J. Membrane Biol. 170, 79–87 (1999)

The Journal of
Membrane
Biology

Transitional Changes in Membrane Potential and Intracellular [Ca^{2+}] in Rat Basophilic Leukemia Cells

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Received: 1 December 1998/Revised: 30 March 1999

Abstract. Using whole-cell current-clamp measurements we have found that thapsigargin-mediated activation of store-regulated Ca^{2+} entry in rat basophilic leukemia cells is accompanied by complex changes in membrane potential. These changes consisted of: (i) an initial slow, small depolarization, (ii) a transitional change in potential to a depolarized value and (iii) transitional changes between a hyperpolarized and a depolarized potential. These complex changes in potential can be explained by the interaction between the endogenous inwardly rectifying K^+ conductance and the generation of a small inward current. To investigate the possible influence of these changes of potential on $[Ca^{2+}]_i$, single cell measurements of fura2 fluorescence were undertaken alone or in combination with current-clamp measurements. Thapsigargin-mediated activation of the store-regulated Ca^{2+} entry pathway was accompanied by a marked increase of $[Ca^{2+}]_i$. During this increase, transient, abrupt declines in $[Ca^{2+}]_i$ were detected in approximately 60% of the cells investigated. These changes of $[Ca^{2+}]_i$ are consistent with the observed changes of membrane potential recorded under current-clamp.

Key words: Ca^{2+} oscillations — Membrane potential — Patch clamp — Current clamp

Introduction

Depletion of intracellular Ca^{2+} stores is a ubiquitous stimulus for the activation of an electrogenic Ca^{2+} influx pathway in nonexcitable cells [store-regulated Ca^{2+} entry pathway; reviewed in 33]. A great deal of information regarding the control and modulation of this pathway is known from fluorimetric measurements of $[Ca^{2+}]_i$ [reviewed in 33] and patch-clamp studies of the transmembrane Ca^{2+} current correlate of store-regulated Ca^{2+} flux (calcium release activated calcium current; I_{CRAC} [10, 11]. As would be expected for an electrogenic Ca^{2+} pathway, changes of $[Ca^{2+}]_i$ brought about by activation of store-regulated Ca^{2+} entry are modulated by membrane potential [17, 25, 29, 35]. This results not only from the influence of driving force but also from the inwardly rectifying nature of the pathway [2, 14]. As a result, depolarization, following activation of store-regulated Ca^{2+} entry, causes a marked decline in Ca^{2+} influx and $[Ca^{2+}]_i$ [17, 25, 29, 35].

To date, little physiological importance has been ascribed to the effect of depolarization on store-regulated Ca^{2+} entry since large depolarizations are not normally observed in nonexcitable cells due to the concomitant activation of Ca^{2+}-activated K^+ channels and the presence of other hyperpolarizing conductances. Only when the Ca^{2+}-activated K^+ conductance is inhibited are store-regulated Ca^{2+}-dependent depolarizations observed [19, 20, 39]. A clear exception to this occurs in rat basophilic leukemia cells, where isotopic and fluorimetric measurements of membrane potential in cell suspensions have documented large depolarizations following activation of store-regulated Ca^{2+} entry under physiological conditions [3, 12, 16, 29]. Rat basophilic leukemia cells represent a class of nonexcitable cells whose membrane potential is set primarily by an inwardly rectifying K^+ current [16, 26, 30]. Other cells falling into this category include rat and rabbit osteoclasts [37, 13] and human and murine macrophages of diverse origin [for review *see* 4]. The conductance-voltage relationship of the inwardly rectifying K^+ channel is such that in many of these cell

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types small changes of inward membrane current can result in large transitional shifts in membrane potential [1, 37]. To date the ability of store-regulated Ca^{2+} entry to induce such membrane potential changes and the effect of these changes on $[Ca^{2+}]_i$ has not been examined at the single cell level.

The present experiments were undertaken (i) to investigate the single cell membrane potential changes induced by activation of store-regulated Ca^{2+} entry in rat basophilic leukemia cells and (ii) to investigate the relationship between membrane potential changes and $[Ca^{2+}]_i$ following activation of store-regulated Ca^{2+} entry.

Materials and Methods

Cell Culture

Rat basophilic leukemia cells of the RBL-1 clone were kindly provided by Dr. S. Ikeda, Guthrie Institute (Sayer, PA) while RBL-2H3 cells were the kind gift of Dr. H. Metzger, NIH, (Bethesda, MD). Both cell lines were propagated in HEPES buffered RPMI 1640 medium (Sigma, St. Louis, MO) containing 25 mM sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin and 10% FBS (Bio-Whitaker) in a humidified 95/5% air/CO_2 atmosphere at 37°C as previously reported by our laboratory [36]. Cultures were propagated by passaging nonadherent cells taken from dense cultures or by removal of adherent cells with trypsin-Versene. No apparent differences in the parameters measured existed between adherent and non-adherent cells. Qualitatively similar results were obtained in both RBL-2H3 and RBL-1 cell lines.

Reagents

Fura2-penta-K^+-salt and fura2-Am (acetoxymethyl ester) were purchased from TeFlabs (Austin, TX) while *bis*-(1,3-diethylthiobarbituric acid) trimethine oxonol (*bis*-oxonol) was purchased from Molecular Probes (Eugene, OR). Thapsigargin was purchased from Calbiochem-Novabiochem (San Diego, CA). N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) was purchased from Calbiochem-Novachem (San Diego, CA) or Sigma (St. Louis, MO). Potassium methylsulfate was purchased from ICN Biochemicals (Aurora, OH). [Ethyleneglycol-*bis*-(β-aminoethyl)-N,N,N′,N′-tetraacetic acid] (EGTA), Na_2GTP and MgATP were purchased from Sigma (St. Louis, MO). Ethyl alcohol and dimethyl sulfoxide (DMSO) were purchased from Aldrich (Milwaukee, WI). $CaCl_2$, $MgCl_2$, NaCl, KCl, HCl, NaOH, KOH, D-glucose and sucrose were purchased from Fisher (Fairlawn, NJ) or Sigma (St. Louis, MO). N-methyl-D-glucamine was purchased from Aldrich (St. Louis, MO). Gramicidin-D was purchased from Sigma (St. Louis, MO) and was made up as a concentrated stock in ethyl alcohol. The pentapotassium salt of fura2 was made up in distilled water while fura2-AM, thapsigargin and *bis*-oxonol were made up as concentrated stocks in DMSO. All reagents were stored at −20°C.

Solutions

Basic Na^+ containing solution had the following composition in mM: 140 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Glucose, 20 Hepes, pH 7.4 with NaOH. When required, extra Ca^{2+} was added directly to this solution. High K^+ solution had the following composition in mM; 143 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Glucose, 20 Hepes, pH 7.4 with KOH. Ca^{2+}-free solutions were made by deleting Ca^{2+} and adding 0.5 mM EGTA. Thapsigargin and gramicidin were added directly to the external solution from concentrated stocks in DMSO. The internal solution employed in voltage- and current-clamp experiments had the following composition in mM; 140 $KMeSO_4$, 0.5 EGTA, 3 $MgCl_2$, 0.1 Na_2GTP, 4 Na_2ATP, 10 HEPES, pH 7.4 with KOH. When required, 50 μM fura2-penta-K^+-salt was added directly to the pipette solution and EGTA omitted to reduce the exogenous Ca^{2+} buffering power. Internal and external solutions were stored at −20°C.

Measurements of Membrane Potential in Cell Suspensions

Membrane potential was measured fluorimetrically in cell suspensions using *bis*-oxonol as previously reported by our laboratory [21, 22, 23, 39]. Cells at a concentration of 1×10^6 cells/ml were equilibrated with 150 nM *bis*-oxonol and fluorescence monitored (Ex 540, 5 nm slit width, Em 580 nm, 10 nM slit width) in a temperature controlled, continually stirred cuvette-based, monochromator-based fluorescence spectrophotometer (Photon Technology International, Monmouth, NJ). External calibration was made by adding gramicidin to cells suspended in media containing varying ratios of Na^+ and NMG^+ and constructing a calibration curve as previously reported [21, 22, 23, 39].

Single Cell FURA2 Fluorescence Measurements

Cells were loaded with fura2 by incubation with the AM derivative of fura2 (4μM for 25 min. @ 37°C) or by dialysis of the cell with a 50 μM fura2-penta K^+ salt containing pipette solution following initiation of conventional tight-seal whole-cell patch-clamp recordings. Measurements of fura2 fluorescence were made on an inverted microscope using a commercially available dual excitation, monochromator based excitation source (Photon Technology International). 340 and 380 nm (±5 nm) excitation wavelengths were directed onto the sample via a 430 nm dichroic mirror. Emission was filtered for a 510 nm wavelength using a 40 nm wide bandpass filter. The fluorescence of a single cell was isolated from surrounding cells using an adjustable diaphragm. For measurements of fura2 fluorescence in intact cells, background fluorescence of the instrument and chamber at 340 and 380 nm was subtracted by measuring fluorescence of an area of the chamber devoid of cells and corresponding to the detection area of the experiment. For measurements of fura2 fluorescence under whole-cell voltage- or current-clamp, the variable diaphragm was positioned around the cell so as to minimize the amount of visible fura2-containing patch pipette. The fluorescence recorded prior to initiation of the whole cell configuration was used for background subtraction. In most experiments, data are presented as qualitative changes in the background subtracted 340/380 fluorescence ratio. In some experiments the 340/380 ratio was converted to an estimate of $[Ca^{2+}]_i$ assuming a K_d of 225 nM and using externally derived values for R_{min}, R_{max} and the ratio of the 380 nm wavelength in the absence and presence of extracellular Ca^{2+} under similar experimental conditions. Fura2 fluorescence was converted to $[Ca^{2+}]$ using the standard algorithm as previously reported [6].

Whole-Cell Recordings

Current recordings were made in the tight seal configuration using an Axopatch 200A or 1C patch-clamp amplifier controlled by Macintosh

based custom software (S3, provided by Dr. S. Ikeda, Guthrie Institute, Sayre, PA) as previously reported by our laboratory [36]. Cells were held at a potential of −40 or 0 mV following establishment of whole cell recording conditions and 255 msec ramps from −125 to +60 mV were delivered every 3 sec immediately following initiation of the whole cell configuration. Current records were filtered at 1 kHz, sampled at 2 kHz and recorded on disk for analysis by commercially available waveform analysis software (Igor, Wavemetrics, Lake Oswego, OR). To minimize K^+ leakage into the chamber, a Hank's/agar bridge containing 5.4 mM K^+ was used to ground the chamber. The data are not corrected for a minor junction potential present under the conditions employed.

Recordings of membrane potential were made using the current-clamp mode of the patch-clamp amplifiers following compensation for residual leakage current. Experiments were performed on cells with membrane potential more hyperpolarized than −70 mV. Potential was filtered at 1 kHz and recorded to disk for analysis or recorded directly to a chart recorder. Sampling paradigms are described in the figure legends.

Solution Changes

For measurements of fura2 fluorescence in intact cells, solution was superfused at a flow rate of 0.9–1.0 ml/min with the aid of a peristaltic pump. This superfusion system had a substantial dead space that accounts for a delay of approximately 40 sec between the change of solution and the arrival of the new solution into the chamber. Solution changes as noted in the figures have been corrected for this delay.

Extracellular solution changes under voltage clamp were made by placing the cell immediately adjacent to a gravity fed superfusion device. This device consisted of six individual inflow lines connected to a single common superfusion line with minimal dead space. Changing from one solution to another resulted in the rapid exchange of extracellular solution without the need to reposition the cell.

Under current clamp, extracellular solution changes were made using the superfusion device described above or by gravity driven bath exchange.

All experiments were performed at 22–25°C unless otherwise noted.

Data are presented as the mean ± SEM.

Results

The characteristics of the whole-cell current-voltage (*I-V*) relationship in RBL cells were investigated under physiological conditions of high intracellular K^+ and low extracellular K^+. Cells were superfused with basic Na^+ solution containing 5 mM Ca^{2+} and conventional whole-cell patch-clamp recordings were initiated immediately following establishment of the whole-cell configuration. Ramps of 250 msec duration from −125 to +60 mV from a holding potential of −40 mV were initiated every three seconds and the membrane currents recorded. With a pipette solution containing 145 mM K^+ and an external solution containing 3 mM K^+, the dominant conductance present was the inwardly rectifying K^+ current as previously reported in RBL cells [18, 26, 30, 34, 40]. The inset of Fig. 1 shows an average non leak-subtracted *I-V* relationship for this K^+ current immediately after break-in ($n = 8$ cells). Changes in extracellular [K^+] in the presence of a constant divalent ion concentration resulted in a concentration dependent shift of the reversal potential with a slope of 53 mV per decade change of extracellular [K^+], consistent with the membrane potential being highly dependent on inwardly rectifying K^+ channels (*data not shown*). Consistent with the characteristics of the inwardly rectifying K^+ current, this inward current was inhibited by extracellular Ba^{2+}, (1 μM - 1 mM; *data now shown*). The main panel in Fig. 1 shows the mean *I-V* relationship between −90 and +60 mV. The *I-V* relationship shows a single zero current potential at approximately −80 mV. Thus, the membrane potential would be expected to sit at −80 mV. However, application of an inward current of slightly greater than 2.5 pA would result in a 2.5 pA offset of the *I-V* relationship and the generation of a new single zero current potential at approximately −15 mV. This arises as a result of (i) the small peak outward current contribution of the *I-V* between −80 and −40 mV (approximately 2.5 pA) and (ii) the decline of the outward current between −60 and −40 mV.

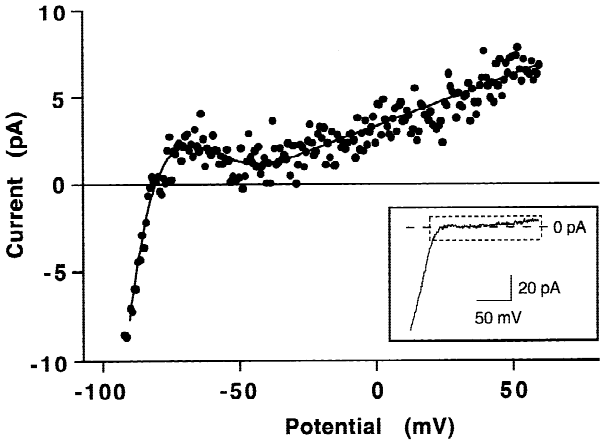

Fig. 1. Low conductance voltage region of the inwardly rectifying K^+ current. RBL-1 cells were whole-cell patch-clamped using a high K^+ internal solution designed to mimic physiological conditions and an external solution containing 3 mM K^+ and 5 mM Ca^{2+} (*See* Materials and Methods). Inset. Mean *I-V* relationship for a voltage ramp of 250 msec duration from −125 to +60 mV from a holding potential of 0 mV ($n = 8$ cells). The *I-V* relationship contained within the dashed box is presented in the main panel of the figure. Main Panel. Low conductance region of the inwardly rectifying K^+ current from −90 to +60 mV.

Experiments were undertaken to determine the characteristics of this postulated membrane potential shift in response to slow, graded inward current injection. Such a situation may be considered to mimic a slowly generating inward current that is constant across all potentials. Establishment of the whole-cell configuration was made under voltage-clamp using internal and external solutions identical to those use in Fig. 1. Upon switching from voltage-clamp to current-clamp mode and compensating for any residual leakage current from the ampli-

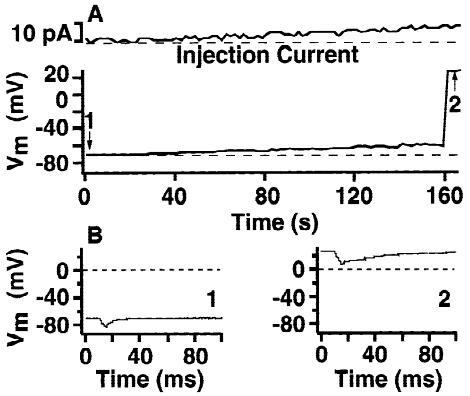

Fig. 2. Effect of injection of inward current on membrane potential under current-clamp. Panel *A.* Increasing depolarizing current was injected into an RBL-1 cell superfused with Na^+ solution containing 3 mM K^+ and 5 mM Ca^{2+} while recording membrane potential under whole-cell current-clamp. Potential was sampled at 5 kHz for 100 msec every 3 sec and potential averaged over the first 10 msec. Panel *B.* Superimposed upon the depolarizing injection current was a brief hyperpolarizing current.

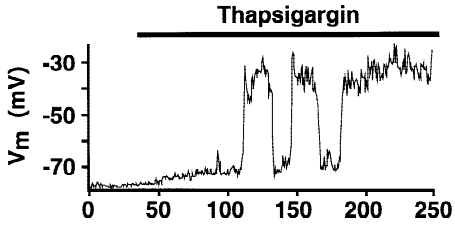

Fig. 3. Transitional changes of membrane potential recorded under current-clamp following addition of thapsigargin. A single RBL-1 cell was placed under whole-cell current-clamp using internal solutions identical to that used in Fig. 2 and superfused with basic Na^+ solution containing 3 mM K^+ and 5 mM Ca^{2+}. Where indicated the cell was superfused with solution containing 500 nM thapsigargin. Potential was sampled every 0.5 sec as an average of 20 points digitized at 2 kHz.

fier, resting membrane potentials in the range of −75 to −85 mV were routinely monitored. Increasing levels of inward injection current were then applied to the cell via the patch pipette. A representative experiment is shown in Fig. 2. A graded increase of injection current was accompanied by a small graded depolarization of approximately 7 mV. This was followed by a rapid transitional change of membrane potential to a value greater than +20 mV. In 4 cells, this rapid transitional change of membrane potential was induced by 5.6 ± 0.4 pA of injection current. Thus, the slow generation of a small inward current results in a biphasic membrane potential change. The first phase is small and dependent upon the rate of development of the inward current. The second phase is rapid and manifests itself as a transitional change of potential. This biphasic response is explained by the fall of the outward current component of the whole-cell *I-V* relationship between −70 and −40 mV (*see* Fig. 1). Once the outward component of the *I-V* relationship is overcome by adequate injection current, the potential will be set by the new zero current potential; which because of the fall of outward current between −70 and −40 mV, will result in a transitional jump of membrane potential to a depolarized value. The value of this new zero current potential will be determined by the interaction between the inward injection current and the amount of endogenous outward current present in the cell above −40 mV.

Superimposed upon the sustained depolarizing injection current in Fig. 2 were brief hyperpolarizing currents to monitor membrane input resistance. Assuming cell capacitance does not change over the course of the experiment, the time constant of the recovery of membrane potential following a brief hyperpolarizing current pulse is proportional to the cell input resistance. It is clear from the slowing of the time course of the potential change upon depolarization (Panel *B*, trace 2) that the cell input resistance was significantly increased, consistent with a potential sensitive conductance decrease of the inwardly rectifying K^+ channel.

On the basis of these data, it can be proposed that activation of an inward current, such as the inwardly rectifying store-regulated Ca^{2+} entry current, would be expected to produce similar effects. To define the effects of the activation of the store-regulated Ca^{2+} current on membrane potential, current-clamp measurements of potential were performed during the activation of this pathway. Store-regulated Ca^{2+} entry was activated by depletion of intracellular Ca^{2+} stores by application of thapsigargin, a selective inhibitor of endosomal Ca^{2+}-ATPase activity. Establishment of the whole-cell configuration was made under voltage-clamp using internal and external solutions identical to those used in Figs. 1 and 2. After verification of a stable *I-V* relationship, the cell was placed in current-clamp mode, amplifier leakage currents were compensated and the membrane potential recorded. Figure 3 shows a representative membrane potential response to application of 500 nM thapsigargin. In this experiment, thapsigargin induced an initial depolarization of approximately 6 mV. This was followed by a rapid, pronounced depolarization to approximately −35 mV, which in turn was followed by rapid transitions between the depolarized and hyperpolarized state. These data reveal that the change of potential has at least two components; a slow depolarization lasting up to many minutes followed by a rapid transitional change of potential to depolarized values. In this experiment the cell underwent a sustained depolarization. In 10 experiments the membrane potential before thapsigargin addition was −76 ± 2 mV. Following thapsigargin addition the resting potential depolarized to −70 ± 3 mV. This was accompanied by rapid transitions of potential to −17 ± 3 mV. In control experiments, no depolarizations were de-

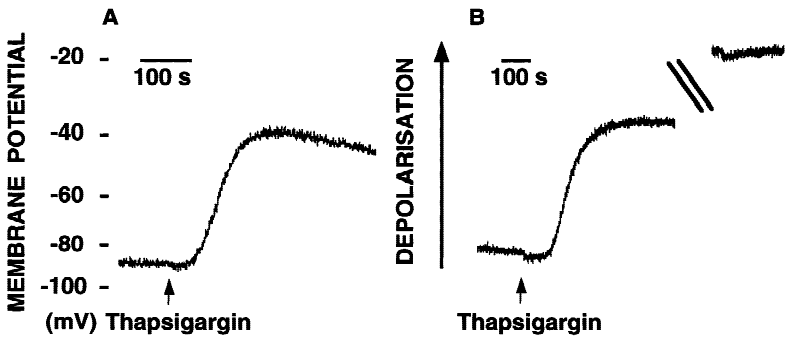

Fig. 4. Membrane potential changes measured with *bis*-oxonol in RBL cell suspensions. Panel *A.* RBL-2H3 cells were equilibrated with *bis*-oxonol at 37°C in Na^+ solution containing 3 mM K^+ and 2 mM Ca^{2+} and fluorescence monitored and calibrated as described in Materials and Methods. Where indicated, 100 nM thapsigargin was added to the cuvette. Panel *B.* The effect of 100 nM thapsigargin on *bis*-oxonol fluorescence of RBL-1 cells at 25°C. Cells were equilibrated with *bis*-oxonol in Na^+ solution containing 3 mM K^+ and 2 mM Ca^{2+} and fluorescence monitored. The break in the trace represents approximately 23 min and corresponds to the addition of agents beyond the scope of these experiments. The fluorescence recorded after the break is that recorded in the presence of 100 nM gramicidin and represents fluorescence at a membrane potential of approximately 0 mV.

tected in the absence of thapsigargin, consistent with events associated with depletion of intracellular Ca^{2+} stores being critical for the initiation of these depolarizations.

Marked depolarizations in response to thapsigargin application were also detected fluorimetrically with *bis*-oxonol is suspensions of intact cells. The result of a representative experiment at 37°C is shown in Fig. 4*A*. In this experiment addition of 100 nM thapsigargin to cells suspended in 1 mM Ca^{2+} containing Na^+ medium induced a depolarization of approximately 55 mV. A large depolarization was also detected at room temperature, suggesting that the events underlying the depolarization are not metabolically labile. A representative result in RBL-1 cells at room temperature is presented in Panel *B.*

Membrane potential measurements in cell suspensions are not representative of changes occurring at the single cell level, since these measurements represent a signal average from millions of cells which mask the complex kinetic changes of membrane potential accompanying activation of store-regulated Ca^{2+} entry, including complex membrane potential oscillations. However, this noninvasive, average measurement of membrane potential does confirm the existence of large membrane depolarizations following depletion of intracellular Ca^{2+} stores and thus, supports the conclusion that the depolarizations detected under current-clamp do not arise from artifactual influences of the use of patch-clamp methods.

Membrane potential changes such as those presented in Fig. 3 would be expected to modulate $[Ca^{2+}]_i$ in a manner consistent with both the effects of potential on driving force and the inwardly rectifying nature of the store-regulated Ca^{2+} influx pathway. A transient depolarization of sufficient duration to allow Ca^{2+} extrusion mechanisms to dominate during the depolarizing interval would be expected to result in a transient decline of $[Ca^{2+}]_i$. To investigate this hypothesis experiments were undertaken to monitor $[Ca^{2+}]_i$ at the single cell level in intact cells following activation of store-regulated Ca^{2+} entry. Intact, fura2 loaded RBL cells were allowed to adhere to a glass coverslip after which the cells were superfused with thapsigargin containing solution and fura2 fluorescence monitored from a single isolated cell. Figure 5 shows a representative response to addition of thapsigargin. In panel *A1*, addition of 500 nM thapsigargin was accompanied by an increase of $[Ca^{2+}]_i$ from approximately 140 nM to a peak of approximately 625 nM after which $[Ca^{2+}]_i$ slowly declined. Superimposed upon this slow fall of $[Ca^{2+}]_i$ were rapid transient declines of $[Ca^{2+}]_i$. For clarity these transient Ca^{2+} responses have been expanded in panel *A2.* These responses were of various magnitudes, with the largest in this experiment being approximately 125 nM. Subsequent removal of extracellular Ca^{2+} was accompanied by a rapid fall of $[Ca^{2+}]_i$ to pre-thapsigargin levels (panel *A1*), consistent with the thapsigargin-induced rise of $[Ca^{2+}]_i$ being a result of sustained Ca^{2+} influx from the extracellular media.

The transient changes of $[Ca^{2+}]_i$ detected following thapsigargin addition were frequently of longer duration as evident in the experiments presented in Fig. 5, panel *B.* In this single cell experiment the data are presented as uncalibrated fura2 fluorescence ratios. For comparison purposes, the transient declines of $[Ca^{2+}]_i$ presented in panels *A1* and *A2* correspond to a fura2 ratio change of approximately 0.5. Approximately 60% (17 of 29 cells) of the cells studied displayed transitional changes of $[Ca^{2+}]_i$. In some cases the transients were not reversible, manifesting themselves as steplike declines of $[Ca^{2+}]_i$ (*data not shown*).

The transient Ca^{2+} responses following thapsigargin addition may have as their underlying mechanism the transient depolarizing responses previously detected under current clamp (Fig. 3). It was important to directly

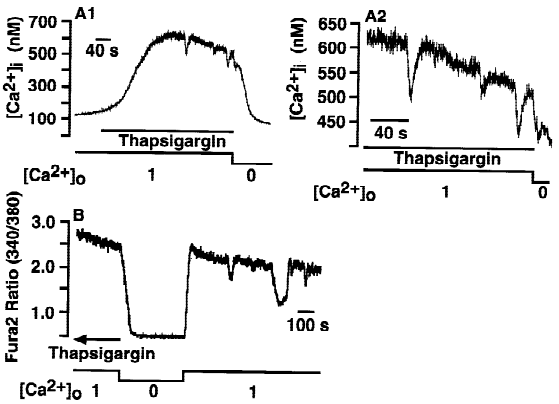

Fig. 5. Reversible, spontaneous changes of $[Ca^{2+}]_i$ following thapsigargin addition. Panel *A1*. A single fura2 loaded RBL-1 cell was superfused with Na^+ solution containing 3 mM K^+ and 1 mM Ca^{2+} and fura2 fluorescence monitored. Where indicated the cell was superfused with 500 nM thapsigargin. Panel *A2*. An expansion of the Ca^{2+} transitions presented in panel *A1*. Panel B. Ca^{2+} transitions in a second RBL-1 cell. Thapsigargin was added prior to the portion of the record displayed as denoted by the arrow.

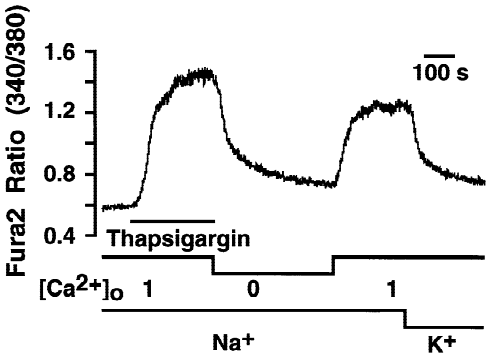

Fig. 6. Effect of K^+-induced membrane depolarization on $[Ca^{2+}]_i$ following thapsigargin addition. A single fura2 loaded RBL-1 cell was superfused with Na^+ or K^+ solution in the presence or absence of extracellular Ca^{2+} as indicated and fura2 fluorescence monitored. Where indicated the cell was superfused with 500 nM thapsigargin.

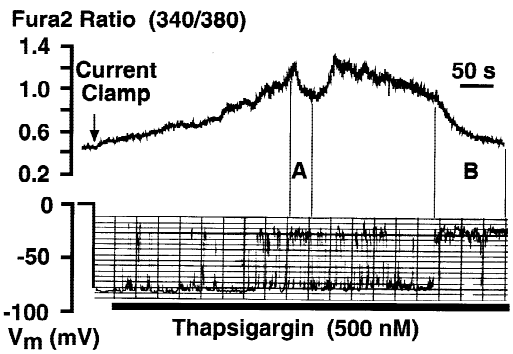

Fig. 7. Simultaneous measurements of membrane potential and fura2 fluorescence during thapsigargin application. A single RBL-2H3 cell superfused with basic Na^+ solution containing 3 mM K^+ and 1 mM Ca^{2+} was placed under current-clamp using conventional tight-seal whole-cell patch-clamp techniques and an internal solution supplemented with fura2. The internal solution was identical to that used in Fig. 2 with the exception that 100 μM EGTA was omitted and replaced with 50 μM fura2. Where indicated the cell was superfused with solution containing 500 nM thapsigargin. Fluorescence was recorded to disk at 5 Hz while the potential was recorded on a chart recorder.

determine the influence of depolarization on $[Ca^{2+}]_i$. To achieve this we have exploited the fact that the membrane potential of RBL cells is set almost exclusively by the inwardly rectifying K^+ conductance. Therefore, elevations of extracellular K^+ from 3 to 150 mM results in depolarization to near 0 mV. The effect of K^+-induced depolarization on $[Ca^{2+}]_i$ following activation of store-regulated Ca^{2+} entry in a single fura2 loaded RBL cell is shown in Fig. 6. The cell was first superfused with Ca^{2+} containing solution supplemented with 500 nM thapsigargin to activate store-regulated Ca^{2+} entry as evident by the rise of the 340/380 fura2 fluorescence ratio. No transient declines of $[Ca^{2+}]_i$ were detected in this experiment. Removal of extracellular Ca^{2+} was accompanied by a rapid fall of $[Ca^{2+}]_i$ which was reversed by readdition of extracellular Ca^{2+}, consistent with a requirement for extracellular Ca^{2+} to sustain the increase of $[Ca^{2+}]_i$. Subsequent replacement of extracellular Na^+ with K^+ was accompanied by a fall of $[Ca^{2+}]_i$ to a value approaching that detected in the absence of extracellular Ca^{2+}. This was not a consequence of removing extracellular Na^+ since replacement of Na^+ with N-methyl-D-glucamine did not induce a fall of intracellular Ca^{2+} (*data not shown*). Given this marked sensitivity to depolarization, it can be proposed that changes of membrane potential underlie the transitional changes of $[Ca^{2+}]_i$ detected following activation of store-regulated Ca^{2+} entry (Fig. 5).

To directly address this hypothesis whole-cell current-clamp recordings were combined with simultaneous measurements of fura2 fluorescence. Such an experiment is shown in Fig. 7. Conventional whole-cell voltage-clamp was initiated using a K^+ containing pipette solution similar to that described previously but devoid of EGTA and supplemented with 50 μM fura2-penta K^+ salt as described in Materials and Methods. After establishing the whole-cell configuration, the cell was held at 0 mV and allowed to load with fura2 by dialysis from the patch pipette. The cell was then placed in current-clamp mode and the membrane potential recorded while superfusing with Na^+ solution containing 1 mM Ca^{2+}. The switch to current clamp was accompanied by a modest increase in the fura2 ratio consistent with a small increase of $[Ca^{2+}]_i$ due to the change of potential from 0 to −83 mV. Subsequent addition of 500 nM thapsigargin in the sustained presence of 1 mM Ca^{2+} was accompanied by a slow increase of $[Ca^{2+}]_i$ and the initiation of brief transitions of membrane potential between −80 and −23

mV. Analysis of the fura2 record reveals a marked decline of $[Ca^{2+}]_i$ that corresponds to rapid oscillations between −80 and −23 mV and an increase in the time spent in the depolarized state (section *A*). Recovery of $[Ca^{2+}]_i$ to pre-decline levels coincided with an alteration of the pattern of membrane potential oscillations and an increase in the time spent in the hyperpolarized state. A second sustained depolarization to approximately −23 mV was accompanied by a sustained decline of $[Ca^{2+}]_i$ to a value virtually identical to that recorded immediately before thapsigargin addition, consistent with a depolarization induced decline of $[Ca^{2+}]_i$.

Discussion

It has previously been demonstrated in experiments using RBL cell suspensions that depletion of intracellular Ca^{2+} stores, induced by inhibition of endosomal Ca^{2+}-ATPase activity or by IgE receptor mediated increases of inositol triphosphate, is accompanied by membrane depolarization [3, 12, 16, 29]. However, the complex changes of potential, the underlying interactions between the membrane conductances responsible for these effects and the role of these changes in modulating $[Ca^{2+}]_i$, at the single cell level, have not been investigated or fully appreciated. The present results demonstrate that the membrane potential change accompanying depletion of intracellular Ca^{2+} stores mediated by thapsigargin are complex and consist of three phases: (i) a slow, modest depolarization of approximately 5 to 10 mV, (ii) a rapid, instantaneous transitional change of potential to a depolarized value, and (iii) nonperiodic oscillations between the depolarized and hyperpolarized potentials.

Membrane potential measurements in single RBL cells have been reported previously. Antigenic stimulation of IgE receptors has been reported to induce a slow membrane depolarization when measured under nystatin perforated-patch conditions [5]. Additionally, approximately 25% of the cells displayed an initial transient depolarization. However, the kinetics of this single, transient depolarization were far slower than those detected in the present experiments following thapsigargin addition. In contrast to the slow changes of membrane potential discussed above, spontaneous, transitional changes of RBL membrane potential similar to those reported in the present experiments have been noted previously in a small population of nonstimulated RBL cells [1, 18]. However, the underlying conductance alterations responsible for these changes and the importance of these changes to Ca^{2+} signaling was not apparent.

The slow initial depolarization and the rapid transitional change of potential to a depolarized value observed in the present experiments can be explained by the slow generation of the inwardly rectifying store-regulated Ca^{2+} entry current. The generation of this current may be sufficient to drive the membrane into the area of low membrane conductance, resulting in the marked transitional depolarization. This hypothesis is supported by our data demonstrating that injection of a small, graded, depolarizing current of less than 10 pA mimics, in part, the membrane potential response to thapsigargin; i.e., an initial slow depolarization followed by a rapid transitional change of potential to a depolarized value. A similar influence of injection current on membrane potential has been reported in osteoclasts, another cell type that relies upon an inwardly rectifying K^+ channel for the maintenance of a hyperpolarized membrane potential [37]. The potential for interaction between small inward currents and the inwardly rectifying K^+ current in the setting of membrane potential of RBL cells has been previously appreciated [27]. The activation of an inward Ca^{2+} current of sufficient magnitude to induce the depolarizations observed following thapsigargin addition has previously been reported in RBL cells following depletion of intracellular Ca^{2+} stores under ionic conditions specifically designed to isolate a Ca^{2+} current [7, 8, 31, 36, 41]. However, the magnitude of this current under the present experimental conditions is unclear and as such it is not possible to definitively ascribe the depolarizations detected in our experiments to the activation of the store-regulated Ca^{2+} entry current. It should be noted that activation of a nonselective cation current in RBL cells by ATP has been reported to induce transient depolarizations [32], a result that may reflect the interaction between the inwardly rectifying K^+ current and the ATP-induced inward current. Our results extend this observation by demonstrating: (i) that activation of a physiological pathway can induce shifts between stable membrane potential values, (ii) that the membrane potential response comprises spontaneous switching between multiple potentials, and (iii) that these shifts of potential can significantly modulate the steady-state level of $[Ca^{2+}]_i$. It is important to note that detection of a transient fall of $[Ca^{2+}]_i$ is dependent upon the depolarizing event being of sufficient duration to sufficiently alter the time spent in the depolarized state. This is particularly evident in Fig. 7 where an obvious fall of $[Ca^{2+}]_i$ is detected only when a marked increase in the time spent in the depolarized state occurs (section *A* or section *B*). Brief depolarizations do not induce detectable changes of $[Ca^{2+}]_i$, presumably because they do not allow sufficient time for Ca^{2+} extrusion mechanisms to lower Ca^{2+} before the cell repolarizes and Ca^{2+} influx is stimulated.

An alternative explanation for the rapid transitional depolarization detected after thapsigargin addition, can be entirely ascribed to inhibition of the constitutively active inwardly rectifying K^+ conductance. It has been reported that the inwardly rectifying K^+ current of RBL and other cells is inhibited by elevations of $[Ca^{2+}]_i$ [15,

30, 38]. Therefore, elevations of $[Ca^{2+}]_i$ brought about by activation of the store-regulated Ca^{2+} influx pathway may induce depolarization via inhibition of the endogenous K^+ conductance. This mechanism is distinct from electrogenic Ca^{2+} influx driving the membrane into a low conductance region as discussed above. In one case the membrane potential is moved into an area of low conductance by the influx of positive charge carried by Ca^{2+}. In the second case, the depolarization is a result of the closure of the inwardly rectifying K^+ channels due to elevations of $[Ca^{2+}]_i$. Interestingly, the depolarization induced by Ca^{2+}-dependent inhibition of the inwardly rectifying K^+ current would be predicted to reverse due to the depolarization-dependent decline of $[Ca^{2+}]_i$. Thus, Ca^{2+}-dependent modulation of the endogenous K^+ current could account for the nonperiodic oscillations of membrane potential if appropriate time lags are incorporated into the scheme. Further experiments are required to address this hypothesis.

Alternatively, nonperiodic oscillations of membrane potential may have as their origin an oscillating inward current. Inhibition of the store-regulated Ca^{2+} entry current during the depolarization phase of the response could be adequate to bring about membrane repolarization. The origin of such an inhibitory effect of depolarization on store-regulated Ca^{2+} entry is speculative. Although Ca^{2+}-dependent inhibition of the current has been demonstrated [42, 43], its role in the present experiments is difficult to reconcile since depolarization results in a marked fall of $[Ca^{2+}]_i$, not the prerequisite sustained increase of $[Ca^{2+}]_i$ required to induce inhibition. However, this discussion does not rule out oscillations of store-regulated Ca^{2+} entry induced by alternative mechanisms.

An increase of the outward current component of the *I-V* relationships could also account for the repolarization. In this regard it is interesting to note that activation of a K^+ current distinct from the inwardly rectifying K^+ current has been proposed to accompany activation of store-regulated Ca^{2+} entry in RBL cells [8]. Furthermore, an outwardly rectifying K^+ current, not normally active in RBL cells, has been reported following G-protein activation [26, 34]. Activation of either of these conductances following thapsigargin exposure could repolarize the membrane and, as such, oscillations of these conductances could account for the nonperiodic oscillations of potential. Further experiments are required to isolate the conductances activated in RBL cells following depletion of $[Ca^{2+}]_i$ stores by thapsigargin.

A single transitional decline of $[Ca^{2+}]_i$ has previously been reported in a small proportion of Jurkat cells exposed to thapsigargin [9]. The underlying mechanism responsible for this change is apparently distinct from that reported in RBL cells since Jurkat cells do not express an inwardly rectifying K^+ channel.

The present results in RBL cells are consistent with transitional changes of $[Ca^{2+}]_i$ being driven exclusively by transitional changes of membrane potential. Previous experiments in RBL cells have reported marked oscillations of $[Ca^{2+}]_i$ following IgE activation [28]. Like Ca^{2+} oscillations in many cell types, the underlying mechanisms driving the Ca^{2+} oscillations are unclear. However, while membrane potential was not measured, oscillations persisted in the presence of valinomycin, which is expected to clamp the membrane potential at or near E_K. Thus, it would appear that membrane potential changes are not necessary for the oscillations of $[Ca^{2+}]_i$ induced by IgE. In this context, it is interesting to note that while Ca^{2+} oscillations of the type observed after IgE stimulation were not detected following elevations of $[Ca^{2+}]_i$ induced by exposure to ionomycin, transitional changes of $[Ca^{2+}]_i$ similar to those reported in the present investigation were obvious in records presented by the authors [28]. Interestingly, ionomycin, like thapsigargin, has been shown to activate store-regulated Ca^{2+} entry in RBL and other cells [10, 24, 36].

The present results demonstrate the importance of membrane potential in modulating $[Ca^{2+}]_i$. While changes of membrane potential do not adequately account for all types of Ca^{2+} oscillations in RBL cells, these experiments highlight the importance of membrane potential changes in the modulation of $[Ca^{2+}]_i$. Furthermore these data raise the question of the role of periodic or nonperiodic oscillations of membrane potential in the generation and/or modulation of oscillatory Ca^{2+} events. The results provide new insights into the control of Ca^{2+} signaling in cells undergoing large membrane potential changes in response to agonist stimulation and have important implications for Ca^{2+} signaling in cells in which the constitutively active, inwardly rectifying K^+ channel is the major hyperpolarizing conductance.

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