Measurements of Ca^{2+} Entry and Sarcoplasmic Reticulum Ca^{2+} Content During the Cardiac Cycle in Guinea Pig and Rat Ventricular Myocytes

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ABSTRACT This study investigates the contribution of Ca^{2+} entry via sarcolemmal (SL) Ca^{2+} channels to the Ca^{2+} transient and its relationship with sarcoplasmic reticulum (SR) Ca^{2+} content during steady-state contraction in guinea pig and rat ventricular myocytes. The action potential clamp technique was used to obtain physiologically relevant changes in membrane potential. A method is shown that allows calculation of Ca^{2+} entry through the SL Ca^{2+} channels by measuring Cd^{2+}-sensitive current during the whole cardiac cycle. SR Ca^{2+} content was calculated from caffeine-induced transient inward current. In guinea pig cardiac myocytes stimulated at 0.5 Hz and 0.2 Hz, Ca^{2+} entry through SL Ca^{2+} channels during a cardiac cycle was ~30% and ~50%, respectively, of the SR Ca^{2+} content. In rat myocytes Ca^{2+} entry via SL Ca^{2+} channels at 0.5 Hz was approximately 3.5% of the SR Ca^{2+} content. In the presence of 500 nM thapsigargin Ca^{2+} entry via SL Ca^{2+} channels in guinea pig cardiac cells was 39% greater than in controls, suggesting a larger contribution of this mechanism to the Ca^{2+} transient when the SR is depleted of Ca^{2+}. These results provide quantitative support to the understanding of the relationship between Ca^{2+} entry and the SR Ca^{2+} content and may help to explain differences in the Ca^{2+} handling observed in different species.

INTRODUCTION

Ca^{2+} released from the sarcoplasmic reticulum (SR) is the major contributor to the transient increase in cytoplasmic Ca^{2+} (Ca^{2+} transient) that is responsible for contraction in mammalian cardiac muscle. Several studies have investigated the role of SR Ca^{2+} on the Ca^{2+} transient under physiological conditions (e.g., Beuckelmann and Wier, 1988; Bassani et al., 1993; Terracciano et al., 1995; Janczewski et al., 1995). The contributions of Ca^{2+} fluxes across the sarcolemma to the Ca^{2+} transient and their relationship with SR Ca^{2+} release have also been widely investigated (e.g., Sutko and Wilkerson, 1980; Bers, 1985; Janczewski and Lakatta, 1993; Negretti et al., 1995; Bassani et al., 1993; Delbridge et al., 1996), but many aspects remain unclear.

Although the ability of Ca^{2+} entering the cell to trigger SR Ca^{2+} release has been clearly demonstrated (Fabiato, 1983), the quantitative contribution made by such Ca^{2+} entry to the Ca^{2+} transient is still under investigation. In studies performed in rabbit and guinea pig cardiac myocytes it has been shown that when the SR is depleted after application of thapsigargin, Ca^{2+} entry through the sarcolemma is still able to support contraction, and under certain conditions such Ca^{2+} entry can be larger than under control conditions (Bassani et al., 1993; Janczewski and Lakatta, 1993). It is clear that a large amount of Ca^{2+} must cross the sarcolemma to support the large contraction observed when the SR is depleted of Ca^{2+}.

Two major routes are likely to be involved in Ca^{2+} entry via the sarcolemma, the sarcolemmal (SL) Ca^{2+} channels and Na^+/Ca^{2+} exchange. Quantitative aspects of Ca^{2+} entry via the SL Ca^{2+} channels during the action potential have been studied with slightly different approaches using action potential clamp techniques (Arreola et al., 1991; Yuan et al., 1996; Grantham and Cannell, 1996). These studies provide useful information on Ca^{2+} flux during physiological changes of membrane voltage. However, one difficulty the authors encountered was the possibility of changes in the SR Ca^{2+} content and in the kinetics of the Na^+/Ca^{2+} exchanger under their experimental conditions. Because these two conditions are extremely important in determining the kinetics and magnitude of the Ca^{2+} transient, and therefore the kinetics of the SL Ca^{2+} channels, difficulties arise in making precise quantitative measurements. In this work we describe a method that allows measurements to be made of the SL Ca^{2+} entry during steady-state contraction and under conditions where the SR Ca^{2+} content and the Na^+/Ca^{2+} exchange are not affected.

The aims of the experiments reported here were 1) to study Ca^{2+} entry through SL Ca^{2+} channels during physiological stimulation (i.e., the cardiac action potential) in guinea pig and rat ventricular myocytes at steady state and without affecting the Na^+/Ca^{2+} exchange contribution or the SR Ca^{2+} content; 2) to assess whether SL Ca^{2+} influx could directly contribute a significant proportion of the Ca^{2+} transient; and 3) to investigate the role of SR Ca^{2+} in regulating this Ca^{2+} entry. The experiments were performed using the action potential clamp technique that allows measurements of currents during physiological changes of membrane potential (Doerr et al., 1990). In addition, the switch-clamp technique was used with high-

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resistance microelectrodes to minimize cell dialysis and avoid large changes in intracellular conditions.

MATERIALS AND METHODS

Cell isolation

The method used for cell isolation has been described in detail elsewhere (Terracciano and MacLeod, 1994; Terracciano et al., 1995). Guinea pig ventricular myocytes were isolated using protease (4 U/ml; Worthington Biochemical Co., Freehold, NJ), then collagenase (0.3 mg/ml; Worthington Biochemical Co.), and hyaluronidase (0.6 mg/ml; Sigma Chemical Co., Poole, England) digestion. Rat myocytes were isolated using only collagenase (1 mg/ml) and hyaluronidase (0.6 mg/ml). After isolation the cells were stored in Dulbecco's minimum essential medium buffered with 25 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES) (Gibco, Uxbridge, Middlesex, England) at room temperature. The experiments were performed within 6–7 h of cell isolation.

Experimental protocol and control experiments

Methods for recording cell shortening and cytoplasmic Ca^{2+} have been described by Terracciano et al. (1995).

Rapid changes in the superfusate were achieved using miniature solenoid valves (Lee Company, Essex, England). These were controlled either manually or by computer. A pipette of 0.2 mm internal diameter placed approximately 1 mm upstream was used to direct the rapidly applied solution onto the cell under investigation.

Current clamp and voltage clamp experiments were performed using an Axoclamp-2B system (Axon Instruments, Foster City, CA). Cells were impaled with borosilicate glass microelectrodes (Clark Electromedical Instruments, Reading, England) with resistances between 20 and 30 MΩ. The microelectrode filling solution contained: KCl 2 M; EGTA 0.1 mM; HEPES 5 mM (pH 7.2). The switching frequency used during voltage clamp was 4–7 kHz. Voltage command, data acquisition, and analysis were performed using pCLAMP6 software (6.0.3; Axon Instruments). The signals, filtered at 100 Hz, were simultaneously recorded on a computer at a digitization rate of 800 Hz and on tape. Unless otherwise specified, data are expressed as mean ± SEM. Significance between means was calculated using Student's *t*-test.

Cells in current clamp were stimulated at 0.5 or at 0.2 Hz with a 1.0-nA pulse of depolarizing current of 10 ms duration. When contraction had reached a steady state, an action potential was recorded for each cell and used as the voltage command in the action potential clamp experiments (at the same rate of stimulation). Cell capacitance was measured by integrating the membrane current obtained by hyperpolarizing steps of 10 mV from a holding potential of −75 mV.

Cd^{2+} (2 mM) was used to block the Ca^{2+} current. This was rapidly applied to the cell between action potentials. One possible problem is that its rapid application only results in an incomplete block of the Ca^{2+} current and this will result in a partial release of Ca^{2+} from the SR. To be sure that this concentration of Cd^{2+} blocked the Ca^{2+} current completely, its effect was tested on each cell with the following protocol. The cell was voltage-clamped at −40 mV, and a train of depolarizing steps to 0 mV and of 300 ms duration was applied at 0.5 Hz. After 15 steps, Cd^{2+} was rapidly applied 500 ms after repolarization of the previous step. The inward current produced by depolarization at the next step was totally abolished, suggesting a complete block of the Ca^{2+} channels in the interval between the two steps. Cells that developed an incomplete block at the next step were discarded.

Cd^{2+}-sensitive currents were obtained by subtraction of the current produced by the first action potential voltage clamp configuration after Cd^{2+} application from the current produced by the action potential immediately preceding Cd^{2+} application. The zero value obtained by this subtraction was used as the baseline for the integration of the Cd^{2+}-sensitive current. Subtraction and integration were performed by pClamp6 software (Axon Instruments).

During the action potential clamp, the continuous change in voltage will produce a changing capacitive current during the action potential. In addition, a leak current of varying amount will also be expected to flow. Because our measurements of Ca^{2+} entry via the SL Ca^{2+} current are obtained by subtraction, any component present before and after Cd^{2+} application is eliminated. Evidence suggesting that the leak current does not change in the presence of Cd^{2+} has been documented by Yuan et al. (1996). Their measurements of leak current in the presence of 1 mM Cd^{2+} were comparable to those using *P/N* leak subtraction. We have no reason to believe that the capacitive current would change in the presence of Cd^{2+}. In summary, although the capacitive and leak currents would obviously show in the records during the action potential clamp, they would be eliminated by the subtraction carried out to determine the Cd^{2+}-sensitive current.

Other conductances that are likely to interfere with the subtraction of Cd^{2+}-sensitive current are those that could be altered by changes in Ca^{2+}. To avoid the possible influence of the Ca^{2+}-sensitive transient outward current carried by Cl^- ions (Kawano et al., 1995), 10 μM niflumic acid (O'Rourke et al., 1996) was continuously present in the superfusing solution. At higher concentrations, niflumic acid has been shown to partially inhibit the L-type Ca^{2+} channels (Sorota, 1994). Experiments were performed to investigate the effects of 10 μM niflumic acid on the Ca^{2+} current. The amplitude of the inward current elicited by step pulses from −40 to 0 mV was not significantly different in the presence or absence of 10 μM niflumic acid (control current amplitude: 6.51 ± 1.2 pA/pF; current amplitude in niflumic acid: 6.57 ± 0.8 pA/pF; $n = 7$). Double exponentials fitted to the inactivation phase of the current were also not affected (τ_1 in control: 123 ± 34 ms; τ_1 in niflumic acid 131 ± 39 ms; τ_2 in control: 11.88 ± 1.9 ms; τ_2 in niflumic acid 11.85 ± 1.9 ms; $n = 7$). No significant differences were found, either in the *I-V* relationship (over the range from −50 to +50 mV) or in the reactivation of the channel measured by depolarizing steps from −40 mV to 0 mV followed by identical steps between 100 ms and 1300 ms later (increments of 200 ms). From these experiments, we conclude that 10 μM niflumic acid has no significant effects on the properties of the SL Ca^{2+} current.

Other Ca^{2+}-activated conductances (Colquhoun et al., 1981; Ehara et al., 1988) could be indirectly affected by Cd^{2+}, but previous experiments where Ca^{2+} was elevated by application of caffeine suggest that in guinea pig cardiac myocytes their importance is minimal (Terracciano and MacLeod, 1994). It is clear that the main Ca^{2+} activated current that will be of functional significance in our experiments will be the transient inward current ascribed to the electrogenic Na^+/Ca^{2+} exchange.

Our assumptions regarding the Cd^{2+}-sensitive current are: 1) During steady-state contraction the same amount of Ca^{2+} entering the cell via outwardly directed Na^+/Ca^{2+} exchanger is extruded by the Na^+/Ca^{2+} exchanger in inward mode. The net effect of this Ca^{2+} movement on the integral of total current measured during the whole cardiac cycle is zero. 2) The inwardly directed Na^+/Ca^{2+} exchange current will contribute to the total integral of the Cd^{2+} subtracted current. 3) Two-thirds of the total integral of the Cd^{2+}-sensitive current is due to Ca^{2+} entry through the sarcolemmal Ca^{2+} channels and one-third to the inward current produced by Na^+/Ca^{2+} exchange in removing this current (Bridge et al., 1990).

From these assumptions it should be emphasized that although the technique allows quantification of Ca^{2+} entry through sarcolemmal Ca^{2+} channels, it does not provide information on the kinetics of this entry.

Solutions

The NT solution contained (in mM): NaCl, 140; KCl, 6; $MgCl_2$, 1; $CaCl_2$, 2; glucose, 10; HEPES, 10 (pH 7.40 ± 0.01). All of the experiments were performed at room temperature (22°C). Niflumic acid was prepared as a 100 mM stock solution in ethanol; thapsigargin was prepared as 1 mM stock solution in dimethyl sulfoxide; nifedipine as 10 mM in dimethyl sulfoxide. Cd^{2+} and caffeine were added as solids to the required solutions. All of the superfusing solutions were prepared

the day of the experiment and kept dark to avoid possible degradation of the compounds.

RESULTS

Fig. 1 shows the effects of the rapid application of 2 mM Cd^{2+} on the membrane current and indo-1 fluorescence elicited by an action potential voltage command of the profile shown in the top trace in Fig. 1 *A*. Because the action potential used as the voltage command was the one recorded from the cell under investigation, one would not expect any compensation current to be required (Doerr et al., 1990). Nevertheless, compensation current could be measured during the action potential (Fig. 1 *A*, *middle trace*). There are several possible reasons for this happening. First, the action potential used was recorded before the application of niflumic acid, whereas the action potential clamp traces are always recorded at least 3 min after starting the perfusion with niflumic acid. Because niflumic acid blocks Ca^{2+}-activated Cl^- channels, it produces changes in action potential shape. Therefore the action potential used as the voltage command is not identical to the one the cell would produce at the time of the action potential clamp recording. Second, as clearly pointed out by Yuan et al. (1996), during action potential clamp there is a continuous change in command potential that will produce capacitive current and changes in leak current. These, together with the niflumic acid-induced current, may be responsible for the current observed. As mentioned in Materials and Methods, there is evidence that the leak current is not affected by Cd^{2+} application (Yuan et al., 1996), and no changes in the capacitative current are expected if the same voltage profiles are used. Consequently, because all of the measurements performed below are obtained by subtracting the compensation current evoked in the presence of Cd^{2+} from that evoked in the absence of Cd^{2+}, all of the non-Cd^{2+}-sensitive components are eliminated and should not affect our calculations.

In the presence of Cd^{2+} (Fig. 1 *A*, *lower traces*), the membrane current was modified and no indo-1 fluorescence changes were detectable. These experiments support the hypothesis that in the presence of 2 mM Cd^{2+}, Ca^{2+} entry through the sarcolemma, via either SL Ca^{2+} channels or Na^+/Ca^{2+} exchange, is inhibited. Consequently, no Ca^{2+} release from the SR is elicited and the SR Ca^{2+} content is not expected to be greatly changed compared with the previous action potential.

Fig. 1 *B* shows the effects of an action potential voltage command on the cell shortening, residual membrane current

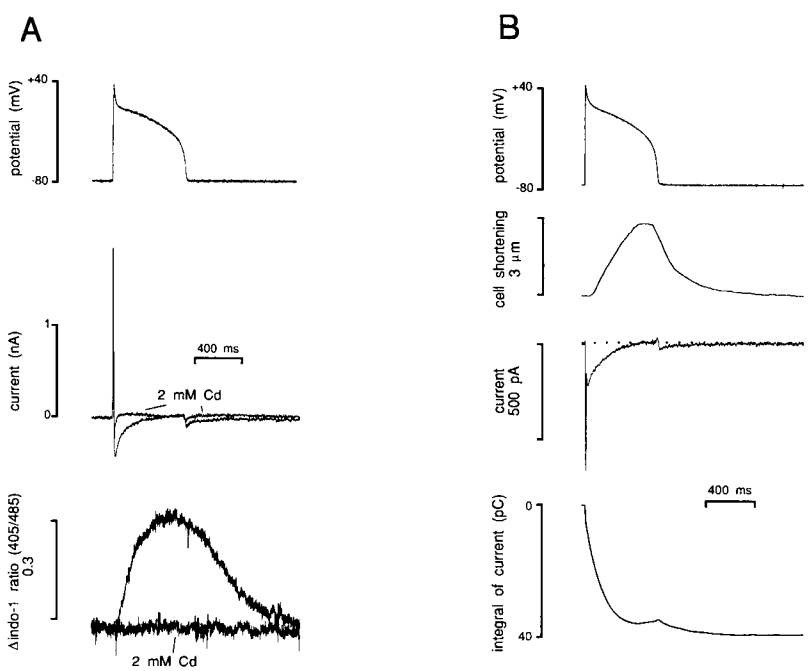

FIGURE 1 (*A*) The effects of rapid application of 2 mM Cd^{2+} on membrane current and indo-1 fluorescence during action potential clamp in a guinea pig ventricular myocyte. The cell was stimulated at 0.5 Hz, and when the contractions had reached a steady state, Cd^{2+} was applied 800 ms after the beginning of the previous action potential. The inward components of the current recorded during the action potential and after repolarization were both reduced (*middle trace*). After application of Cd^{2+} the transient increase in indo-1 fluorescence was abolished (*bottom trace*). (*B*) The Cd^{2+}-sensitive current recorded in another guinea pig myocyte during action potential clamp. The experimental conditions were similar to the experiment described in *A*, except that the cell was not loaded with indo-1. The second trace from the top shows cell shortening before the application of 2 mM Cd^{2+}. During Cd^{2+} no shortening of the cell was detectable. The Cd^{2+}-sensitive current (*third trace from top*) was calculated by subtracting the current obtained during Cd^{2+} application from the one elicited before Cd^{2+} application. The bottom trace shows the integral of the current. The dotted line represents zero level of current. This current, measured during the whole cardiac cycle (from the beginning of one action potential to the same point at the next), can be used to calculate Ca^{2+} entry via SL Ca^{2+} channels.

(i.e., after subtraction of the non-Cd^{2+}-sensitive current) and its integral. In this and in all other experiments described, indo-1 fluorescence was not measured, to avoid the buffering effect of the indicator on cytoplasmic Ca^{2+}. We emphasize that the third trace from the top shows the result obtained by subtraction of the total current evoked between the upstrokes of the first and second action potentials after application of Cd^{2+} from that produced over an identical cycle before Cd^{2+} application. This Cd^{2+}-sensitive current displayed an inwardly directed spike during the early part of the action potential. Because this was also observed in four cells in which Cd^{2+} was substituted by 30 μM nifedipine (not shown), we suggest that this component of the Cd^{2+}-sensitive current represents inward Ca^{2+} flux through the SL L-type Ca^{2+} channels.

The integral of the current has been obtained over the whole cardiac cycle (i.e., from the beginning of one action potential to the same point at the beginning of the next one). In 24 cells under steady-state contraction at 0.5 Hz, the integral was 82 ± 5 pC. The cell capacitance was 130 ± 9 pF. The total integral of the Cd^{2+}-sensitive current calculated as described above was 0.65 ± 0.05 pC/pF. Assuming a conversion factor of 5 pF/pl for guinea pig cells (Page, 1978), we calculated a cell volume of 26 ± 2 pl (n = 24). The integral of Cd^{2+}-sensitive current measured over the whole cardiac cycle can be used to calculate Ca^{2+} entry through the sarcolemma (see Discussion).

Fig. 2 shows the effects of frequency of stimulation on the Cd^{2+}-sensitive current described above (Fig. 2 *A*) and on the current elicited by the fast application of caffeine (Fig 2 *B*). The latter has been used to calculate the SR Ca^{2+} content (Varro et al., 1993; Terracciano et al., 1995; Delbridge et al., 1996). The cells were stimulated at 0.5 Hz and 0.2 Hz. Both frequencies were tested in every cell. In this cell the action potential recorded and used as voltage command at 0.2 Hz was prolonged compared with the one at 0.5 Hz, but in general the action potential duration at 90% repolarization (APD90) was not statistically different at the two frequencies of stimulation in six cells (APD90 at 0.5 Hz = 532 ± 14 ms; APD90 at 0.2 Hz = 576 ± 32 ms). Although in traces from the cell shown in Fig. 2 the integral of the Cd^{2+}-sensitive current at the end of the cardiac cycle was smaller at 0.5 Hz compared with the one elicited at 0.2 Hz, no significant difference was detected in six cells (80.2 ± 5.2 pC at 0.5 Hz and 91.6 ± 5.7 pC at 0.2 Hz). In contrast, at 0.5 Hz the charge carried by the caffeine-induced transient inward current was larger than the one recorded after stimulation at 0.2 Hz (61 ± 3.2 pC at 0.2 Hz and 91.1 ± 5.7 pC at 0.5 Hz; n = 6; p = 0.0011), suggesting a larger amount of caffeine-releasable Ca^{2+} at 0.5 Hz in guinea pig cardiac cells.

It should be noted that in this figure and in Figs. 3 and 4 the integral of the current is still increasing at the end of the trace. One could speculate whether the assessment of the baseline was correct. As stated in Materials and Methods, the baseline for the calculation of the current integral is the zero value produced by the subtraction of Cd^{2+}-sensitive current. In many situations no changes in the membrane current were detected throughout the diastolic period during Cd^{2+} application, and therefore the zero level corresponded

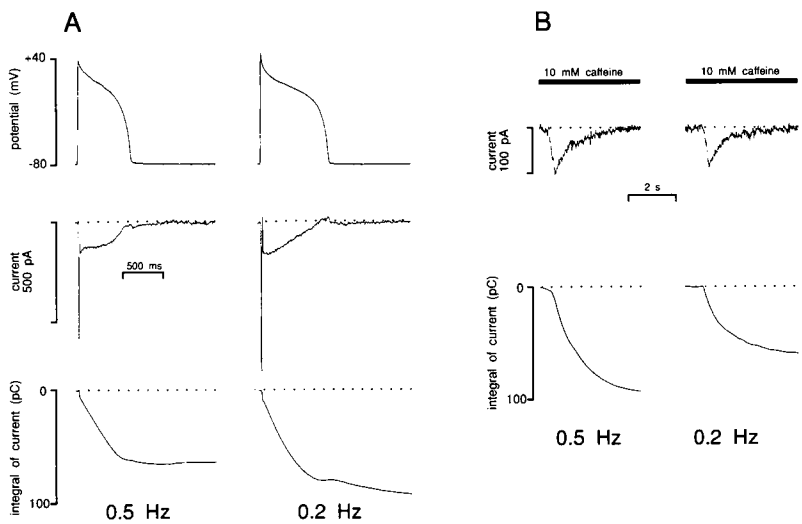

FIGURE 2 The effects of the frequency of stimulation on the Cd^{2+}-sensitive current and on the caffeine-dependent transient inward current are shown. In the same cell, action potentials at 0.5 and 0.2 Hz were recorded and then used as voltage commands. A protocol identical to the one described in Fig. 1 *B* was used. This cell was first stimulated at 0.5 Hz. The Cd^{2+}-sensitive current was calculated (*A*, *left*). Cd^{2+} was withdrawn, and when the contraction was completely restored stimulation was stopped and, after a 2-s rest, 10 mM caffeine was rapidly applied. A transient inward current ascribed to the electrogenic Na^{+}/Ca^{2+} exchange was recorded (*B*, *left*) and used to calculate the SR Ca^{2+} content. The cell was then stimulated at 0.2 Hz, using the 0.2-Hz action potential voltage command and the protocol repeated. The Cd^{2+}-sensitive current was calculated (*A*, *right*). After reaching steady state again, stimulation was stopped for 5 s and caffeine was applied (*B*, *right*). The bottom traces of both panels show the integral of the currents. It should be stressed again that for the calculation of the Ca^{2+} entry via SL Ca^{2+} channels, the Cd^{2+}-sensitive current has been integrated during the whole cardiac cycle. The dotted lines represent the zero level.

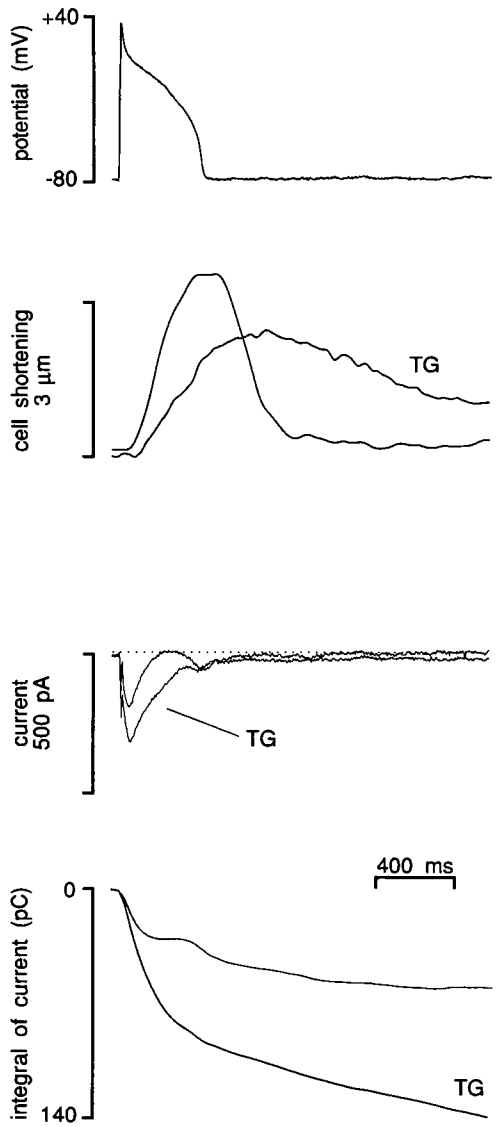

FIGURE 3 The effects of 500 nM thapsigargin (TG) on cell shortening and the Cd^{2+}-sensitive current during action potential clamp. The voltage command recorded in control conditions was also used during application of thapsigargin (*top trace*). Cells were stimulated at 0.2 Hz. When the cell had recovered from Cd^{2+} application, thapsigargin was applied for 5 min. At the end of this period cell shortening was still approximately 70% of control, although it had a slower time course (*second trace from top*). The Cd^{2+}-sensitive current in thapsigargin was calculated (*third trace from top*), and the integral of this current was larger than that elicited in control conditions. The dotted line indicates zero level of current.

to the current flowing during the resting membrane potential. In other circumstances, Cd^{2+} application produced a small outwardly directed current consistent with inhibition of Na^+/Ca^{2+} exchange by Cd^{2+}. In guinea pig cells Na^+/Ca^{2+} exchange extrudes Ca^{2+} from the cells during rest (rest decay) (Bers, 1991) and produces an inward current. Its inhibition therefore produces a shift in the compensation current in the outward direction. Except for the very first part of the action potential, the exchanger is probably continuously extruding Ca^{2+} in varying quantities, depending on the membrane potential and the intra- and extracellular concentrations of Na^+ and Ca^{2+}. We speculate that in some cells this extrusion is not complete before the next action potential is applied, so that a significant current will be detected, and this would give rise to a continuous decline in the current integral. At the same time it should be noted that, although the final value of the Cd^{2+}-sensitive current has always been calculated during the whole cardiac cycle, for clarity not all of this cycle is shown in the figures and therefore the integral could reach a plateau later.

In an attempt to investigate further the relationship between the Cd^{2+}-sensitive current and the SR Ca^{2+} content in guinea pig cardiac myocytes, the experiments described in Fig. 3 were performed. The SR was depleted of Ca^{2+} by application of 500 nM thapsigargin for 5 min. To enable complete relaxation of the cell during application of thapsigargin, cells were stimulated at 0.2 Hz. Rapid application of caffeine after this exposure to thapsigargin failed to elicit a transient inward current and a transient contraction, suggesting that the SR was depleted of Ca^{2+}. The action potential recorded at 0.2 Hz at the beginning of the experiment was used throughout the protocol (Fig. 3, *top trace*). In the presence of thapsigargin, a large contraction was still present (Fig. 3, *second trace from top*), and the Cd^{2+}-sensitive current was larger than in controls. In nine cells the charge carried by this current increased by 39.6 ± 5% in the presence of thapsigargin (control: 77.1 ± 7.3 pC; thapsigargin: 107.5 ± 11 pC; $p = 0.0003$; control: 0.61 ± 0.02 pC/pF; thapsigargin: 0.72 ± 0.02 pC/pF). In this figure the integral of the current in the presence of thapsigargin is still increasing. We suggest that this is due to continued Na^+/Ca^{2+} exchange current, which is the main way Ca^{2+} is expelled from the cell in this condition.

Ca^{2+} entry through the SL Ca^{2+} channels in rat cardiac myocytes was also investigated for comparison with the results obtained in guinea pig cells. Fig. 4 *A* shows the Cd^{2+}-sensitive current during action potential clamp measured in guinea pig and rat ventricular myocytes. The charge carried by the Cd^{2+}-sensitive current recorded in rat myocytes was 26.3 ± 3 pC ($n = 10$) (0.11 ± 0.01 pC/pF), considerably smaller than the one recorded in guinea pig cells. The caffeine-induced transient inward current in rat myocytes carried a charge of 168.5 ± 21 pC ($n = 10$) (0.67 ± 0.03 pC/pF).

Fig. 4 *B* (*upper graph*) compares the calculated Ca^{2+} entry through the SL Ca^{2+} channel with the SR Ca^{2+} content in rat and guinea pig ventricular myocytes. SR Ca^{2+} content has been calculated as described by Callewaert et al. (1989), Varro et al. (1993), Terracciano and MacLeod (1994), and Delbridge et al. (1996). The capacitance-volume ratio used in rat myocytes was 8.4 pF/pl (Satoh et al., 1996). Non-Na^+/Ca^{2+} exchange-mediated Ca^{2+} extrusion corrections in rat myocytes were: 1/0.67 (Varro et al., 1993) and 1/0.87 (Bassani et al., 1994). In rat ventricular myocytes the SR Ca^{2+} content was larger than in guinea pig ventricular myocytes. In addition, the Ca^{2+} entry through the SL was smaller in rat myocytes, particularly if compared with

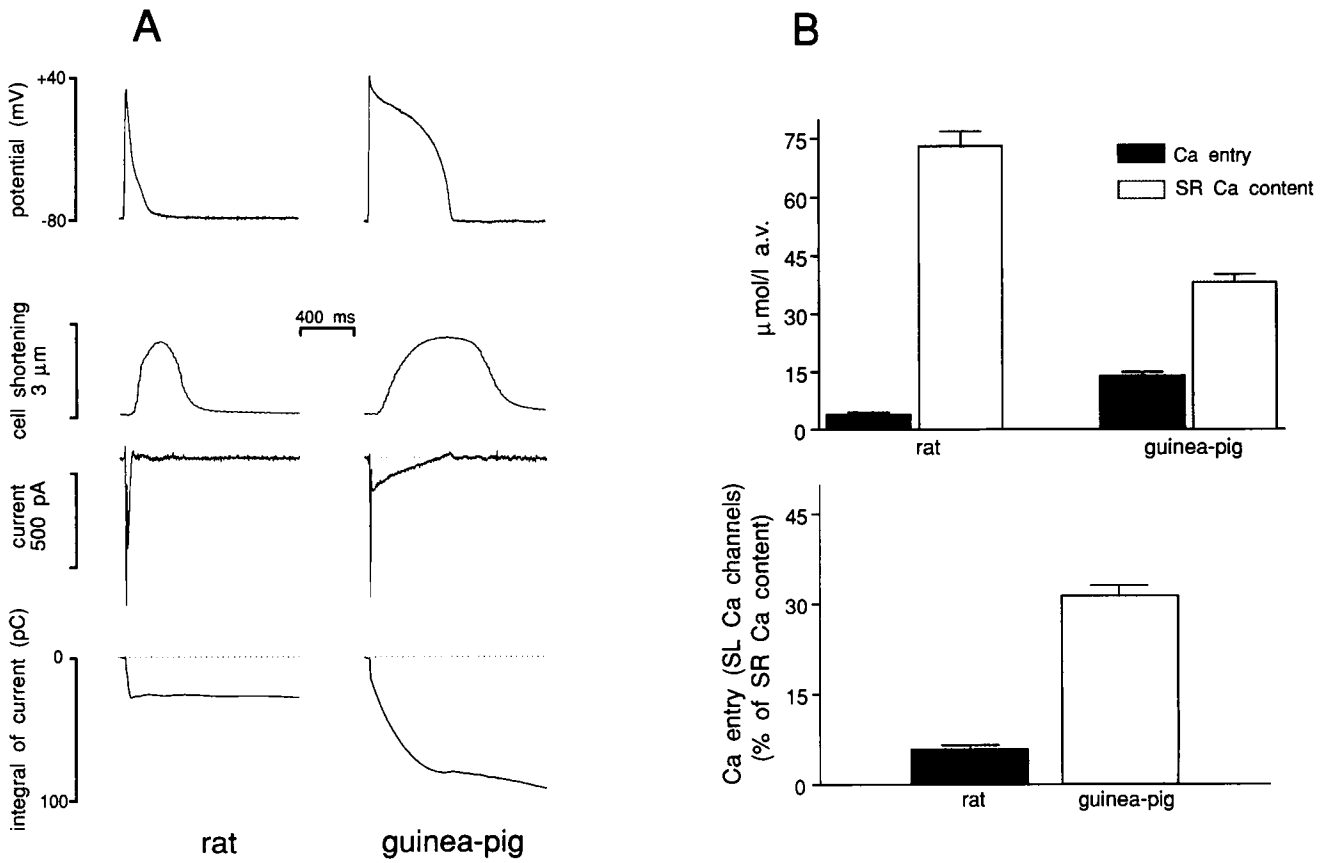

FIGURE 4 (*A*) The Cd^{2+}-sensitive current recorded in rat myocytes (*left*) compared with the one obtained in guinea pig myocytes (*right*). The cells were of similar volume (32 pl). The caffeine-sensitive transient inward current in rat myocytes was measured, and the SR Ca^{2+} content was calculated. (*B*) Upper graph: Values for SR Ca^{2+} content plotted together with the calculated Ca^{2+} entry through the SL Ca^{2+} channels. Lower graph: Ca^{2+} entry through the SL Ca^{2+} channels normalized to SR Ca^{2+} content.

SR Ca^{2+} content (*lower graph*) (rat myocytes, 5.7 ± 0.7%, $n = 6$; guinea pig myocytes, 31.4 ± 1.7%, $n = 11$).

DISCUSSION

Assumptions and limitations of the study

Some assumptions must be made so that the Cd^{2+}-sensitive current described above can be used to calculate Ca^{2+} entry via SL Ca^{2+} channels:

1. When contraction has reached a steady state, no net change in the Ca^{2+} content of intracellular compartments is expected. Ca^{2+} entering the cell through Ca^{2+} channels and outward Na^+/Ca^{2+} exchange is removed from the cytoplasm, mainly via inward Na^+/Ca^{2+} exchange. In cardiac myocytes it has been shown that the contribution of other mechanisms, such as the SL Ca^{2+} ATPase, are less important (Terracciano and MacLeod, 1994; Negretti et al., 1993). Nevertheless, in rat myocytes, this contribution could be significantly larger when the SR does not take part in cytoplasmic Ca^{2+} removal (Negretti et al., 1993; Bassani et al., 1994), and it should be taken into account.

2. During steady-state contraction the same amount of Ca^{2+} entering the cell via outwardly directed Na^+/Ca^{2+} exchanger is extruded by the Na^+/Ca^{2+} exchanger in inward mode. The net effect of this Ca^{2+} movement on the integral of total current measured during the whole cardiac cycle is zero.

3. A major Ca^{2+}-activated conductance is likely to be abolished by superfusing the cell with a Cl^- channel blocker. It has recently been shown that in cardiac myocytes the Ca^{2+}-sensitive and 4-aminopyridine-insensitive transient outward current ($I_{to(Ca)}$) is carried by Cl^- ions (Kawano et al., 1995). For this reason all of the experiments described above were performed in the presence of the Cl^- current blocker niflumic acid. Other Ca^{2+}-activated conductances (Colquhoun et al., 1981; Ehara et al., 1988) could be indirectly affected by Cd^{2+}, but previous experiments where Ca^{2+} was elevated by application of caffeine suggest that in guinea pig cardiac myocytes their importance is minimal (Terracciano and MacLeod, 1994). It is clear that the main Ca^{2+}-activated current is the transient inward current ascribed to the electrogenic Na^+/Ca^{2+} exchange. This current is taken into account in the calculation because it is part of the Cd^{2+}-sensitive current described below.

4. In the presence of 2 mM Cd^{2+} the contribution to Ca^{2+} entry via T-type Ca^{2+} channels in ventricular myocytes is minimal (Bean, 1989). This is supported by the absence of changes in indo-1 fluorescence in the presence of Cd^{2+} during stimulation (Fig. 1 *A*).

5. The stoichiometry of the Na^+/Ca^{2+} exchanger is 3 Na^+:1 Ca^{2+}, so that for one Ca^{2+} ion extruded, one net charge enters the cell, producing an inward current. The total effect of a Ca^{2+} ion entering the cell via the SL Ca^{2+} channels and being extruded via Na^+/Ca^{2+} exchange on the membrane current is an inward movement of three charges.

6. Two-thirds of the total integral of the Cd^{2+}-sensitive current is due to the Ca^{2+} entry through the sarcolemmal Ca^{2+} channels and one-third to the inward current produced by Na^+/Ca^{2+} exchange in removing this current (Bridge et al., 1990).

Quantifying Ca^{2+} entry via SL Ca^{2+} channels

In the presence of 2 mM Cd^{2+}, both the Ca^{2+} current and the Na^+/Ca^{2+} exchange inward and outward currents are inhibited (Kimura et al., 1987; Hobai et al., 1996). Therefore, with the assumptions made above, the current measured during the whole cardiac cycle (from the beginning of one action potential to the same point at the next) and resulting from the subtraction reported in Fig. 1 *B* is the sum of the inward current produced by Ca^{2+} flowing through the Ca^{2+} channels and the inward current produced by the electrogenic Na^+/Ca^{2+} exchange. The outward Na^+/Ca^{2+} exchange current flowing during the action potential is also included in the Cd^{2+}-sensitive current. The Ca^{2+} entering the cell in this way would be extruded by the Na^+/Ca^{2+} exchange in the opposite mode so that, as noted in assumption 2, at the end of the cardiac cycle the net effect of the outward Na^+/Ca^{2+} exchange current on the total integral of the Cd^{2+}-sensitive current is zero. Using these assumptions, the Ca^{2+} entering via the outward Na^+/Ca^{2+} exchange and removed by the inward Na^+/Ca^{2+} exchange cannot be calculated, and no information about its kinetics during the cardiac cycle can be obtained. Nevertheless, quantitative information can be derived from the experimental conditions. Two-thirds of the total integral of the Cd^{2+}-sensitive current is due to the Ca^{2+} entry through the sarcolemmal Ca^{2+} channels, and one-third to the inward current produced by Na^+/Ca^{2+} exchange in removing this Ca^{2+} (Bridge et al., 1990).

We calculated the SL Ca^{2+} entry via the Ca^{2+} channels by taking two-thirds of the Cd^{2+}-sensitive current and normalized to cell volume. The value was then corrected, assuming 25% of the cell volume was occupied by mitochondria. (14 ± 1 μmol of Ca^{2+} per liter of non-mitochondrial cell volume (a.v.) (n = 24) enters guinea pig ventricular myocytes paced at 0.5 Hz via Ca^{2+} channels during a cardiac cycle (and is extruded via inward Na^+/Ca^{2+} exchange).

Relative contribution of Ca^{2+} entry through the SL Ca^{2+} channels to the Ca^{2+} transient

The SR Ca^{2+} content at 0.5 Hz under these conditions was 38.2 ± 2 μM a.v. (n = 11). Ca^{2+} entry via Ca^{2+} channels therefore was 31.4 ± 1.7% (n = 11) of the SR Ca^{2+} content. This suggests that, in guinea pig cardiac myocytes, even if the whole SR Ca^{2+} content is released during a twitch, Ca^{2+} entry through the SL Ca^{2+} channels represents a large component of the Ca^{2+} transient. It should be stressed again that these experiments do not give information about the Ca^{2+} influx via the outward Na^+/Ca^{2+} exchange.

Role of Ca^{2+} entry via SL Ca^{2+} channels in the relationship between stimulation rate and Ca^{2+} transient

In guinea pig ventricular myocytes a positive staircase phenomenon can be observed. The experiments described in Fig. 2 show that, although the SR Ca^{2+} content was larger at 0.5 Hz (0.5 Hz: 39.8 ± 2.3 μM a.v.; 0.2 Hz: 27 ± 2.5 μM a.v.; n = 6; p = 0.0037), no significant difference in the Ca^{2+} entry via SL Ca^{2+} channels could be detected at 0.5 or 0.2 Hz (Ca^{2+} entry at 0.5 Hz: 12 ± 1.3 μM a.v.; at 0.2 Hz: 13.5 ± 1.2 μM a.v.; n = 6; p = 0.416). Ca^{2+} entry via SL Ca^{2+} channels may not, therefore, play a large role in determining the positive staircase phenomenon in guinea pig cardiac myocytes within the range of frequencies used in these experiments.

Role of Ca^{2+} entry via SL Ca^{2+} channels in the absence of Ca^{2+} release from the SR

The experiments performed in the presence of thapsigargin show that Ca^{2+} entry via SL Ca^{2+} channels in the absence of SR Ca^{2+} release increases by approximately 39% (from 13.1 ± 0.4 μM a.v. to 18.3 ± 0.7 μM a.v.; n = 9) in guinea pig cardiac myocytes. We speculate that this larger Ca^{2+} influx, possibly together with an increase in Ca^{2+} entry via the outward Na^+/Ca^{2+} exchange, supports the large contractions still observed in conditions when the SR is depleted of Ca^{2+}.

Species differences in the Ca^{2+} entry through the SL Ca^{2+} channels during the action potential

Measurements were made of Ca^{2+} entry via the SL Ca^{2+} channels and SR Ca^{2+} content in rat cardiac myocytes. Cell capacitance in 10 rat myocytes was 265 ± 33 pF. At 0.5 Hz the SR Ca^{2+} content (corrected for cell volume using a factor of 8.4 pF/pl for rat cells; Satoh et al., 1996) was larger than in guinea pig cells (73 ± 3.7 μM a.v.; n = 6). This was even larger if corrected for non-Na^+/Ca^{2+} exchange mechanisms (117 ± 1.3 μM a.v. (1/0.67; Varro et al., 1993); 91 ± 2.7 μM a.v. (1/0.87; Bassani et al., 1994)). Ca^{2+} entry via SL Ca^{2+} channels in rat was 3.99 ± 0.5 μM a.v. (n =

9) or 5.76 ± 0.7% of the SR Ca^{2+} content (or 3.5 ± 0.4% for 1/0.67 correction and 4.5 ± 0.5% for 1/0.87 correction used). In contrast, in guinea pig cells under similar conditions, Ca^{2+} entry via SL Ca^{2+} channels was approximately 30% of the SR Ca^{2+} content. Note that in this species we have not corrected for non-Na^+/Ca^{2+} exchange mechanisms because their role in Ca^{2+} extrusion is minimal (Terracciano and MacLeod, 1994). These results compare favorably with other studies in rat myocytes, where a small contribution of the Ca^{2+} current to the Ca^{2+} transient was estimated (Negretti et al., 1995).

In conclusion, the experiments described were performed to quantify the fluxes of Ca^{2+} across the sarcolemmal Ca^{2+} channels during normal action potential and steady-state contraction in cardiac myocytes with minimal perturbation of the intracellular medium. The two main mechanisms that regulate Ca^{2+} handling, SR and Na^+/Ca^{2+} exchange, were unaffected by the experimental technique, allowing a further step in the understanding of the Ca^{2+} regulation during normal stimulation and contraction. We have shown significant changes in the contribution of this entry to the Ca^{2+} transient, particularly in conditions where the SR Ca^{2+} content is low. These results quantitatively support previous observations regarding species differences in the various contributions of the Ca^{2+} regulation mechanisms to contraction in cardiac myocytes.

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