Temperature-dependent Block of Capacitative Ca\textsuperscript{2+} Influx in the Human Leukemic Cell Line KU-812*

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The mechanism by which depletion of intracellular Ca\textsuperscript{2+} stores activates Ca\textsuperscript{2+} influx is not understood. We recently showed that primaquine, an inhibitor of vesicular transport, blocks the activation of the calcium release-activated calcium current (I\textsubscript{CRAC}) in rat megakaryocytes (Somasundaram, B., Norman, J. C., and Mahaut-Smith, M. P. (1995) Biochem. J. 309, 725–729). Since it is well established that vesicular transport is temperature-sensitive, we have investigated the effect of temperature on both the activation and maintenance of store-mediated Ca\textsuperscript{2+} and Mn\textsuperscript{2+} influx in the human leukemic cell line KU-812 using a combination of whole cell I\textsubscript{CRAC} recordings and measurements of Mn\textsuperscript{2+} photoquench of fura-2. Activation of I\textsubscript{CRAC} was temperature-sensitive, showing a nonlinear reduction when the temperature was lowered from 27 to 17 °C with an abrupt change at 21–22 °C and complete inhibition at 17 °C. Once activated, I\textsubscript{CRAC} also displayed an abrupt reduction at 21–22 °C but was not completely blocked even when the temperature was reduced to 14 °C, suggesting that at least one of the temperature-sensitive components is exclusively involved in I\textsubscript{CRAC} activation. Activation of store-mediated Mn\textsuperscript{2+} influx also showed similar nonlinear temperature sensitivity and complete inhibition at 19 °C. However, in contrast to I\textsubscript{CRAC} measurements, lowering the temperature following maximal activation of the influx pathway at 37 °C did not result in any detectable residual Mn\textsuperscript{2+} entry below 19 °C. We conclude that the mechanism of store-mediated Ca\textsuperscript{2+} influx involves temperature-dependent steps in both its maintenance and activation, suggesting dependence on a lipid membrane environment.

In many nonexcitable cells, depletion of intracellular Ca\textsuperscript{2+} stores by inositol 1,4,5-trisphosphate activates Ca\textsuperscript{2+} influx across the plasma membrane, a phenomenon termed “capacitative Ca\textsuperscript{2+} entry” (Putney, 1986). This Ca\textsuperscript{2+} entry pathway was first characterized electrophysiologically in mast cells and named “calcium release-activated calcium current” (I\textsubscript{CRAC})

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1 The abbreviations used are: I\textsubscript{CRAC}, calcium release-activated calcium current; GTP\textsuperscript{Y}S, guanosine 5’-O-(thiotriphosphate); [Ca\textsuperscript{2+}]\textsubscript{i}, free cytosolic Ca\textsuperscript{2+} concentration; TG, thapsigargin; BAPTA, 1,2-bis(2-aminophenoxo)ethane-N,N’,N’-tetracetic acid; Q\textsubscript{Eo}, temperature coefficient; E\textsubscript{a}, activation energy; ETH, (−)-[R,R]-N,N’-bis-[11-ethoxy-carbonyl]undecyl]-N,N’-4,5-tetramethyl-3,6-dioxaoctane-diamide, diethyl-N,N’-{[(4R5R)-4,5-dimethyl-1,8-dioxo-3,6-dionachemethylene]-bis(12-methylaminodecanoate).
ability of large numbers of cells for population studies of Mn^{2+} quench.

**MATERIALS AND METHODS**

**Cells and Reagents—** KU-812 cells were obtained from the European Collection of Animal Cell Cultures (Centre for Applied Microbiology and Research, Porton Down, UK) and cultured in a humified atmosphere at 37°C and 5% CO₂ in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 2 mM glutamine, 10 units/ml penicillin, and 10 mg/ml streptomycin. Cells were harvested by centrifugation, washed, and resuspended in a standard external solution (see below). Thapsigargin (TG), valinomycin, and ionomycin were obtained from Sigma, and fura-2/AM, pluronic acid P81, and from Molecular Probes, Inc., (Eugene, OR). All these agents were prepared as stocks in dimethyl sulfoxide. Cs⁺-BAPTA was from Molecular Probes (Eugene OR), and all other reagents were from Sigma.

**Electrophysiology—** Patch clamp experiments were performed in the conventional whole cell configuration (Hamill et al., 1981) by means of an Axopatch 200A patch clamp amplifier (Axon Instruments, Inc., Foster City, CA). Pipettes were pulled from borosilicate glass tubing (Clark Electromedical Instruments) and had filled resistances of 2–3 megohms. Series resistances were in the range of 10–30 megohms, and 40–70% series resistance compensation was used. The cells were held at 23–24°C in a standard external solution containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM glucose, and 10 mM HEPES, pH 7.4 (adjusted with Tris), and fura-2/AM (approximately 50 μg/ml free Ca²⁺ (25 mM VpCa²⁺-unit)). Cells were washed in a solution containing 120 mM KCl, 25 mM HEPES, 3 mM MgCl₂, and 1 mM EGTA, washed again in the same solution without EGTA, and finally resuspended in this non-EGTA-containing solution at a final concentration of 2 × 10⁶ cells/ml. The cells were then stored on ice until use. For each experiment, a cell was warmed to 37°C and was added to give a final concentration of 40 μM and incubated for 2 min. The cell suspension was made up to 60 μl with non-EGTA-containing solution, phosphocreatine (10 mM), creatine kinase (20 units/ml), and 3 mM ATP and then transferred to form a droplet into which the calcium electrode tip was placed. The temperature was adjusted as required before experimentation.

**Fluorescence Recording—** Fura-2 fluorescence measurements were carried out on cell populations in a final volume of 1.5 ml (10⁶ cells/ml) using a Cairn spectrophotometer system. Excitation wavelengths of 340, 360, and 380 nm were provided by a filter wheel rotating at 35 Hz in the light path. Emitted light was filtered by a 485 nm long pass filter and samples averaged to give a data point every 500 ms. The background corrected 540/380 ratio was multiplied by constants for fura-2 at different temperatures obtained from Shuttlesworth and Thompson (1991) to give an indication of [Ca²⁺]. The isosbestic excitation wavelength (360 nm) was used to monitor the fura-2 photoquench by Mn²⁺; an established measure of influx through the Ca²⁺ store-activated influx pathway. Cells were loaded with fura-2 by incubation with 1 μM fura-2/AM and 0.1% Pluronic F127 for 30 min at 24°C in standard external solution containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM glucose, 2 mg/ml apyrase, 10 mM HEPES, pH 7.4 (adjusted with Tris), and 1 mM CaCl₂. The apyrase was used to minimize purinergic receptor activation, which may be caused by ATP or ADP released from damaged cells. The cells were spun down and resuspended in the standard external solution with 2 mM CaCl₂ and split into 100-μl aliquots each containing 10⁶ cells and stored at room temperature until use. Prior to experimentation the cells were spun and resuspended in 100 μl of nominally Ca²⁺-free standard external medium with the K⁺ ionophore valinomycin (1 μM). Valinomycin was then present throughout the experiment allowing the cell membrane potential to be “clamped” close to the reversal potential of K⁺ (approximately −80 mV) through the Ca²⁺ this resulted in a shift in the photoquench curve when valinomycin was added immediately or 6 min before measurement of Mn²⁺ photoquench (n = 3). To study the effect of temperature on activation of store-dependent Mn²⁺ influx, the 100-μl cell aliquot was added to 1.4 ml of medium at the required temperature. After a 30-s delay, 100 μM MnCl₂ was added, followed 6 min later by 100 μM Mn²⁺⁻. To measure basal Mn²⁺ influx, 100 μM MnCl₂ was added in place of TG, after a 30-s incubation at the required temperature. To study the effects of temperature on the maintenance of the store-mediated Mn²⁺ influx pathway, the stores were emptied first by adding 100 μM TG to the 100-μl cell suspension in nominally Ca²⁺-free standard external medium and incubated for 6 min at 37°C. The cell suspension was then added to the cuvette containing 1.4 ml of nominally Ca²⁺-free standard external medium at the required temperature. After a 30-s delay, 100 μM MnCl₂ was added, and the quench was measured. In controls, the 100-μl cell suspensions were incubated at 37°C without TG for the same length of time but with 1 mM added Ca²⁺ to prevent any passive store depletion during incubation. To quantify Mn²⁺ entry, the slopes of the fura-2 photoquench over the first 15 s after Mn²⁺ addition were determined. The very initial, sharp drop in fluorescence observed in some experiments, due to external free dye, was not included in the calculations.

The degree of TG-induced store depletion was assessed at both 34 and 18°C by comparing the ionomycin-induced (5 μM) Ca²⁺ rise before and after a 6-min incubation with TG. To eliminate the contribution of Ca²⁺ influx to these measurements, 0.1 mM EGTA was added to the external medium immediately before addition of ionomycin.

**Measurement of Cytosolic ATP—** Cytosolic ATP concentrations were measured using an ATP assay kit (Calbiochem) based on the firefly luciferase-catalyzed oxidation of β-nitrophenyl in the presence of ATP-magnesium salt and oxygen. A cell suspension (3 × 10⁶ cells/ml) in standard external medium was incubated at the desired temperature

**Temperature Effects on ICRAC**

I_{CRAC} was determined with a Ca²⁺-sensitive minielectrode suspended in 60 μl of permeabilized cell suspension stirred by a magnetic agitator. The Ca²⁺ minielectrodes were prepared using a protocol similar to that of Clapper and Lee (1985). The electrode membrane was made with a Ca²⁺ electrode mixture (Fluka Chemical Corp.) containing 4.15 mM of ETH 1001 Ca²⁺ ionophore, 37 mg of 5-nitrophenyl ether, 0.42 mg of sodium tetraphenyl borate, and 24 mg of polyvinyl chloride dissolved in 240 μl of triethylthefluoro. The electrode was filled with a 10 mM CaCl₂ solution and connected to a Genway 3040 pH and ion analyzer. The minireference (3 mM KCl) electrode used (ULTRA-WICK®) was purchased from World Precision Instruments, Inc. The electrode was calibrated at 15, 20, and 30°C using various Ca²⁺-EGTA buffers displayed in a linear response within the range of 300 μM free Ca²⁺ (25 mM VpCa²⁺-unit). Cells were washed in a solution containing 120 mM KCl, 25 mM HEPES, 3 mM MgCl₂, and 1 mM EGTA, washed again in the same solution without EGTA, and finally resuspended in this non-EGTA-containing solution at a final concentration of 2 × 10⁶ cells/ml. The cells were then stored on ice until use. For each experiment, a cell was warmed to 37°C and was added to give a final concentration of 40 μM and incubated for 2 min. The cell suspension was made up to 60 μl with non-EGTA-containing solution, phosphocreatine (10 mM), creatine kinase (20 units/ml), and 3 mM ATP and then transferred to form a droplet into which the calcium electrode tip was placed. The temperature was adjusted as required before experimentation.
Temperature Effects on $I_{\text{CRAC}}$

**RESULTS**

**Effect of Temperature on $I_{\text{CRAC}}$ Activation**—To study $I_{\text{CRAC}}$ in KU-812 cells, conditions were selected that have been shown to enhance this small current and largely eliminate contributions from other ionic currents (Somasundaram et al., 1995; see “Materials and Methods”). Briefly, the external medium contained 2 mM Ca$^{2+}$ and no K$^+$ or Na$^+$, and any outward K$^+$ currents were blocked by replacing the internal K$^+$ with Cs$^+$. The [Ca$^{2+}$]$_i$ was strongly buffered with 20 mM BAPTA. Under whole cell voltage clamp, depletion of internal Ca$^{2+}$ stores by 3 $\mu$M TG, an endoplasmic Ca-ATPase inhibitor, evoked an inward current at a holding potential of $-50$ mV (Fig. 1A). The currents generated by voltage ramps from $-140$ to $+50$ mV before TG application and at various times during the development of the inward current are shown in Fig. 1B. This inwardly rectifying current showed little or no reversal within the voltage range studied, developed without detectable single channel events, was selective for Ca$^{2+}$ (data not shown), and was blocked by 1 mM Zn$^{2+}$ (data not shown), all of which are characteristic of $I_{\text{CRAC}}$ reported in other cells (Fasalato et al., 1994; Somasundaram et al., 1995). To study the effect of temperature on the activation of $I_{\text{CRAC}}$, cells were held at various temperatures ranging from 15 to 27°C, and the current evoked by TG was recorded. The currents activated at a holding potential of $-20$ mV at all four different temperatures are shown in Fig. 1C. The maximum TG-induced current density was reduced with decreasing temperature such that no measurable current was activated at or below 17°C. The speed of development of the current was also reduced, and the times to half-maximum current were 82 ± 12, 72 ± 8, 55 ± 9, and 50 ± 7 s at 19, 20, 23, and 26°C, respectively. At maximal activation, the current-voltage relationship generated by voltage ramps from $-140$ mV to $+50$ mV at the different temperatures did not show any obvious shift in the reversal potential along the x axis (Fig. 1D). It should be noted that it was not technically possible to perform whole cell patch clamp recordings from cells held at temperatures greater than 27°C due to rapid deterioration of glass membrane seals.

The TG-evoked $I_{\text{CRAC}}$ densities, measured at $-120$ mV, in individual cells at different temperatures are shown in Fig. 2A. Despite the considerable variation in current density between cells, there is a clear positive correlation between current density and temperature. This relationship is further illustrated in Fig. 2B, in which the mean current density values at $-120$, $-70$, and $-20$ mV are plotted against temperature. Over the temperature range of 27 to 21°C the current density at all three voltages displayed little change, whereas further lowering of temperature caused a significant decrease in current, which became blocked completely at 17°C. To describe quan-
Temperature Effects on \( I_{\text{CRAC}} \)

![Temperature vs. Time Graph](image)

**Fig. 3. Activation of \( I_{\text{CRAC}} \) in store-depleted cells by raising the temperature.** A, effect of temperature (top trace) on the whole cell current (bottom trace) after the application of 3 \( \mu \text{g} \) TG at a holding potential of -50 mV. Bar, TG application at 17 °C. B, Membrane currents generated by voltage ramps from -120 to +60 mV during the experiment shown in A before (a) and at various temperatures (b, 17 °C; c, 20 °C; d, 28 °C; e, 20 °C) after the application of TG.

TG application at 17 °C resulted in \( I_{\text{CRAC}} \) activation, as shown by the experiment in Fig. 3. The current-voltage relationships at 17 °C 2 min after TG application (Fig. 3B, trace b) showed no evidence of a store-dependent current, whereas this current slowly developed following a temperature increase to 20 °C (Fig. 3B, trace c). A further abrupt increase in temperature to 28 °C produced a parallel increase in \( I_{\text{CRAC}} \) (Fig. 3B, trace d), and furthermore, this temperature-dependent effect was reversible, as shown by the currents obtained at 20 °C before and after increasing the temperature to 28 °C (Fig. 3B, traces c and e).

**Effect of Temperature on \( I_{\text{CRAC}} \) Maintenance**—We next studied the effect of temperature on \( I_{\text{CRAC}} \) following full activation at 24 °C, to determine whether the temperature dependence arose primarily from an effect on the activation mechanism of the current or via a direct effect on the influx pathway itself. In the experiments of Fig. 4, \( I_{\text{CRAC}} \) was maximally activated by TG-induced store depletion at 24.5 °C before the temperature was lowered to 14.5 °C at a rate of 0.1 °C/s and then returned to 24.5 °C. A continuous record of \( I_{\text{CRAC}} \) at a holding potential of -60 mV is shown in Fig. 4A, and current-voltage relationships at different temperatures are shown in Fig. 4B. Fig. 4C shows the average current density at -120 and -70 mV calculated from 10 cells at different temperatures following prolonged incubation at 14 °C.

When the temperature was dropped from 24.5 to 22 °C, the inhibition of current density was of a level (Fig. 5A) very similar to that observed when measuring temperature effects on \( I_{\text{CRAC}} \) activation (Fig. 2B). However, reduction in temperature from 22 to 14.5 °C resulted in incomplete inhibition of current. Even at 14.5 °C, the \( I_{\text{CRAC}} \) current density remained at 44% of maximum, in contrast to the complete inhibition at this temperature observed for \( I_{\text{CRAC}} \) activation (Fig. 2B). Indeed, even after prolonged incubations of up to 3 min at 14 °C, cells...
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**FIG. 4. Effect of temperature on the maintenance of I_{CRAC}.** A, effect of temperature (top trace) on the whole cell current (bottom trace) after maximal activation at 23°C by 3 µM TG at a holding potential of −40 mV. B, membrane currents generated by voltage ramps from −140 to +40 mV at different times during the experiments shown in A: a, at 23°C before TG; b, after maximal activation of I_{CRAC} at 23°C; c, after lowering the temperature to 16°C; and d, after reheating to 23°C. C, mean I_{CRAC} densities at −120 and −70 mV at different temperatures following activation by 3 µM TG at 24.5°C. The data were obtained from similar experiments as described in A and B and were corrected for background current measured prior to TG application. Each column is the mean from 10 cells. Bars, S.E.; pF, picofarad.

still maintained substantial amounts of I_{CRAC} (data not shown). This was in contrast to the complete block of I_{CRAC} activation observed at this temperature (Fig. 2A). To quantitatively describe the temperature dependence of I_{CRAC} maintenance, the mean current densities were plotted against temperature in the form of an Arrhenius plot (Fig. 5B) from which $E_a$ values were calculated for temperatures from 24.5 to 22°C of 5 kcal/mol ($Q_{10} = 1.4$) at −120 mV and 2.2 kcal/mol ($Q_{10} = 1.1$) at −70 mV. From 22 to 14.5°C, the $E_a$ values increased to 23 kcal/mol ($Q_{10} = 3.5$) at −120 mV and 35 kcal/mol ($Q_{10} = 5.6$) at −70 mV.

**FIG. 5. Analysis of the temperature dependence on the maintenance of I_{CRAC}.** A, mean I_{CRAC} densities at −120 and −70 mV as a function of temperature following maximal activation of I_{CRAC} at 24.5°C. The current densities were corrected for background current measured prior to TG application. Bars, S.E. B, Arrhenius plots of the data in A. Curves in A and B are the result of a four-variable logistic fit. pF, picofarad.

whole cell patch configuration, dialysis of the cytosol may lead to removal of intracellular components controlling Ca²⁺ influx. These potential problems were minimized by investigating the effect of temperature on store-mediated Ca²⁺ influx in intact cells using Mn²⁺ as a surrogate permeable ion and measuring the rate of the Mn²⁺-induced photoquench of cytosolic fura-2 at different temperatures. The experimental protocol is illustrated in Fig. 6A and shows a greatly increased Mn²⁺ quench in cells depleted at 37°C than in cells depleted at 16°C. TG-induced store release led to a substantial increase in Mn²⁺ influx compared with nondepleted cells at 32°C (Fig. 6B, traces c and d). At 10°C, however, the photoquench in store-depleted cells was indistinguishable from that in nondepleted cells (Fig. 6B, traces a and b). The differences in the Mn²⁺ photoquench between store-depleted and nondepleted cells at 10, 16, 22, and 32°C are shown in Fig. 6C. From this basal-subtracted photoquench, the rate of store-mediated Mn²⁺ entry at different temperatures was calculated (Fig. 6D). When the temperature was decreased from 37 to 28°C, there was a small reduction in the rate of the Mn²⁺ quench, but on decreasing the temperature further from 28 to 10°C, there was a drastic reduction in the rate of Mn²⁺ influx, with a complete block at 18°C. To examine the temperature effects on Mn²⁺ influx quantitatively, the rate of the store-mediated Mn²⁺ quench was plotted as an Arrhenius plot (Fig. 6E). The store-mediated Mn²⁺ influx shows a linear decrease in the rate of influx from 37 to 25°C with a $E_a$ of 2 kcal/mol ($Q_{10} = 1.2$). At about 25°C there is an abrupt increase in the sensitivity of Mn²⁺ influx to temperature. Below this temperature, the $E_a$ increases to 86 kcal/mol ($Q_{10} = 51$), reflecting a much higher energy barrier and similar to the increase observed when Ca²⁺ influx was measured as I_{CRAC}.

To establish whether this temperature sensitivity was a result of its effect on the activation mechanism of influx or on the influx pathway itself, we depleted the stores at 37°C prior...
to measuring the rate of Mn$^{2+}$ influx at various temperatures. The difference in the rate of the Mn$^{2+}$ photoquench between store-depleted and nondepleted cells measured in this way is shown in Fig. 7A. The maintenance of Mn$^{2+}$ influx was affected by temperature in a manner similar to activation. The Arrhenius plot (Fig. 7B) of the rate of the store-mediated Mn$^{2+}$ quench component shows a change of $E_a$ from 1.3 kcal/mol ($Q_{10} = 1$) between 37 and 23 °C to 119 kcal/mol ($Q_{10} = 61$) between 23 and 10 °C. Thus, in contrast to the patch clamp recordings, in which there was found to be a temperature effect on both activation and maintenance, using the Mn$^{2+}$ photoquench we could only detect a temperature-dependent block on the maintenance of the store-mediated influx pathway.

**Effect of Temperature on TG-induced Store Release**—The observed temperature-dependent block of store-mediated Ca$^{2+}$ entry may have been a consequence of temperature sensitivity in the ability of TG to release Ca$^{2+}$ from internal stores. This appears unlikely, however, since the temperature effects on Mn$^{2+}$ influx were equivalent whether TG was added at 37 °C or at lower temperatures (compare Fig. 6, D and E, with Fig. 7, A and B). To further assess whether Ca$^{2+}$ stores were being emptied at low temperatures, we used both fura-2 and a Ca$^{2+}$ minielectrode to measure TG-induced Ca$^{2+}$ release at different temperatures. As illustrated in Fig. 6A, TG was added to cells in nominally Ca$^{2+}$-free external medium at different temperatures, and the peak [Ca$^{2+}$], during the subsequent 6 min was assessed using the 340:380-nm fura-2 ratio, corrected (see “Materials and Methods”) for temperature effects on the $K_d$ of the dye (Fig. 8C). These results suggest that there was no significant difference in the level of Ca$^{2+}$ released at different temperatures by TG over 6 min. Further confirmation of this is illustrated in Fig. 8D. The initial pool size was assessed by measuring the peak [Ca$^{2+}$] following the addition of 5 μM ionomycin at both 18 and 34 °C. The degree of pool depletion (as a percentage of initial pool content) was measured by the ionomycin-induced [Ca$^{2+}$] rise following a 6-min incubation with TG and was not shown to be significantly different at these two temperatures. Since fura-2 measurements only indicate the net balance between Ca$^{2+}$ influx and efflux, we resorted to Ca$^{2+}$-sensitive minielectrode measurements on digitonin-permeabilized cells to directly assess TG-induced Ca$^{2+}$ store release at different temperatures. The protocol by which this was carried out is shown in Fig. 8A (also see “Materials and Methods”). The amount of Ca$^{2+}$ released by TG at different temperatures was measured as a percentage of the Ca$^{2+}$ released by ionomycin and is shown in Fig. 8B. There was no significant reduction in the percentage of Ca$^{2+}$ released by TG at lower temperatures. However, this method may also have limitations, since ionomycin may show some temperature sensitivity. Taken together, these two independent methods of testing the temperature sensitivity of TG-induced store release suggest that there is no significant difference in the degree of store release within the temperature range studied, and, hence, the temperature effects on store-mediated Ca$^{2+}$ and

\[ \text{Effect of temperature on store-dependent Mn}^{2+}\text{ entry.} A, \text{ fluorescence signals at } 360 \text{ nm (top traces, } F_{360}\text{) and } 340:380 \text{ fluorescence ratio (bottom traces, } R_{340:380}\text{) at 10 and 37 °C. Bars, timing of addition of 100 nM TG and 100 μM Mn}^{2+}. B, \text{ basal Mn}^{2+}\text{ photoquench of fura 2 at 10 °C (a) and 37 °C (c) and TG-induced Mn}^{2+}\text{ photoquench of fura 2 at 10 °C (b) and 37 °C (d). C, store-mediated component of Mn}^{2+}\text{ photoquench at different temperatures obtained by subtraction of basal from TG-induced Mn}^{2+}\text{ photoquench shown in B. D, mean rate of store-mediated Mn}^{2+}\text{ entry as a function of activation temperature. The rate of Mn}^{2+}\text{ entry was calculated from the initial slope (regression for the first 30 s) of the difference between TG-induced and basal Mn}^{2+}\text{ photoquench as shown in C. The rate of quench is in arbitrary units. Bars, S.E.; } n = 4 E, \text{ Arrhenius plot of the mean rate of Mn}^{2+}\text{ entry from data in D. Curves in D and E are the result of a for-variable logistic fit. All experiments were carried out in nominally Ca}^{2+}\text{-free saline.} \]
Temperature Effects on I_{CRAC}

Mn^{2+} influx are unlikely to be due to incomplete store release. The effect of temperature on I_{CRAC} and Mn^{2+} influx observed when the temperature was lowered. Most ion channels are known to be sensitive to temperature but exhibit a linear change in activity with change in temperature and have low activation energies (Bamburg and Lauger, 1974; Zeidel et al., 1992). Among Ca^{2+} channels, the voltage-gated channels from ventricular myocytes (Cavaliere et al., 1985) and sensory neurons (Nobile et al., 1990) also show a linear increase in current with increasing temperature due to an increase in channel probability. However, some ion channels, including the acetylcholine channel (Fischbach and Lass, 1978) and Ca^{2+} channels in neuroblastoma cells (Narahashi et al., 1987), show a nonlinear decrease in conductance when the temperature is lowered, with a transition around 20 °C, and it has been suggested that such transition effects are indicative of membrane-dependent regulation of channel gating (Romey et al., 1980). The nonlinearity in the Arrhenius plots of various membrane functions have been correlated with phase transition or lateral phase separation of membrane phospholipids (Raison 1972; Linden et al., 1973; Warren et al., 1975; Chapman, 1975). In the present study, the observed transition of I_{CRAC} and Mn^{2+} influx at 21–24 °C indicates that this influx pathway may also be in close association with a lipid membrane environment. A similar transition of Mn^{2+} influx at 21 °C has also been observed in rat parotid acinar cells (Lockwich et al., 1994). The store-mediated influx pathway shows a much greater increase in E_{c} compared with the acetylcholine channel or the neuroblastoma channels. It should be also noted that such transitional changes also apply to other membrane transport systems, such as transporters and pumps (Rega, 1986), and in view of the fact that the

**Fig. 7.** Effect of temperature on the maintenance of store-mediated Mn^{2+} influx. A, mean rate of Mn^{2+} entry as a function of temperature. B, Arrhenius plot of the data in A. The rate of Mn^{2+} photoquench was calculated as described in Fig. 6, B and C; however, the Ca^{2+} stores were first emptied at 37 °C using TG before changing to the desired temperature at which the Mn^{2+} quench was measured (see “Materials and Methods”). Curves in A and B are the result of a four-variable logistic fit.

The main finding of the present study is the nonlinear reduction of I_{CRAC} and Mn^{2+} influx following store depletion at 37 °C. The inhibitory effects of temperature on Ca^{2+} influx are unlikely to be due to partial emptying of stores by TG at lower temperatures. Both fura-2 and the Ca^{2+}-sensitive minielectrode measurements demonstrate that, following a 6-min incubation with TG, the Ca^{2+} released from stores did not vary significantly over the temperature range studied (10–37 °C). Furthermore, the temperature-dependent inhibition of Mn^{2+} influx was equivalent whether TG addition was carried out at low temperatures or at 37 °C, suggesting equivalent store depletion after 6 min at different temperatures.

Another concern was whether the observed temperature-dependent block of store-dependent Ca^{2+} influx was due to depletion of cytosolic ATP, since Gamberucci et al. (1994) have shown that a 5% reduction in cytosolic ATP concentration can inhibit store-mediated Ca^{2+} influx by 50%. However, our results show that cytosolic ATP concentrations at temperatures at which I_{CRAC} and Mn^{2+} influx were blocked (4 mM at 17–18 °C) were in fact greater than at higher temperatures (3 mM at 37 °C), at which the influx was fully activated, implying a temperature-dependent reduction in ATP hydrolysis.

The main discrepancy between the two methods may be due to a number of factors. In contrast to Mn^{2+} influx measurements, patch clamp recordings will lead to dialysis of the cytosol with two possible consequences. The use of internal solutions low in Na^{+}, K^{+}, and Cl^{-} and high in Ca^{2+} may alter temperature-dependent lipid rearrangements, which could explain the incomplete inhibition of I_{CRAC} seen on cooling following activation at 24 °C. In addition, any soluble factors that may inhibit I_{CRAC} will be dialyzed out. One such factor, ATP, which will be at much higher levels in intact cells, has recently been shown to inactivate I_{CRAC} possibly via protein kinase C activation (Parekh and Penner, 1995). This may explain the complete temperature-dependent inhibition of Mn^{2+} entry following store depletion at 37 °C.

DISCUSSION

The present study demonstrates that, in KU-812 cells, store-regulated Ca^{2+} and Mn^{2+} entry is strongly dependent on temperature. It is clear from patch clamp recordings that both the activation of I_{CRAC} and its maintenance are temperature-sensitive. The specific temperature effect on activation is apparent from the fact that development of I_{CRAC} is blocked at 17 °C, although once activated, lowering the temperature even to 14 °C does not totally inhibit the current. The temperature effects on the maintenance of I_{CRAC} are demonstrated by the nonlinear decrease when the temperature is lowered, with an abrupt transition around 22 °C. Although Mn^{2+} quench measurements also showed equivalent temperature-dependent decreases in influx with an abrupt transition temperature, we could only detect temperature effects on maintenance of influx, since these were equivalent regardless of whether the temperature was changed before or after store release.

This discrepancy between the two methods may be due to two possible consequences. The use of internal solutions low in Na^{+}, K^{+}, and Cl^{-} and high in Ca^{2+} may alter temperature-dependent lipid rearrangements, which could explain the incomplete inhibition of I_{CRAC} seen on cooling following activation at 24 °C. In addition, any soluble factors that may inhibit I_{CRAC} will be dialyzed out. One such factor, ATP, which will be at much higher levels in intact cells, has recently been shown to inactivate I_{CRAC} possibly via protein kinase C activation (Parekh and Penner, 1995). This may explain the complete temperature-dependent inhibition of Mn^{2+} entry following store depletion at 37 °C.

The inhibitory effects of temperature on Ca^{2+} influx are unlikely to be due to partial emptying of stores by TG at lower temperatures. Both fura-2 and the Ca^{2+}-sensitive minielectrode measurements demonstrate that, following a 6-min incubation with TG, the Ca^{2+} released from stores did not vary significantly over the temperature range studied (10–37 °C). Furthermore, the temperature-dependent inhibition of Mn^{2+} influx was equivalent whether TG addition was carried out at low temperatures or at 37 °C, suggesting equivalent store depletion after 6 min at different temperatures.

Another concern was whether the observed temperature-dependent block of store-dependent Ca^{2+} influx was due to depletion of cytosolic ATP, since Gamberucci et al. (1994) have shown that a 5% reduction in cytosolic ATP concentration can inhibit store-mediated Ca^{2+} influx by 50%. However, our results show that cytosolic ATP concentrations at temperatures at which I_{CRAC} and Mn^{2+} influx were blocked (4 mM at 17–18 °C) were in fact greater than at higher temperatures (3 mM at 37 °C), at which the influx was fully activated, implying a temperature-dependent reduction in ATP hydrolysis.

The main finding of the present study is the nonlinear reduction of I_{CRAC} and Mn^{2+} influx observed when the temperature was lowered. Most ion channels are known to be sensitive to temperature but exhibit a linear change in activity with change in temperature and have low activation energies (Bamburg and Lauger, 1974; Zeidel et al., 1992). Among Ca^{2+} channels, the voltage-gated channels from ventricular myocytes (Cavaliere et al., 1985) and sensory neurons (Nobile et al., 1990) also show a linear increase in current with increasing temperature due to an increase in open channel probability. However, some ion channels, including the acetylcholine channel (Fischbach and Lass, 1978) and Ca^{2+} channels in neuroblastoma cells (Narahashi et al., 1987), show a nonlinear decrease in conductance when the temperature is lowered, with a transition around 20 °C, and it has been suggested that such transition effects are indicative of membrane-dependent regulation of channel gating (Romey et al., 1980). The nonlinearity in the Arrhenius plots of various membrane functions have been correlated with phase transition or lateral phase separation of membrane phospholipids (Raison 1972; Linden et al., 1973; Warren et al., 1975; Chapman, 1975). In the present study, the observed transition of I_{CRAC} and Mn^{2+} influx at 21–24 °C indicates that this influx pathway may also be in close association with a lipid membrane environment. A similar transition of Mn^{2+} influx at 21 °C has also been observed in rat parotid acinar cells (Lockwich et al., 1994). The store-mediated influx pathway shows a much greater increase in E_{c} compared with the acetylcholine channel or the neuroblastoma channels. It should be also noted that such transitional changes also apply to other membrane transport systems, such as transporters and pumps (Rega, 1986), and in view of the fact that the
The store-dependent Ca\textsuperscript{2+} influx pathway has been shown to be of very low conductance with no single channel noise, the possibility of a transporter being involved in capacitative Ca\textsuperscript{2+} influx should not be ruled out. The possibility also exists that abrupt breaks in Arrhenius plots may reflect intrinsic changes in the protein conformation independent of changes in the membrane phospholipid (Dean and Tanford, 1978; Sondergaard, 1979; Hoffman et al., 1979). This may be occurring in the store-dependent Ca\textsuperscript{2+} influx pathway, in which a possible conformational coupling between stores and plasma membrane has been postulated (Irvine, 1992; Berridge, 1995).

The other interesting finding is that \textit{I\textsubscript{CRAC}} activation is completely inhibited at 17 °C but that, once activated, the current can be maintained even at lower temperatures. These observations suggest that, unlike the effect of temperature on Mn\textsuperscript{2+} entry, there is an additional effect of temperature specifically on \textit{I\textsubscript{CRAC}} activation. Although this could be a consequence of intracellular dialysis, it is consistent with the possible involvement of vesicular transport in the activation pathway of \textit{I\textsubscript{CRAC}}. It is established that low temperatures, between 15 and 22 °C, block the \textit{in vivo} transport of membrane constituents at different points along the endoplasmic reticulum-golgi apparatus-plasma membrane pathway (Matlin and Simons 1983; Tartakoff, 1986; Saraste et al., 1986; Moreau and Cassagne, 1994). This transition temperature is dependent on the various chain lengths of the phospholipid, and typically in animal cells, the golgi apparatus-plasma membrane pathway mediates the transfer of C\textsubscript{20}–C\textsubscript{24}-containing phospholipids, which is blocked at 16 °C (Moreau and Cassagne, 1994). Furthermore, this may also explain the fact that the maintenance of \textit{I\textsubscript{CRAC}}, although reduced, is not totally inhibited by lowering the temperature. Once the activation of the influx pathway via a vesicular transport process has been achieved (recruitment of channels or regulators of channels to the plasma membrane), then blocking this process should not inhibit Ca\textsuperscript{2+} influx, since the channels, or channel regulators, are already on the plasma membrane.

FIG. 8. Effect of temperature on TG-induced release of stored Ca\textsuperscript{2+}. A, percentage Ca\textsuperscript{2+} released by TG (3 μM) and ionomycin (10 μM) in digitonin-permeabilized cell suspensions at 15 and 30 °C. The cells were suspended in an internal medium containing an ATP-regenerating system, and Ca\textsuperscript{2+} was measured by a mini-Ca\textsuperscript{2+} electrode (see "Materials and Methods"). B, TG-induced Ca\textsuperscript{2+} store release measured from experiments as in A, given as a mean percentage of ionomycin (Iono)-induced Ca\textsuperscript{2+} release, plotted as a function of temperature. Bars, S.D.; n = 3. C, effect of temperature on the mean TG-induced peak [Ca\textsuperscript{2+}], measured as 340:380 fluorescence ratio (\textit{R\textsubscript{340:380}}). The ratio was multiplied by the \textit{K\textsubscript{d}} of fura-2 at different temperatures (see "Materials and Methods"). Bars, S.D. D, TG-induced Ca\textsuperscript{2+} release at 18 and 34 °C calculated from the difference between peak [Ca\textsuperscript{2+}], rise induced by ionomycin before and 6 min after TG addition (n = 3). This is expressed as a percentage of the ionomycin-induced peak [Ca\textsuperscript{2+}], rise before TG addition.

FIG. 9. Effect of temperature on cytosolic [ATP]. The cytosolic ATP concentration is plotted as a function of incubation temperature; each point is the mean of two experiments. Cell suspensions were incubated for 6 min at different temperatures in a standard external medium containing 2 mM Ca\textsuperscript{2+} before cytosolic [ATP] was determined using a luciferin/luciferase assay (see "Materials and Methods").
Temperature Effects on $I_{\text{CRAC}}$

In conclusion, we have provided the first direct evidence demonstrating that both activation and maintenance of the store-mediated Ca\(^{2+}\) influx pathway are exquisitely temperature-sensitive, suggesting a very intimate association with the lipid membrane environment.

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