland). Series resistance compensation was used to minimize voltage error due to access resistance. The cellular action potentials (AP) were recorded with the same amplifier set to the current-clamp mode.

All signals (APs, currents, Indo 1-AM fluorescence, and contractions) were passed through the computer interface, digitized at 2 kHz and stored on a disc for off-line analysis. They were also directly recorded on tape and with the Gould TA 240 chart recorder.

### *Solutions*

For cell isolation and throughout the experiments we used a Tyrode solution of the following composition (in mM): 144 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, and 5.0 sodium pyruvate. 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 2.0 mM. The patch pipettes were filled with a solution of the following composition (in mM): 100.0 potassium aspartate, 35.0 KCl, 2.0 $MgCl_2$, 10.0 NaCl and 10.0 HEPES. The pH was adjusted with KOH to 7.20.

Nifedipine, Nystatin, DMSO, Indo 1, $CdCl_2$ and thapsigargin was obtained from Sigma Chemical Company USA.

### *Data processing and statistical analysis*

Quanitative data are presented as Means ± SE. Charge transfered was calculated from the $Ca^{2+}$ current by the data aquisition and processing V-clamp software mentioned above in "Electrophysiological investigation". Correlation between the charge transfered and amplitude of $Ca^{2+}$ transients was calculated by least square method by Jandel Scientific Table Curve program.

## RESULTS

$Ca^{2+}$ influx by two main routes: sarcolemmal $Ca^{2+}$ channels and reversed Na/Ca exchange may contribute to activation of contraction. The following series of experiments addressed the question whether relation of their importance would change in the cells treated with TG with respect to the normal cells. To this effect we designed the experimental conditions which enabled the selective or diverse changes in $Ca^{2+}$ channels and/or Na/Ca exchange and investigated their effect in normal cells and in cells pretreated with TG.

*The effect of nifedipine on APs and contractions in normal cells and in cells pretreated with TG*

20 μM nifedipine was superfused during steady state stimulation at the rate of 30/min and APs and contractions recorded. As shown in *Fig. 1B* nifedipine largely reduced duration of AP and inhibited contractions in normal cells. Fifteen min of superfusion with $10^{-6}$ M TG resulted in slight increase in duration of APs, decrease in amplitude of contraction by 30% and large increase in time to peak shortening (*Fig. 1C*). These effects of TG have been described in detail previously (6). The effects of nifedipine in these cells did not differ from those in the control cells (*Fig. 1D*).

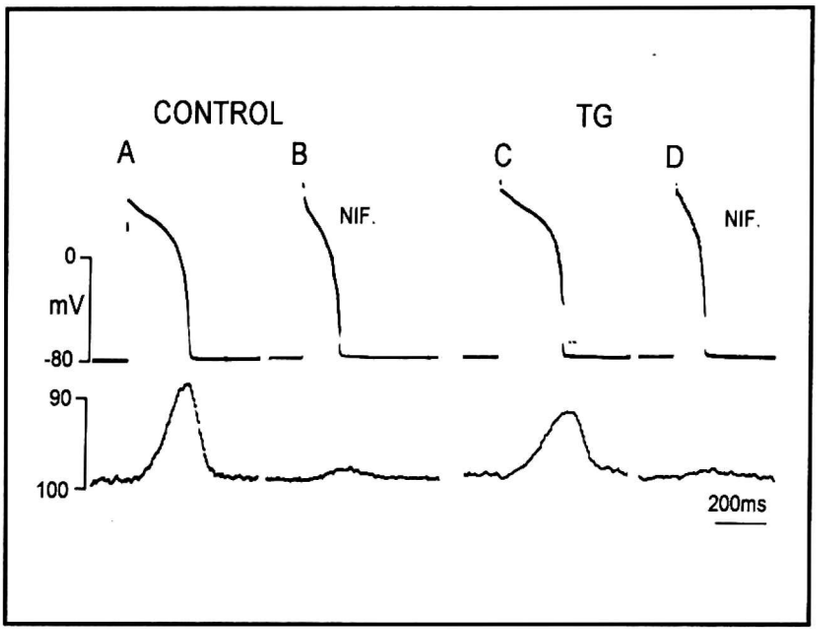


*Fig. 1.* The effect of 20 μM nfedipine on action potentials (top) and contractions of single myocytes of guinea-pig heart. A-control; B-5 min of superfusion with nifedipine; C-10 min of superfusion with $10^{-6}$ M thapsigargin (TG) of a cell different than that in A and B; D-same cell, 5 min of superfusion with nifedipine.

Large change in the time course of APs due to inhibition of $Ca^{2+}$ current could affect various links of excitation-contraction coupling. Therefore the next series of experiments were performed in the voltage clamped cells in which the time course of depolarization is rigorously controled.

*The effect of blocking of $Ca^{2+}$ channels on I—$V_m$, $Ca^{2+}$ transient-$V_m$ and contraction-$V_m$ relation in normal cells*

The peak $Ca^{2+}$ current showed typical relation to $V_m$ (*Fig. 2 and 3*). It begane to activate at about −30 mV, reached its maximum at 0 mV and declined to 0 between +50 and 60 mV. The amplitudes of $Ca^{2+}$ transients and contractions showed similar relation to $V_m$, however, with some important modifications. The peak of both of them was shifted to more positive voltages by 15 mV with respect to the peak of the current. The decline of $Ca^{+2}$ transient and contractions at positive potentials was far less complete than that of the current. At +70 mV, where the current was practically absent, $Ca^{2+}$ transient

dropped by only 20% (*Fig. 2 and 3*). At positive potentials activation of inward $Ca^{2+}$ current is diminished or absent, whereas $Ca^{2+}$ influx by reversed Na/Ca exchange may be enhanced. This could activate directly contractile proteins and/or induce $Ca^{2+}$ release from SR.

20 μM nifedipine reduced the peak $Ca^{2+}$ current by 75%, proportionally at all voltages (*Fig. 2 and 3*). So the I—V relation did not change except for decreased amplitudes. Nifedipine reduced largely amplitude of $Ca^{2+}$ transients. It reached its maximum between −10 and 0 mV and only slighty declined when the voltages were increased gradually to +70 mV. So the maximum reduction (by 70%) of $Ca^{2+}$ transient by nifedipine was at 0 mV i.e. when the $Ca^{2+}$ current is the largest. At +70 mV amplitude of $Ca^{2+}$ transient was reduced by 55% (*Fig. 3*).

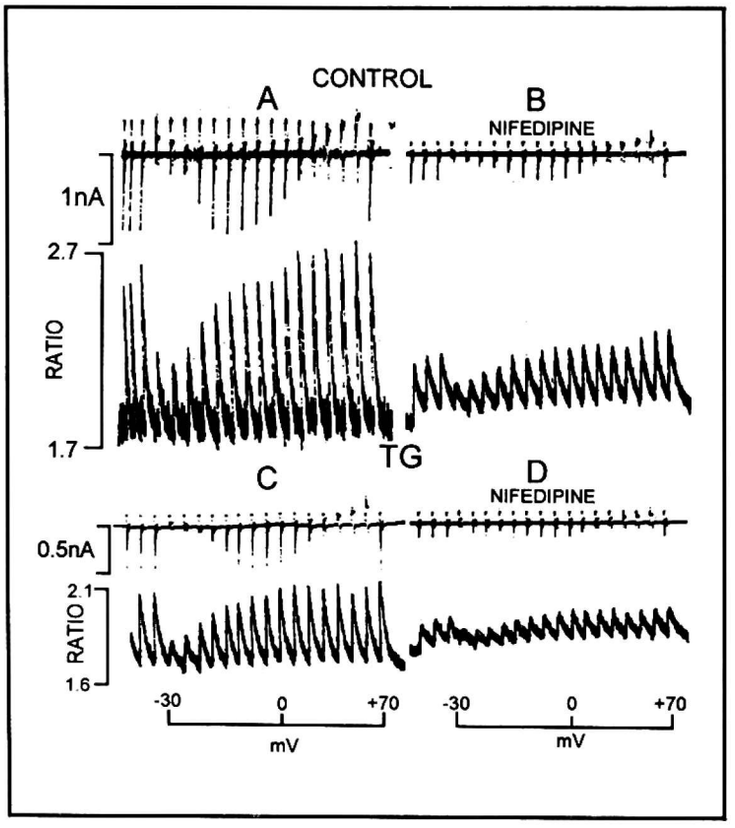


*Fig. 2.* The effect of 20μM nifedipine on Ca current (top, downward deflections) and Indo-1 fluorescence of single myocytes of guinea-pig heart. An example of the original records from which the graphs shown in *Fig. 3* have been constructed. A-current-voltage (top) and $Ca^{2+}$ transient-voltage relation in control cell; B-same cell superfused for 5 min with nifedipine; C-another cell pretreatd for 15 min with $10^{-6}$ M thapsigargin (TG); D-same cell as in C after 5 min of superfusion with nifedipine. The lower amplitudes of the currents and Ca transients in C and D than in A and B result from the smaller size of the cell and not from the effect of TG.

As shown in *Fig. 2 and 3* it was very difficult to block completely $Ca^{2+}$ current with nifidipine even at concentration as high as 20 μM. This resulted mostly from its use- dependent effect and partial inactivation by UV light. We had to stop pulsing for few seconds when changing from steady state stimulation to investigation of I—V relation and the conditioning pulses did not usually reduce the $Ca^{2+}$ current to the level reached at steady state. Therefore in the next series of experiments we used 20 μM $Cd^{2+}$. It blocks the $Ca^{2+}$ channels but at this concentration it does not block the Na/Ca exchange. Its effect is not use- dependent.

$Cd^{2+}$ inhibited $Ca^{2+}$ current much stronger than nifedipine. Its maximum did not exceede 7% of control and was shifted to −15 mV. At positive

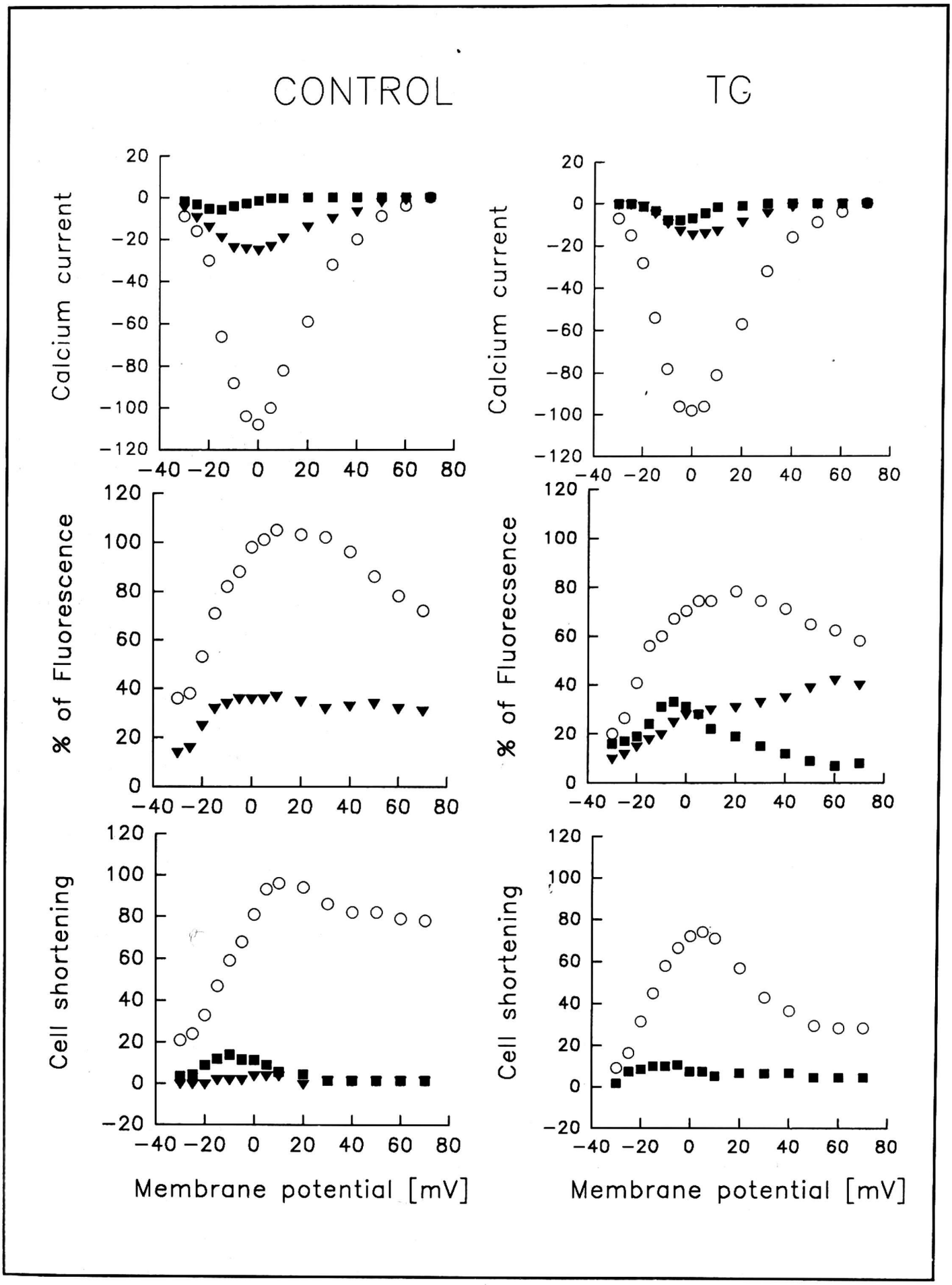


*Fig. 3.* The effect of 20 μM nifedipine (triangles) or 20 μM Cd (squares) on current-voltage relation, $Ca^{2+}$ transient-voltage relation or contraction-voltage relation in single myocytes of guinea-pig heart. Open symbols–controls. TG–15 min of superfusion with $10^{-6}$ M thapsigargin. Means, n = 5—8.

potentials the current was not visible. Small contractions appeared at negative potentials with the peak amplitude of 18% of control at −15 mV (*Fig. 3*). At positive potentials practically no contractions were noted. Since $Cd^{2+}$ accelerated leak of Indo 1 from cells we did not perform the control runs with $Ca^{2+}$ transients in these experments in order to treat them with TG before they lose their dye.

*The effect of blocking of $Ca^{2+}$ channels on I—$V_m$, $Ca^{+2}$ -$V_m$, and contraction-$V_m$ relation in cells pretreated with TG*

The effects of TG on I—$V_m$ relation were small and statistically not significant (*Fig. 3*), which is consistent with our previous results (6). The amplitude of $Ca^{2+}$ transients decreased mostly at negative to +20 mV potentials. Their peak amplitude was reduced by 20%. Peak amplitude of contractions was reduced by 30%. The effects of nifedipine and $Cd^{2+}$ on I—$V_m$ relation were very similar to those in the control cells. Differences are not significant statistically. The effect of nifedipine on $Ca^{2+}$ transients was also similar to hat in the controls cells. Their amplitude decreased to 35% of control at 0 mV and slightly increased at more positive potentials. At +70 mV it reached 67% of control. The further increase at positive potential is the only difference between the normal and pretreated with TG cells (*Fig. 3*). It could mean that Na/Ca exchange transports more $Ca^{2+}$ into the cells under the effect of TG. However, due to relatively large dispersion of individual results, the difference is not significant statistically.

The effect of $Cd^{2+}$ on $Ca^{2+}$ transients was at negative potentials similar to that of nifedipine. However, after the peak at −15 mV they declined at more positive potentials reaching almost 0 at +50 mV. The residual transients were subthreshold for contractile proteins since contractions were at all potentials hardly visible (*Fig. 3*).

*The effect of duration of depolarizing pulses on $Ca^{2+}$ transients in normal cells and in cells pretreated with TG*

Cells were pulsed at the steady rate of 30/min. Each 20 depolarizations one of te 200 ms pulses was substituted with the pulse of the duration of 5, 10, 20, 30, 50, 100 or 300 ms and the membrane currents and $Ca^{2+}$ transients were recorded. Early repolarization inactivated prematurely $Ca^{2+}$ current thereby reducing the amount of $Ca^{2+}$ diffusing into cell. This was calculated as the total charge transfred with the $Ca^{2+}$ current. In normal cells shortening of the depolarizing pulse had little effect on amplitude of $Ca^{2+}$ transient, however, its duration was reduced proportionally to the reduction of the duration of the pluse (*Fig. 4A*). In cells pretreated

with TG $Ca^{2+}$ transient was absent when 5 or 10 ms pulses were applied (not shown) and was hardly visible when 20 ms pulse was used. Its amplitude correlated linearly with the charge transfered with the $Ca^{2+}$ current as duration of the pulses was increased (*Fig. 4B and Fig. 5*).

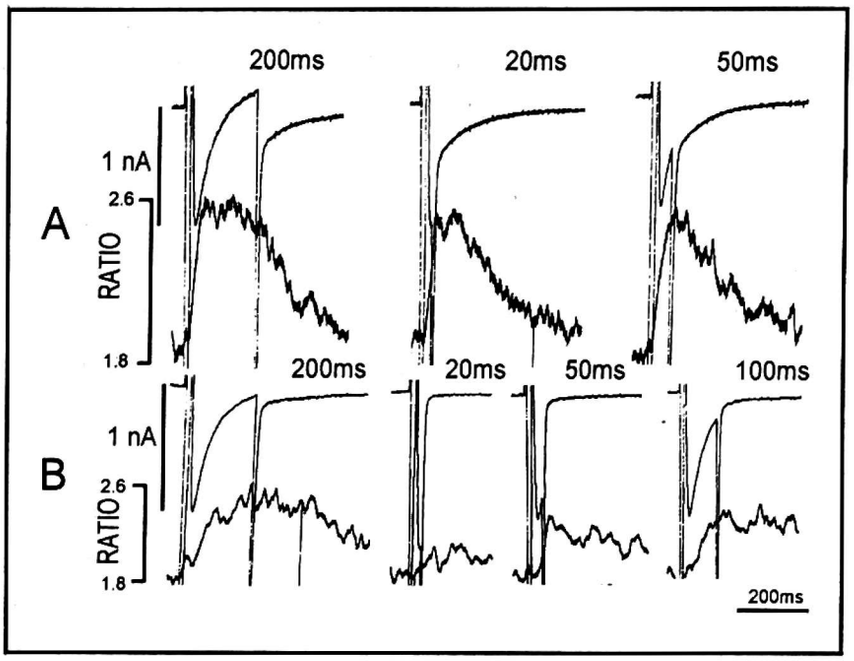


*Fig. 4.*The effect of premature inactivation of $Ca^{2+}$ current (top records) on Ca transients in normal cell (A), and in the cell pretreated for 15 min with $10^{-6}$ M thapsigargin (B).

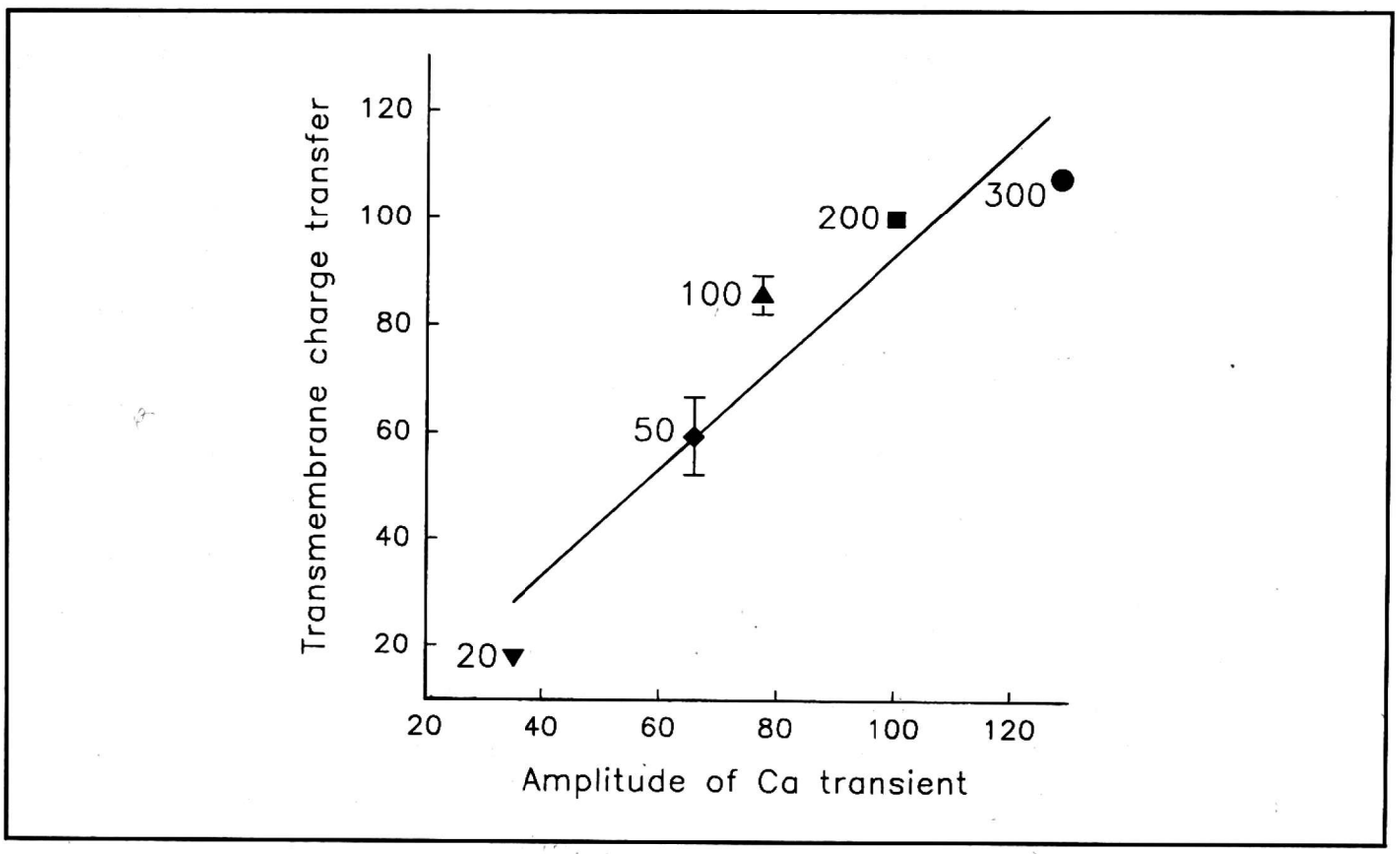


*Fig. 5.* Relation between the amplitude of $Ca^{2+}$ transient and integral of $Ca^{2+}$ current (charge transfered) in guinea-pig myocytes pretreated for 15 min with $10^{-6}$ M thapsigargin. Integral of $Ca^{2+}$ current changed by shortening of depolarizing pulses as shown in *Fig. 4.* All values expressed as % of integral of the current and amplitude of the transient initiated by depolarizing pulse of 200 ms duration. Numbers accompanying the symbols indicate duration of depolarizing pulse. Means $\pm$ SE, n = 4. Linear correlation coefficient r = 0.901.

## DISCUSSION

In normal cells shortening of depolarizing pulses did not affect significantly the amplitude of $Ca^{2+}$ transients, although it decreased their duration. In cells pretreated with TG shortening of depolarizing pulses to 5—20 ms reduced amplitude of the transients almost to 0. When duration of the interposed short pulses was increased, the amplitude of the transient was linearly related to the charge transfered with the $Ca^{2+}$ current. Early repolarization results in premature inactivation of sarcolemmal $Ca^{2+}$ current thereby reducing the amount of $Ca^{2+}$ diffusing into cell. Thus these results suggest that in normal cell activation of contraction is not dependent directly on $Ca^{2+}$ influx, whereas in cells pretreated with TG activation of contraction is directly dependent on the influx. TG selectively inhibits $Ca^{2+}$-ATPase of the SR (15) which results in complete depletion of the SR $Ca^{2+}$ (5, 6, 16, 17). Thus our results show that the direct source of activator $Ca^{2+}$ in normal cells is SR. Evidently $Ca^{2+}$ current flowing over the initial miliseconds of depolarization is sufficient to activate the $Ca^{2+}$ release channels of the SR. This is consistent with the recent results obtained by means of combining the fluorescent dyes technique with confocal microscopy. They showed that the local $Ca^{2+}$ transients (called $Ca^{2+}$ sparks) resulting from $Ca^{2+}$ release from the single clusters of the SR release channels appeare with the delay equal to the waiting time of activation of the single sarcolemmal $Ca^{2+}$ channels (18, 19). $Ca^{2+}$ influx in cells pretreated with TG results in direct activation of $Ca^{2+}$ transients and contractions the amplitude of which is not much reduced with respect to normal cells, albeit their kinetics is changed. The most likely route of the influx are the sarcolemmal $Ca^{2+}$ channels which are early inactivated by premature repolarization. The other effect of very early repolarization may be an early inhibition of $Ca^{2+}$ influx by Na/Ca exchange reversed over the initial part of depolarization. Na/Ca exchange is supposed to reverse during the 0 phase of AP or at the beginning of a clamp to $+5$ mV due to rendering $V_m$ positive to Na/Ca exchange equilibrium potential (7, 8). The exchange remains reversed until sarcoplasmic $[Ca^{2+}]$ reaches the level at which equilibrium potential becomes positive to $V_m$. In the normal cell this happens 20—30 ms after the beginning of phase 0 of AP or beginning of depolarizing pulse. In cells pretreated with TG sarcoplasmic $[Ca^{2+}]$ rises much more slowly than in the normal cells (*Fig. 4*). Therefore reversal of Na/Ca exchange may last longer in these cells i.e. more $Ca^{2+}$ may enter the cell by this route. Early repolarization would interrupt the influx and activate outward $Ca^{2+}$ transport by Na/Ca exchange working now in "$Ca^{2+}$ out" mode. Despite this theoretical possibility our experiments, in which we investigated the effect of blocking the sarcolemmal $Ca^{2+}$ channels on $Ca^{2+}$ transients and contractions, suggest that reversed Na/Ca exchange was not more important for activation of contraction in the cells pretreated with TG than in the normal cells.

In normal cells $Ca^{2+}$ transients and contractions showed at negative potentials the dependence on $V_m$ similar to that of $Ca^{2+}$ current. However, their peak amplitude was shifted to positive potentials with respect to that of the current and their decline at positive potentials was negligible despite decline of the current to O (*Fig. 2 and 3*). At very positive potentials, like +70 mV in our experiments, Na/Ca exchange is reversed. So it may be supposed that at the positive potentials the $Ca^{2+}$ transients and contractions are initiated by $Ca^{2+}$ influx by this route either due to $Ca^{2+}$-induced $Ca^{2+}$ release from SR as proposed by several groups of investigators (11—14) or directly. Experiments with TG would suggest the second possibility because the transients and contractions initiated at positive potentials were not blocked despite depletion of the SR $Ca^{2+}$ by the compound (5, 6). However, the blockers of sarcolemmal $Ca^{2+}$ channels, nifedipine and $Cd^{2+}$, decreased the amplitude of the transients and contractions at positive potentials $Cd^{2+}$, which blocked the $Ca^{2+}$ current more completely, being more effective than nifedipine (*Fig. 3*). This result contradicts the supposition that $Ca^{2+}$ transients and contractions were initiated at positive potentials mostly by $Ca^{2+}$ influx by the reversed Na/Ca exchange. In normal cells they could result from $Ca^{2+}$ release from the SR by feeble $Ca^{2+}$ current, which was not visible in our recordes. However, in cells pretreated with TG this could not be the case since the current, if present, was too small to activate contractions directly. So the mechanism of activation of the $Ca^{2+}$ transients and contractions at positive potentials is not clear and deserves further experimentation. Neverthless in cells pretreated with TG it did not differ significantly from that in normal cells. The only noted difference was less inhibition of $Ca^{2+}$ transients at positive potentials by nifedipine, however, $Cd^{2+}$ which blocked the $Ca^{2+}$ current more completely, inhibited them in cells treated with TG as strongly as in normal cells.

This work and our previous papers (5, 6) provide results which seem to be conflicting. In the normal cells the direct source of activator $Ca^{2+}$is SR. This is consistent with the current experience and notion that $Ca^{2+}$ influx through the sarcolemmal $Ca^{2+}$ channels is too small to activate significant contraction (2). However, in cells in which SR has been depleted of its $Ca^{2+}$, the $Ca^{2+}$ transients and contractions seem to be activated directly by $Ca^{2+}$ influx mostly through the sarcolemmal $Ca^{2+}$ channels. This and the previous paper (6) provided evidence that $Ca^{2+}$ influx is not increased in the cells pretreated with TG. So why it is so effective? We propose the following working hypothesis. We immagin that in the normal cells all $Ca^{2+}$ influx is traped by SR. Janczewski and Lakatta (20) had provided recently evidence that this may be reall the case. $Ca^{2+}$ trapped by SR is stored till the next depolarization when it is released to activate contraction. Thereafter it is transported out of the cell by Na/Ca exchangers in the amount equal to the influx and in the same amount

trapped by SR (recent influx). In the cells treated with TG contractions seem to be activated directly by $Ca^{2+}$ influx which is not increased with respect to that in normal cells. There are some possible reasons why, despite of this, it is so effective. One of them may be that the $Ca^{2+}$ buffering capacity of the cell pretreated with TG is much less than that in the normal cell. This may result from the inhibition of SR $Ca^{2+}$-ATPase (no trapping) and increased saturation of other $Ca^{2+}$ binding sites. The other possible explanation of effectiveness of $Ca^{2+}$ influx in the cells treated with TG is some additional, yet not identified source of activator $Ca^{2+}$. The present knowledge of the cell biology points to the internal leaflet of sarcolemma. It has been shown that it contains the negatively charged phospholipids which may bind large percentage of the cellular $Ca^{2+}$ content (21—23). It is a metter of further experimentation whether this $Ca^{2+}$ could be released by $Ca^{2+}$ influx through the sarcolemmal $Ca^{2+}$ channels (a kind of additional $Ca^{2+}$-induced $Ca^{2+}$ release). If this is the case, this $Ca^{2+}$ could be also trapped by SR, released to activate contraction and re-bound by sarcolemma upon relaxation. In cells treated with TG sarcolemmal $Ca^{2+}$ together with $Ca^{2+}$ diffusing through the sarcolemmal $Ca^{2+}$ channels would directly activate contraction and be re-bound upon repolarisation. Similar mechanism has been proposed by Lulmann et al (24).

In conclusion, we propose that in guinea-pig cardiac myocytes poisoned with TG the direct source of $Ca^{2+}$ activating contraction is sarcolemmal $Ca^{2+}$ influx or, that there is an additional $Ca^{2+}$ source (internal leaflet of sarcolemma?) from which it could be released upon depolarisation.

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