Original articles

B. LEWARTOWSKI, K. EMANUEL AND G. A. LANGER *

DIBUCAINE DISPLACEABLE SARCOLEMMAL Ca^{2+} FRACTION IN GUINEA-PIG CARDIAC MYOCYTES

Department of Clinical Physiology, Medical Center of Postgraduate Education, Warsaw and * Cardiovascular Laboratories, School of Medicine, UCLA, Los Angeles.

In previous work we found that Ca^{2+} bound to the internal leaflet of sarcolemma of the neonatal cultured rat cardiomyocytes may be diasplaced by a local anesthetic, dibucaine (DBC). This resulted in inhibition of Na/Ca exchange. Now we found that the DBC displaceable Ca^{2+} fraction may be demonstrated also in single, enzymatically isolated cardiomyocytes of adult guinea pigs. Only cell length was used as an index of the free sarcoplasmic Ca^{2+} concentration since we found that DBC is fluorescent when irradiated with UV light, the wave length ovarlapping that of Indo 1 fluorescence. DBC (0.5 mM) induced a contracture whose amplitude ranged from 30%—100% of that of the electrically stimulated twitch. Blocking of Na/Ca exchange with 0.5 mM Ni^{2+}, or $0Na^+$, $0Ca^{2+}$ solution did not affect the amplitude nor the time course of the contracture. Caffeine (15 mM) superfused during exposure of the cell to DBC initiated contraction which relaxed despite continued caffeine superfusion. DBC also initiated contracture in cells pretreated with thapsigargin, a blocker of sarcoplasmic reticulum Ca^{2+} uptake. DBC did not intiate contracture in skinned myocytes and decreased their sensitivity to Ca^{2+}. These results suggest that DBC displaces Ca^{2+} from sarcolemma and that the displacement results in inhibition of Na/Ca exchange. Inhibition may, however, be overcome by high subsarcolemmal Ca^{2+} concentration induced by its SR release with caffeine. DBC rapidly blocked sodium and calcium curents in voltage clamped cells. Calcium curent recovered within about a minute upon washout of DBC, whereas recovery of sodium current started after about 8 min and was completed within about 12 min. Rapid depolarisation from a holding potential of -80 mV to 0 or $+20$ mV potentiated the response of the cell to DBC. This suggests that the Ca^{2+} fraction which is displaced by DBC is distinct from that released by depolarisation, and therefore does not contribute to activation of a normal, electrically stimulated contraction.

K e y   w o r d s: *cardiac myocytes, sarcolemma, calcium, Na/Ca exchange, dibucaine, calcium binding, diadic cleft.*

INTRODUCTION

The internal leaflet of sarcolemma contains a large amount of anionic Ca-binding sites, predominantly phospholipid in nature (1, 2). These binding sites remain in equilibrium with Ca^{2+} contained within the narrow space between the membranes of the terminal cisternae of sarcoplasmic reticulum

(SR) and the walls of the transverse tubules (T) or external sarcolemma (SR-SL clefts) (3). The content of Ca^{2+} within these clefts depends on Ca^{2+} influx by activated sarcolemmal Ca^{2+} channels and reversed Na/Ca exchange, on Ca^{2+} release from the SR calcium release channels, on outward Ca^{2+} transport by Na/Ca exchange working in the "Ca^{2+} out mode" and on Ca^{2+} diffusion into the bulk sarcoplasm (3). Calcium bound to the internal leaflet of sarcolemma and remaining in equilibrium with Ca^{2+} within the SR-SL cleft form a fraction, the properties of which may be investigated in the intact cultured neonatal rat cells by isotopic ^{45}Ca washout technique (4—6). It has been found that efflux of this fraction critically depends on the activity of the Na/Ca exchangers and that interventions which affect its Ca^{2+} content, affect the rate of the Na/Ca exchange (6, 7). Thus it seems that the subsarcolemmal/sarcolemmal Ca^{2+} fraction provides the direct source of Ca^{2+} for the Na/Ca exchangers working in the Ca^{2+} out mode. In this respect buffering of Ca^{2+} within the SR-SL cleft by phospholipid binding sites of the internal leaflet of sarcolemma would play an important role in the promotion outward Ca^{2+} transport by delaying the decay of Ca^{2+} concentration within the cleft after its rapid release from the SR in the stimulated cell (3) as well as after its slow release by the single ryanodine receptors between beats (7). This way the exchangers would be exposed to high Ca^{2+} concentration for a longer time enabling a high rate of operation despite their high K_m ~6 µM (8). In fact Lewartowski et al. (9) found that the rate of Na/Ca exchange reflected by the tail current is higher during the upstroke than during the later phases of the total Ca^{2+} transient whereas the bulk Ca^{2+} concentration is lower. This finding is consistent with the idea that the Na/Ca exchangers are exposed for at least the initial ~50 ms of the transient to a Ca^{2+} concentration much higher than that in the bulk sarcoplasm.

Recently it has been found that the Na/Ca exchange dependent Ca^{2+} fraction may by displaced from the sarcolemma isolated from cultured neonatal rat cells as well as from intact cells by the local anaesthetic dibucaine (10). The displacement led to inhibition of Na/Ca exchange. In this paper we report that the dibucaine displaceable Ca^{2+} fraction may be found also in venticular myocytes isolated from adult guinea-pigs and that its properties are similar to the properties of that found in neonatal rat cells. We also tested the hypothesis of Lulmann et al. (11) according to which the Ca^{2+} bound to the internal leaflet of sarcolemma may be released by cell depolarisation to contribute to the activation of contraction.

METHODS

Cell isolation and superfusion and recording of their length.

Guinea-pigs of both sexes weighing 300—350 g were anesthetised with an overdose of Nembutal. The chest was opened, the heart rapidly excised and perfused through the aorta by the

Langendorf method. Five min of perfusion with nominally Ca^{2+} free Tyrode solution (for composition of the solutions see below) was followed by 15 min of perfusion with Tyrode solution containing 20 mg of collagenase type B (Boehringer) and 3 mg of protease (Sigma). Thereafter the ventricles were agitated in the same enzyme containing solution, the cells filtered through the nylon mesh and allowed to sediment. The supernatant was discarded and the cells washed with Tyrode solution containing Ca^{2+} the concentration of which was gradually increased to 1.0 mM. The yield of living myocytes ranged from ~45% to 80%. About 0.5 ml of cell suspension forming a large drop was put on a coverslip glued to the margin of the round hole cut in the bottom of plastic Petri dish fixed on the stage of an inverted microscope (Diaphot, Nicon). When the cells bacame attached to the glass, the ends of the inlet and suction tubes of the modified rapid superfusion system originally designed by Rich *et al.* (12) were immersed in the drop. Thus the cells were positioned in the stream of the solution flowing between the inlet and suction tubes. Miniature magnetic valves allowed change of the composition of the superfusing solution within ~300 msec. Platinum wire electrodes immersed in the stream of superfusate enabled electrical stimulation of the cell. A TV camera mounted in the side port of the microscope and fitted to the edge tracking system designed and built by John Parker (Cardiovascular Laboratories, School of Medicine, UCLA) enabled recording of the cell length by the Gould TA chart recorder.

Skinned myocytes.

In 5 experiments isolated myocytes were twice washed with the relaxing solution (13) (for composition see below) and skinned by 5 min exposure to 0.05% Triton X 100 dissolved in the same relaxing solution. After washing, skinned cells were rapidly superfused by relaxing solution or relaxing solution containing 0.5 mM DBC as described above. Since they attached poorly to the glass and easily washed away we had to hold a chosen myocyte in place by pressing it lightly to the coverslip by a glass micropipette (diameter 2—3 μm). The effect of DBC on the response of skinned myocytes to elevated Ca^{2+} concentration was tested by bolus injections of small volumes (10—80 μl) of 10 mM Ca^{2+} dissolved in the relaxing solution into the superfusion lines. After reproducible contractions were obtained, superfusion solution was switched to that containing 0.5 mM dibucaine and the injections repeated.

Voltage clamping.

Cells superfused as described in the preceding paragraph were voltage clamped in the whole cell mode by the Axopatch-1D 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). The microelectrodes were drown from borosilicate glass tubing (World Precision Instruments, USA). When filled with the internal solution they had a resistance between 2.5 and 3.5 MΩ. The cell membrane under the electrode was disrupted by suction. Series resistance compensation was used to reduce the voltage error due to access resistance.

Solutions.

For cell isolation and throughout the experiments was used 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, and 5.0 sodium pyruvate. The pH of the solution was adjusted with NaOH to 7.3 for isolation of cells and to 7.4 for

experiments. In the experiments $CaCl_2$ was added to concentration of 3.0 mM. In the 0 Na^+ 0 Ca^{2+} solution these ions were replaced with equimolar Li^+. The patch pipettes were filled with the solution containing (in mM): 100.0 potassium aspartate, 35.0 KCl, 10.0 NaCl and 10.0 HEPES. The pH was adjusted to 7.2 with KOH. For preparation and superfusion of the skinned cells we used a relaxing solution (13) of the following composition (in mM): potassium methylsulfate (KMS) 56.4, ethylene glycol-bis (β-amino ethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) 10, $CaCl_2$, 0.02, $MgCl_2$ 6.9, MgATP 10, creatinphosphate (CP) 15, (N,N-bis[2-hydroxyethyl]-2-aminoethane-sulfonic acid (BES) 50, KOH 1.8, pH 7.2. All experiments were performed at 24°C.

Thapsigargin, dibucaine, and constituents of the superfusion solutions were purchased from Sigma.

RESULTS

When beginning these experiments we attempted to use the intracellular free $[Ca^{2+}]$ assessed by fluorescence of Indo-1 loaded to the cells as an index of displacement of sarcolemmal Ca^{2+} by dibucaine. Unfortunately, we found that dibucaine is fluorescent under the effect of the ultraviolet irradiation used for Indo-1 and that its wave overlaps that of Indo-1 fluorescence. Therefore we could not use our Indo-1 system in these experiments and the only index of displacement of Ca^{2+} by dibucaine was cell shortening.

The effect of dibucaine on resting cell length.

Cells were stimulated at the rate of 30/min. After steady state had been attained, stimulation was stopped and 5 sec later superfusate was switched to that containing 0.5 mM dibucaine. Electrically stimulated cells shortened by $11 \pm 3\%$ (mean $\pm$ SD, n = 48) of their resting length. Superfusion of dibucaine initiated a contracture reaching 3—11% of resting cell length (*Fig. 1*). In a few cases the contracture was preceded by a spontaneous phasic contraction (*Fig. 2*). The second superfusion of dibucaine (without electrical stimulation in between) resulted in a contracture of amplitude only slightly lower than that of the first (not shown). The cells were inexcitable for 15—10 s after dibucaine washout. When excitability recovered, the amplitude of contractions gradually increased and for about 60 s was larger than before DBC (not shown).

We tested the effect of inhibition of the Na/Ca exchange on the dibucaine stimulated contractures in 18 cells. For this purpose cells were superfused with normal Tyrode solution containing 5.0 mM Ni^{2+} (11 cells, Fig. 1) or by no Na^+, no Ca^{2+} solution (+100 μM EGTA) (7 cells, not shown) 5 sec prior to dibucaine which was also dissolved in one of these solutions. Inhibition of Na/Ca exchange had no consistent effect on cell contracture initiated by superfusion with dibucaine or on their relaxation upon its washout (*Fig. 1*).
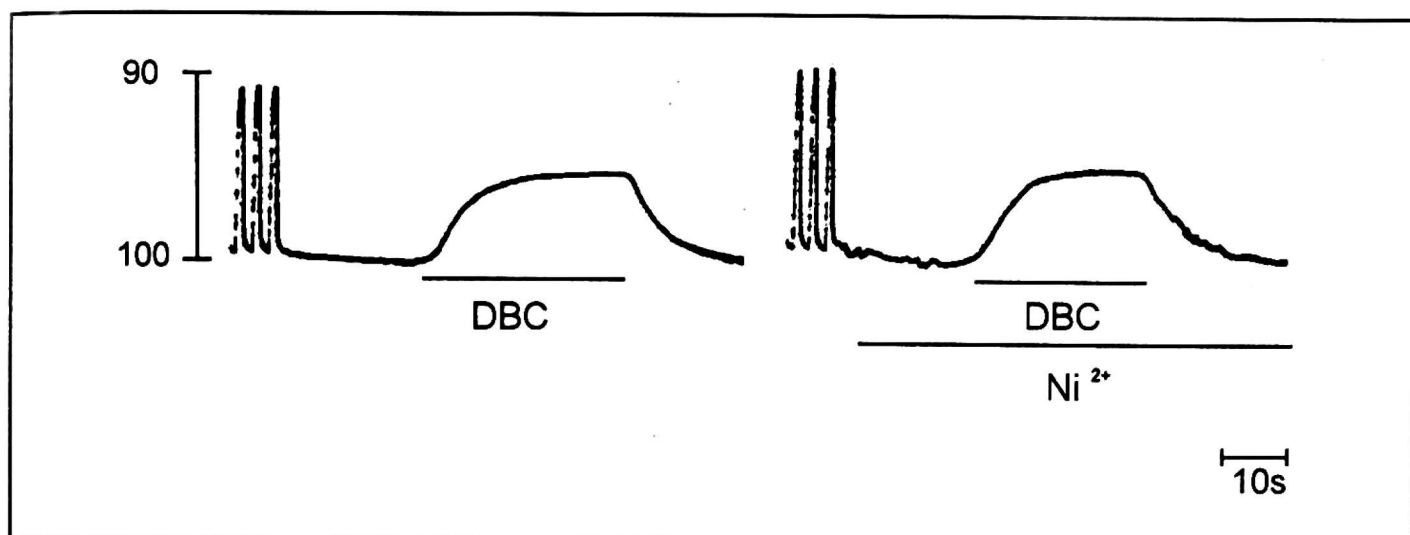
Fig. 1. Electrically stimulated contractions and contractile response to 0.5 mM dibucaine (DBC) of a single myocyte of guinea-pig heart. Right panel: 5.0 mM Ni^{2+} superfused prior to DBC. Left scale in this and following figures — % of the resting cell length.
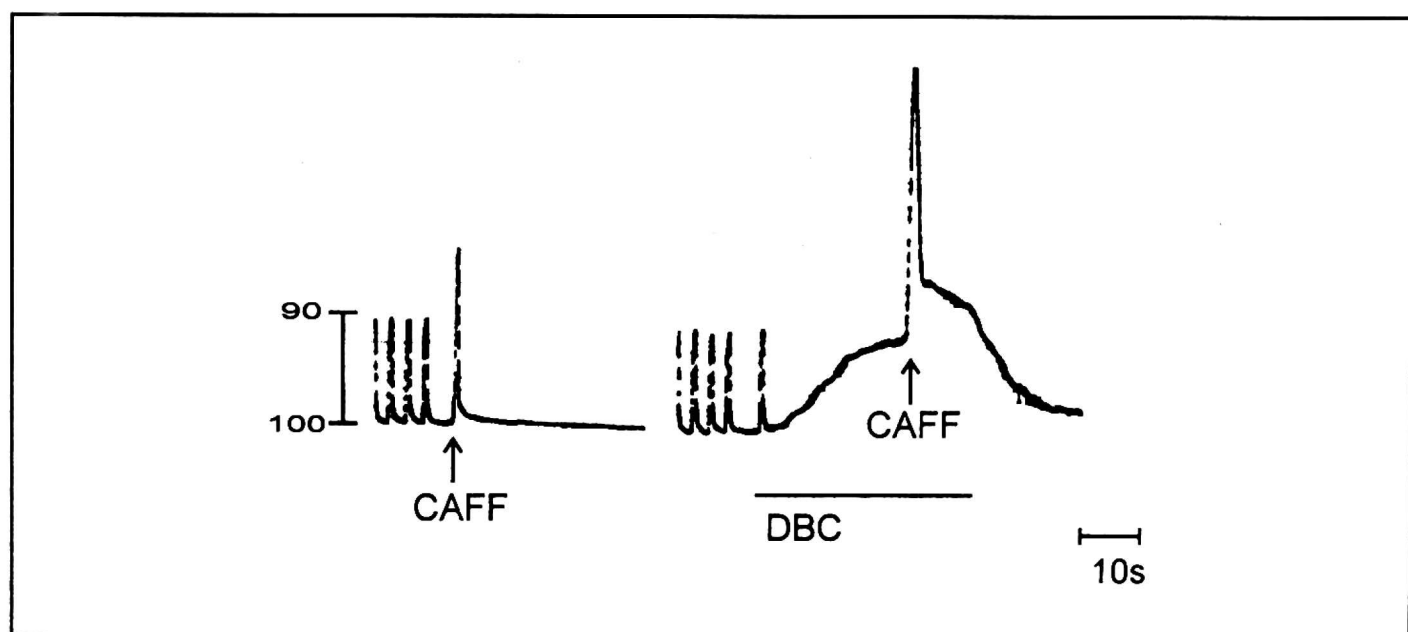

Fig. 2. Electrically stimulated contractions and contractile responses to 1 s superfusion of 15 mM caffeine of a single myocyte of guinea-pig heart. DBC — 0.5 mM dibucaine.

These results suggest that Ca^{2+} displaced by dibucaine does not leave the cell and is rebound by the same sites upon dibucaine washout.

Dibucaine does not displace Ca^{2+} from the SR.

The SR Ca^{2+} content was tested by 1 s superfusion of the Tyrode solution containing 15 mM caffeine. This resulted in a short contracture of the amplitude of $15 \pm 6\%$ of the resting cell length. The contractile response to caffeine of the cell superfused with dibucaine was greatly potentiated (*Fig. 2*). Thus dibucaine does not release SR Ca^{2+}.

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The above conclusion was supported in the experiments in which dibucaine was superfused over 6 cells pretreated for 30 min with 10^{-6} M thapsigargin (TG). These cells showed the slow kinetics of otherwise unaltered contraction (*Fig. 3*) and did not respond to caffeine superfusion (not shown). In these cells dibucaine produced contractures whose amplitude and time course did not differ from those in normal cells (*Fig. 3*). Also in these experiments the contractile response to dibucaine was not affected by superfusion of the cells with 5.0 mM Ni^{2+} (not shown).
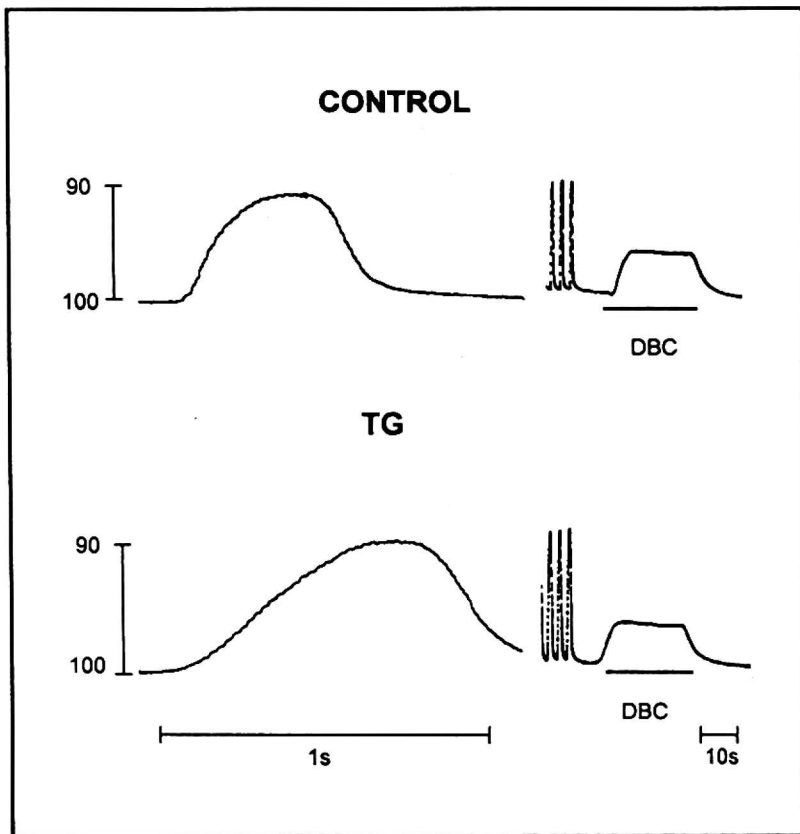

Fig. 3. Electrically stimulated contractions and responses to 0.5 mM dibucaine (DBC) of single myocyte of guinea-pig heart. TG: 10^{-3} M thapsigargin. Please, note the differences in time scale between the left and right panels.

The effect of dibucaine on Na/Ca exchange

Increase in the contractile response to caffeine by dibucaine may be due to the displacement of Ca^{2+} from an intracellular site to the SR or inhibition by dibucaine of Na/Ca exchange. The latter could be due to a direct effect on the exchangers or indirectly by displacement of that Ca^{2+} destined for Na/Ca exchange. These possibilities were tested in the experiments performed according to the following protocols published by Negretti *et al.* (14) and Bassani *et al.* (15). Relaxation from electrically stimulated contractions depends on reuptake of Ca^{2+} by the SR, on the outward Ca^{2+} transport by Na/Ca exchange and sarcolemmal Ca^{2+}-ATPase and on mitochondrial Ca^{2+} uptake. Long lasting superfusion with caffeine releases the SR Ca^{2+} and prevents its reuptake by the SR. Thus comparison of the rate of relaxation from electrically stimulated contraction and contraction initiated by long superfusion with

caffeine may help to assess the contribution of the SR to the former. Inhibition of Na/Ca exchange prior to and during caffeine superfusion indicates a major contribution of this exchange to relaxation from caffeine contraction which then depends on sarcolemmal Ca^{2+} ATPase and mitochondria. *Fig. 4A* shows electrically stimulated twitches, the response of a cell to 10 s caffeine superfusion and the response to 10 s caffeine superfusion of the same cell pretreated with no Na^+, no Ca^{2+} solution containing EGTA. We did not measure the rate constant of relaxation in this work since it was not necessary for our purposes. Neverthless it can be seen that inhibition of the reuptake of Ca^{2+} by the SR little affects the rate of relaxation which depends largely on the remaining relaxing mechanisms. However, inhibition of Na/Ca exchange markedly delays relaxation which shows that, in guinea-pig, it depends mostly on outward Ca^{2+} transport by the exchangers. *Fig. 4B* shows
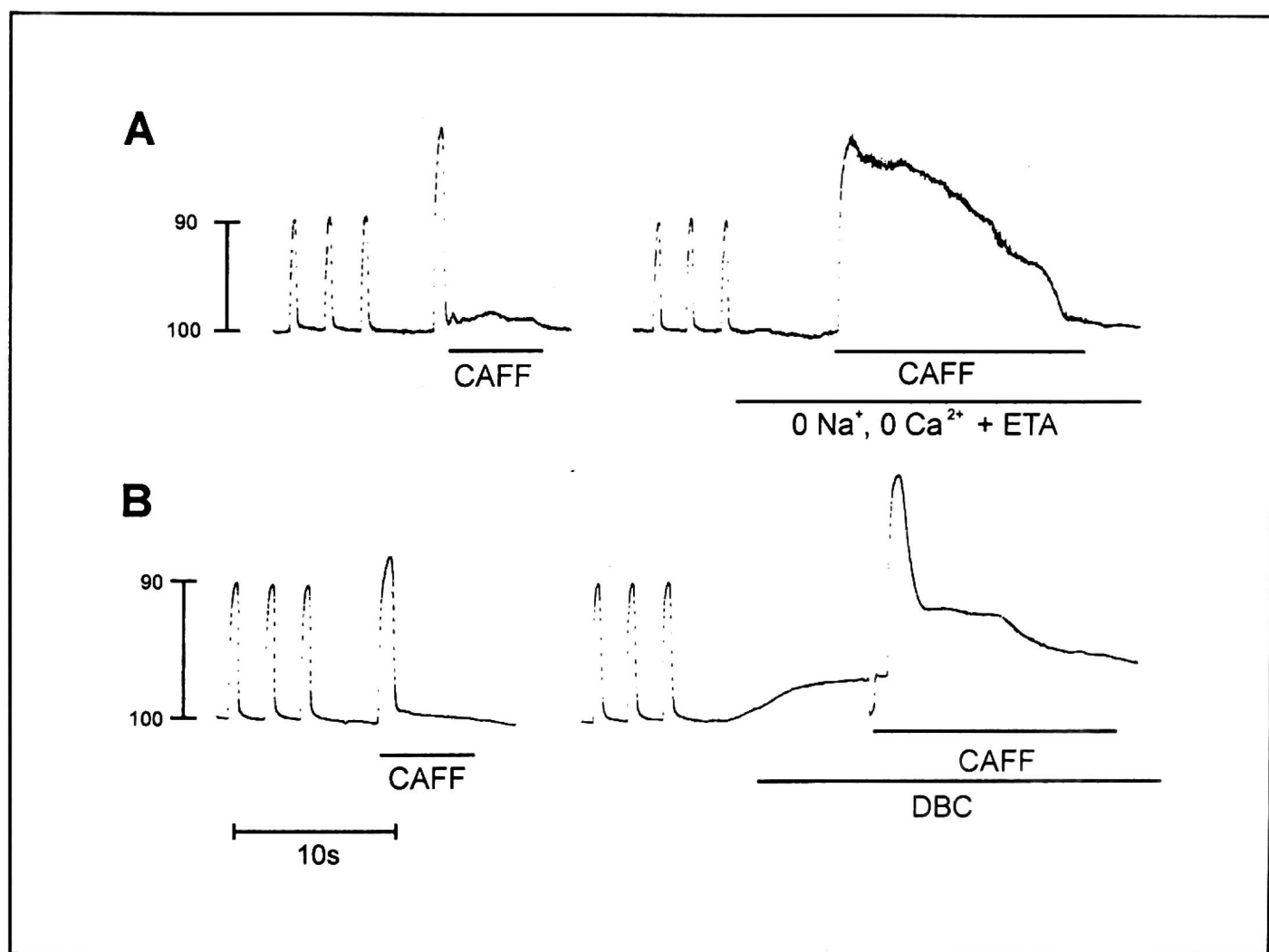

Fig. 4A, left panel: Electrically stimulated contractions and response to 10 s superfusion of 15.0 mM caffeine of a single myocyte of guinea-pig heart. A, right panel: same cell, response to caffeine superfused for 20 s during superfusion of 0 Na, $^+$0 Ca^{2+} + EGTA solution in order to inhibit Na/Ca exchange. B, left panel: like left panel in A. B, right panel: response of the same cell to caffeine superfused during 0.5 mM dibucaine (DBC) superfusion. Note the difference between the effects of inhibition of the Na/Ca exchange and dibucaine on cell relaxation from caffeine contraction (see text for further discussion).

again the electrically stimulated twitches, the reponse of the cell to 10 s superfusion with caffeine and the response to caffeine of the same cell pretreated with dibucaine. Dibucaine increased the amplitude of the response and somewhat delayed relaxation, however, the slowing was not comparable to that due to inhibition of the Na/Ca exchange shown in *Fig. 4A*. The cell did not relax to the initial length as long as caffeine + dibucaine was superfused. Similar results were obtained in 6 other cells.

Dibucaine does not affect the length of the skinned myocytes and decreases their sensitivity to Ca^{2+}.

Since we could use only the contractile response of cell as an index of expected Ca^{2+} displacement from sarcolemma by dibucaine, we had to check whether this compound has any direct effect on the contractile proteins or on their sensitivity to Ca^{2+}. To this end we performed experiments on myocytes skinned with Triton X-100.

Skinned myocytes superfused with the relaxing solution had normal shape, although they were more transparent then normal and their contours were less sharp. They showed striations, which were, however, less sharp than in normal cells. Dibucaine (0.5 mM) rapidly superfused over these myocytes did not affect their length (*Fig. 5*). The cells were very sensitive to small variations in the amount of injected Ca^{2+} solution so that apparently a small increase of $[Ca^{2+}]$ killed them very often (they became irreversibly rounded). Therefore it was very difficult to test responses to several concentrations of Ca^{2+} in one cell. Instead, we chose myocytes which repeatedly responded with a slight suprathreshold contractions (2 cells, *Fig. 5A*), with contractions of the amplitude of that of electrically stimulated twitches ($\sim 10\%$ of cell length, 2 cells, *Fig. 5B*) and very strong contractions reducing the cell length by $\sim 40\%$ (2 cells, *Fig. 5C*). Dibucaine increased the amount of Ca^{2+} necessary to produce the control contraction by 30—100% in 5 cells. In one cell in which a medium size contraction was produced no effect of DBC was observed. Sensitivity of cells to Ca^{2+} increased after DBC washout so that even the amounts of Ca^{2+} producing moderate contractions evoked irreversible maximal contracture (cells became rounded). The last effect explains the mechanism of increase in the electrically stimulated contractions of intact cells above control upon DBC washout. These results show that dibucaine did not cause sarcomeric contraction directly nor by increasing their sensitivity to Ca^{2+}.
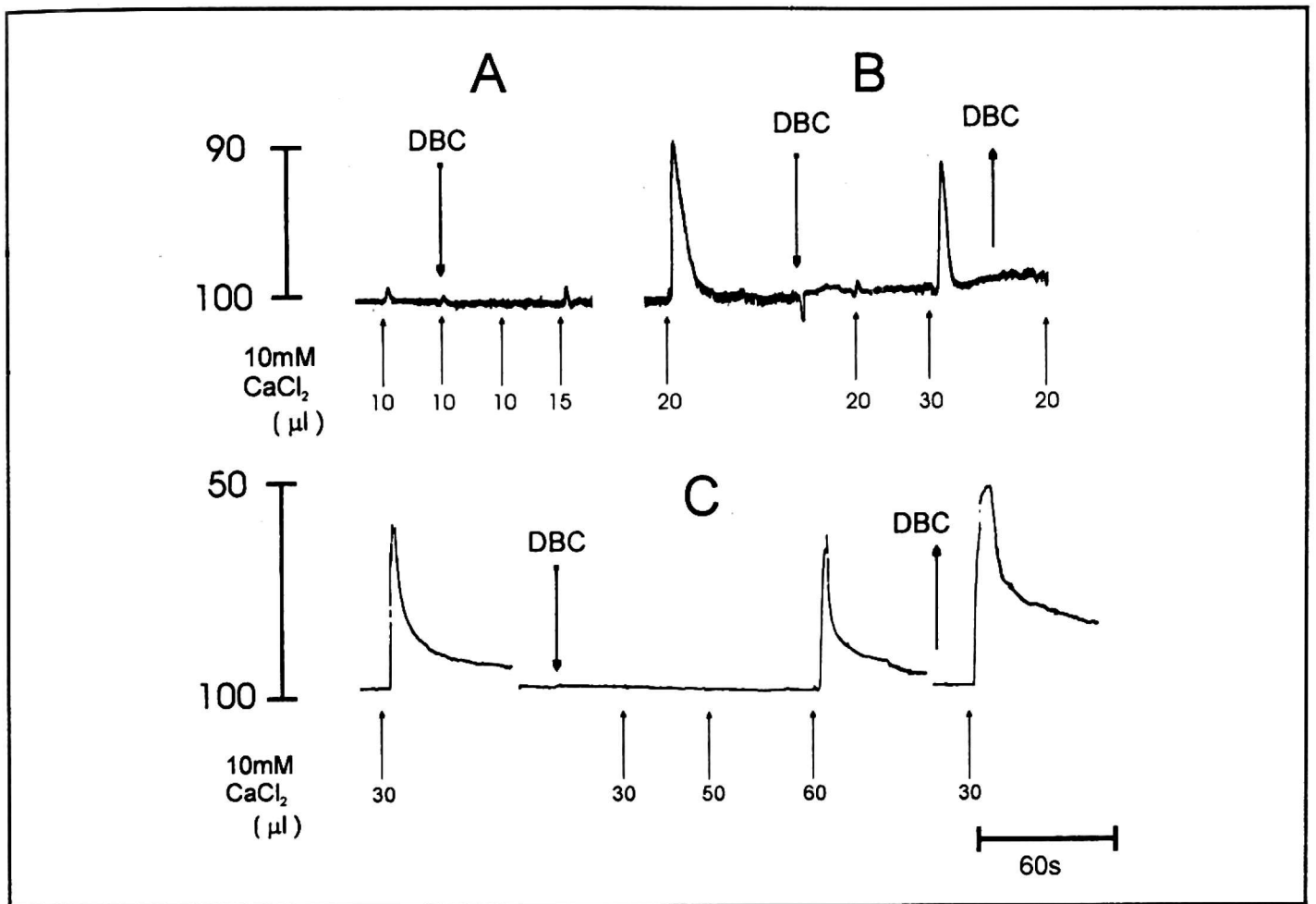
Fig. 5. The effect of 0.5 mM dibucaine (DBC) on length of the skinned myocytes and their responsiveness to bolus injections of 10—60 μl 10 mM Ca^{2+} dissolved in the relaxing solution into the superfusion lines. A: threshold contractile response; B: another cell — contraction amplitude comparable to that of electrically stimulated twitch in normal cell. C: another cell — nearly maximal contractile responses. Left scales: the diastolic cell length. In B 20 μl of Ca^{2+} solution killed the cell after DBC washout.

The effects of dibucaine in the voltage clamped cells.

Lulmann *et al.* (11) proposed that sarcolemmal-bound Ca^{2+} may be displaced by depolarisation to contribute to the activation of contraction. Therefore in the next experiments we tested whether the dibucaine displaceable fraction of Ca^{2+} may be displaced by depolarisation imposed on voltage clamped cells. If this is the case depolarisation to potentials close to the plateau of an action potential should initiate some contraction even if the Ca^{2+} channels and Na/Ca exchange are blocked. Also depolarisation should inhibit or attenuate the response to dibucaine. The experiments were performed in cells pretreated for 30 min with 10^{-6} M thapsigargin in order to avoid interference by Ca^{2+} released from the SR. Cells were stimulated by the pulses from a holding potential of −80mV to −40 mV (duration 20 ms) followed by steps to +5 mV (duration 200 ms) at the rate of 30/min. The prepulses activated and inactivated the Na^+ current (I_{Na}) whereas the steps to +5 mV activated the Ca^{2+} current (I_{Ca}) *(Fig. 7A)*.

First we tested the effect of long lasting depolarisation on the membrane currents and cell length. For this purpose stimulation was stopped and the holding potential was switched from -80 mV to 0 mV or to $+20$ mV. This resulted in a rapid strong phasic inward current followed by a much slower sustained outward current and a phasic contraction followed by a contracture sustained up to the end of depolarisation (*Fig. 6A*). When the same cell was pretreated with 5.0 mM Ni^{2+}, the outward current was much less, there was no phasic contraction and the sustained contracture was negligible (*Fig. 6B*). The difference of 51 ± 13 nA (n = 5) between the outward currents before and after the Ni^{2+} superfusion reflects the intensity of the reversed Na/Ca exchange in the depolarised cell. The phasic contraction was probably mostly initiated by I_{Ca} activated by depolarisation whereas the sustained contracture was mostly due to the Ca^{2+} influx by reversed Na/Ca exchange.
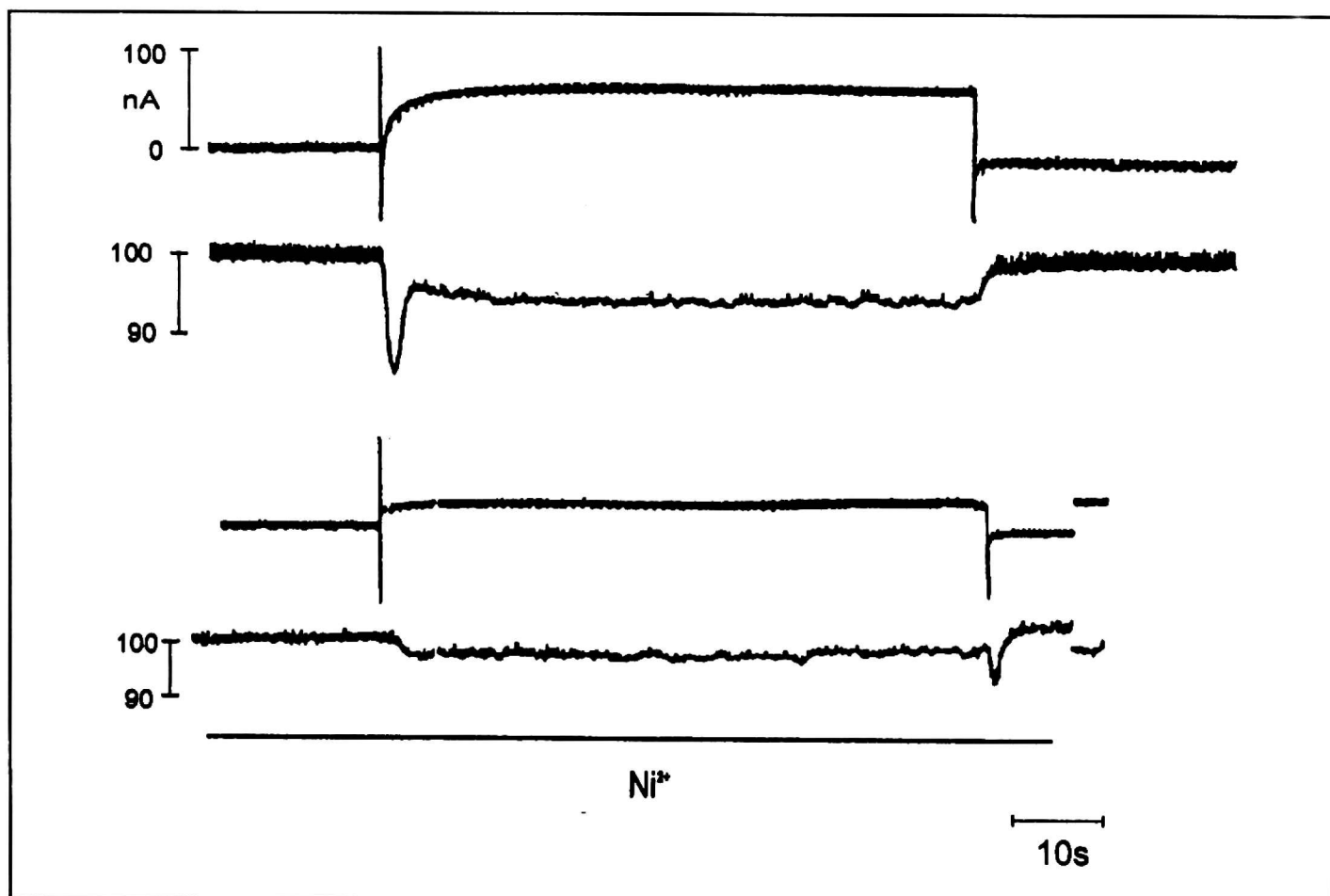

Fig. 6. Membrane currents (upper records) and cell length activated by switching of the holding potential from -80 mV to $+20$ mV. Inward currents off scale. A: control; B: same cell pretreated with 5.0 mM Ni^{2+}.

Dibucaine superfused over the stimulated cells blocked both the I_{Na} and I_{Ca} within a few seconds. Upon washout I_{Ca} recovered completely within about 1 min, however, I_{Na} started to reappear about 60 s later (*Fig. 7A, B*). In some cells its recovery took as long as ~ 180 sec. Thus there was a time window upon washout in which dibucaine selectively blocked I_{Na}.
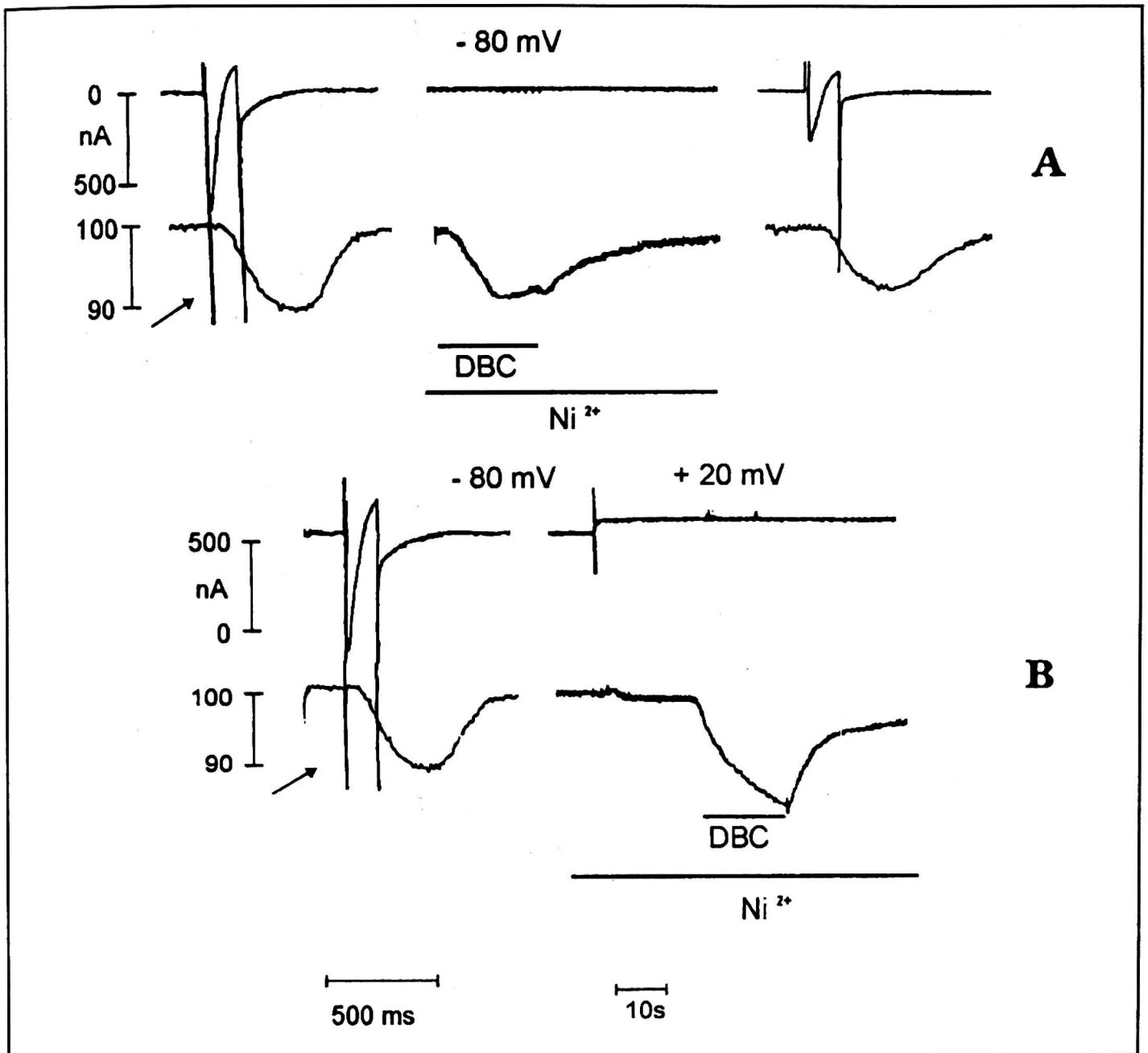
Fig. 7. Membrane currents (upper records) and the length of a single myocyte of guinea-pig heart pretreated with thapsigargin. A, left panel: cell pulsed at the rate of 30/min by prepulses from -80 mV to -40 mV (20 ms) followed by pulses to $+5$ mV (for 200 ms). I_{Na} (arrow, out of scale) followed by I_{Ca}. A, middle panel: stimulation stopped for 60 s and cell clamped at -80 mV superfused with 5.0 mM Ni^{2+} and 0.5 mM dibucaine (DBC). A, right panel: partial recovery of I_{Ca} upon washout of DBC, I_{Na} still blocked. B, left panel: same cell, complete recovery after 3 min washout of DBC. B, right panel: stimulation stopped and the holding potential switched from -80 mV to $+20$ mV. Please, notice the difference in contractile response to DBC of the cell clamped at -80 mV and the same cell clamped to $+20$ mV.

Dibucaine superfused over the resting cell clamped at ~ 80 mV and pretreated with Ni^{2+} initiated contracture which did not differ in amplitude and time course from that in the unclamped cells (*Fig. 7A*). Despite apparent elevation in the sarcoplasmic $[Ca^{2+}]$ we never observed any change in the membrane current during dibucaine superfusion in cells in which Na/Ca exchange had not been inhibited (not shown). This is in accordance with the

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above results which suggested that Na/Ca exchange is not active during dibucaine superfusion.

In the next experiments we compared the effect of dibucaine in 6 cells superfused with Ni^{2+}, clamped at -80 mV with that in the same cells a few seconds after the holding potential had been switched to 0 or to $+20$ mV. In all depolarised cells the response was at least doubled with respect to the polarised cells (*Fig. 7A, B*).

DISCUSSION

In this work we found that a sarcolemmal, dibucaine displaceable Ca^{2+} fraction demonstrated previously in the neaonatal cultured rat cells (10) is present also in the myocytes of adult guinea-pig heart. Its displacement results in inhibition of Na/Ca exchange. Reversibility and reproducibility of the cells responses to dibucaine permitted us investigation of its properties. Unfortunately, fluoresence of dibucaine illuminated by ultrafiolet light precluded use of our Indo 1 system and forced us to rely on cell length as an indirect index of displacement of sarcolemmal Ca^{2+} by dibucaine. Dibucaine, when superfused over previously stimulated cells induced sustained contracture of amplitude ranging from $\sim 30 - \sim 100\%$ of that of an electrically stimulated twitch. Cells promptly relaxed upon dibucaine washout. The Ca^{2+} activating this contracture was not released from the SR since caffeine superfused during the dibucaine superfusion still elicited a strong phasic contraction and the response to dibucaine was not inhibited by TG, a blocker of SR Ca^{2+} uptake (16—19). This results is consistent with that of Wang *et al.* (10) who also found that caffeine stimulated strong contraction in neonatal rat myocytes pretreated with dibucaine. In addition this contracture also appeared in the absence of extracellular Ca^{2+}.

In the present experiments dibucaine did not contract cells skinned with Triton X-100 although they responded with contraction to a transient increase in Ca^{2+} concentration. This result shows that contractures produced in normal cells by dibucaine did not result from its direct effect on the contractile proteins and that the sarcolemma was its primary target. Moreover, dibucaine attenuated the contractile response of the skinned cells to increased Ca^{2+} concentration. These results taken together with the results of experiments with caffeine, suggest that contractures produced by dibucaine in normal cells were initiated by increase in sarcoplasmic Ca^{2+} concentration due to its displacement from sarcolemma.

The response of cells to a second dibucaine superfusion without any intervening stimulation, was only slightly less than the initial response. Moreover, preperfusion of cells with the Na^+ and Ca^{2+} deficient solution or by normal Tyrode solution containing 5.0 mM Ni^{2+} neither affected the

contractile response of cells to dibucaine nor their relaxation upon its washout. Both interventions inhibit Na/Ca exchange (20), which is the main route of Ca^{2+} extrusion in cardiac myocytes. These results suggest that most of the Ca^{2+} displaced by dibucaine does not leave the cell but cycles between sarcolemmal binding and the cytosol. These results also suggest that Na/Ca exchange is inhibited during dibucaine superfusion despite apparently elevated sarcoplasmic Ca^{2+} concentration. These findings are consistent with those of Wang et al. (10) who also found that displacement of sarcolemmal Ca^{2+} by dibucaine inhibits Na/Ca exchange. They proposed that the dibucaine effect was due to its inhibition of inner sarcolemmal Ca^{2+} binding leading to more rapid diffusion of Ca^{2+} from the diadic clefts. Such rapid loss from the cleft spaces is predicted to markedly diminish Ca^{2+} efflux via Na/Ca exchange (3). However, dibucaine which, as shown in the present study, strongly inhibits the sarcolemmal ionic channels could also directly inhibit the Na/Ca exchangers. This possibility was tested in the experiments in which cells were exposed to long superfusion with caffeine. Dibucaine only slightly delayed relaxation from the caffeine contracture which, in the continued presence of caffeine in the superfusing solution, depends mostly on Na/Ca exchange (14, 15) whereas inhibition of the exchange by the 0 Na^+, 0 Ca^{2+} solution delayed the relaxation many fold (Fig. 4). Thus the inhibition of exchange by dibucaine was negligible when the sarcoplasmic Ca^{2+} concentration was increased by caffeine whereas the exchange was inhibited when cytosolic Ca^{2+} was increased following its displacement from the sarcolemma. We propose the following explanation for this difference. Free Ca^{2+} concentration within the diadic clefts remains in equilibrium with Ca^{2+} bound to the internal leaflet of sarcolemma. This results in slow drop of Ca^{2+} within the cleft after its release from the SR Ca^{2+} channels. Displacement of Ca^{2+} from sarcolemma and prevention of its binding by dibucaine results in rapid drop of its concentration within the cleft (3). Both processes cause that Ca^{2+} concentration as sensed by the Na/Ca exchangers, which are mostly localised to the transverse tubules (21), to rapidly fall to levels far below their K_D. This results in inhibition of Na/Ca exchange despite the elevated bulk sarcoplasmic Ca^{2+} concentration. Caffeine releases large amounts of Ca^{2+} into the clefts and transiently increases the subsarcolemmal Ca^{2+} concentration. This stimulates Na/Ca exchange as shown by Wang et al. (10) which results in prompt relaxation. However, the cleft Ca^{2+} concentration does decline and the exchangers are again exposed to low levels of Ca^{2+} concentration which inhibits its further efflux with consequent inhibition of relaxation at a level higher than before caffeine superfusion as shown in Fig. 4. Thus stimulation of Na/Ca exchange by high subsarcolemmal Ca^{2+} concentration in the presence of dibucaine (Fig. 4B) shows that its inactivation did not result from the direct inhibitory effect of dibucaine on the exchangers as already pointed out by Wang et al. (10).

The above results are consistent with the hypothesis according to which inner sarcolemmal leaflet Ca^{2+} binding with its effect on subsarcolemmal Ca^{2+} diffusion plays a significant role in the facilitation of Na/Ca exchange (3, 10). However, theoretically, sarcolemmal bound Ca^{2+} might play another important physiological role. According to Lullmann's *et al.* (11) hypothesis Ca^{2+} bound to the internal leaflet of sarcolemma may be released by depolarisation of the cell, due to increase in subsarcolemmal proton concentration. In this way the sarcolemma would provide an additional source of Ca^{2+} activating contraction. Therefore we tested whether the dibucaine displaceable Ca^{2+} may be also displaced by depolarisation. If so depolarisation should produce some contraction independent of activation of sarcolemmal Ca^{2+} channels or reversed Na/Ca exchange, and the response to dibucaine in the already depolarised cell should be decreased or blocked. In voltage clamped cells, in which Ca^{2+} channels and Na/Ca exchange were blocked by 5.0 mM Ni^{2+}, rapid switching of the holding potential from -80 mV to 0 mV or $+20$ mV did not produce significant contraction. The contractile response to dibucaine was in all cells increased. The reason of the increase is not clear. The Ca^{2+} activating the contracture could not be released from the SR since the experiments were performed in the cells pretreated with TG. Disregarding the mechanism of increase, these experiments show that previous depolarisation does not deplete the sarcolemmal bound Ca^{2+} which may be then displaced by dibucaine. Thus the dibucaine displaceable Ca^{2+} fraction is not released upon depolarisation and therefore it cannot be an additional source of Ca^{2+} activating contraction.

In conclusion, we found that in guinea pig ventricular myocytes dibucaine displaces calcium ions most probably from the inner leaflet of sarcolemma and that also in these cells the displacement results in inhibition of Na/Ca exchange.

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