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Isolation and Characterization of Membrane Potential Changes Associated With Release of Calcium from Intracellular Stores in Rat Thymic Lymphocytes

O.I. Wilson,[1] I. Marriott,[1] M.P. Mahaut-Smith,[2] L.J. Hymel,[1] M.J. Mason[1]

[1]Department of Physiology, Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, Louisiana 70112
[2]Physiological Laboratory, Cambridge University, Cambridge, England CB2 3EG

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Abstract. Membrane potential changes accompanying Ca^{2+} influx stimulated by release of Ca^{2+} from intracellular stores (store-regulated Ca^{2+} uptake) were monitored in BAPTA-loaded rat thymic lymphocytes using the fluorescent indicator *bis*(1,3-diethylthiobarbituric acid)trimethine oxonol. Depletion of $[Ca^{2+}]_i$ stores by the application of thapsigargin, ionomycin or cyclopiazonic acid induced a depolarization which was (i) dependent upon BAPTA-loading, (ii) dependent upon extracellular Ca^{2+}, (iii) independent of extracellular Na^+ and (iv) abolished by 5 mM extracellular Ni^{2+}. This depolarization was followed by a charybdotoxin-sensitive repolarization and subsequent hyperpolarization to values approximating the K^+ equilibrium potential, consistent with secondary activation of a K^+ conductance. These membrane potential changes temporally correlated with Ca^{2+} influx from the extracellular medium as measured fluorimetrically with indo-1. The divalent cation permeability sequence was investigated by monitoring the magnitude of the depolarization observed following the addition of 4 mM Ca^{2+}, Mn^{2+}, Ba^{2+} or Sr^{2+} to cells pretreated with doses of thapsigargin or ionomycin known to activate the store-regulated calcium uptake pathway. On the basis of these experiments, we conclude that the store-regulated Ca^{2+} uptake pathway has the following permeability sequence: $Ca^{2+} > Mn^{2+} >> Ba^{2+}$, Sr^{2+} with Mn^{2+} displaying significant permeability relative to Ca^{2+}. This pathway is distinguishable from other divalent cation uptake pathways reported in other cells types on the basis of its activation by thapsigargin and its high Mn^{2+} permeability.

Correspondence to: M.J. Mason

Key words: Membrane potential — Ca^{2+} channels — Thapsigargin — T-lymphocytes — Mn^{2+}

Introduction

One of the earliest ionic events following mitogenic stimulation of T-lymphocytes is a rise in $[Ca^{2+}]_i$. When measured in cell suspensions, this increase in $[Ca^{2+}]_i$ is composed of two components. The first is a release of Ca^{2+} from intracellular stores followed by a second component resulting from a sustained electrogenic influx of Ca^{2+} across the plasma membrane from extracellular sources. The combination of these two mechanisms results in a biphasic rise in $[Ca^{2+}]_i$ with a transient peak due to release from stores subsiding to a lower, sustained plateau phase maintained by the electrogenic influx of Ca^{2+} from the extracellular medium.

The mechanism responsible for the initial Ca^{2+} peak following mitogenic stimulation is well characterized and is generally accepted to be due to IP_3-mediated release of Ca^{2+} from an intracellular Ca^{2+} pool (IP_3-sensitive Ca^{2+} pool) [34]. It is well documented that the secondary sustained rise in $[Ca^{2+}]_i$ is due to influx of Ca^{2+} from the extracellular medium inasmuch as it is stringently dependent upon the availability of extracellular Ca^{2+} [2, 35], is blocked by membrane impermeant inorganic antagonists such as La^{3+} and Ni^{2+} [3, 24, 30] and is associated with an increase in unidirectional divalent cation uptake measured isotopically with $^{45}Ca^{2+}$ [6, 24].

Uptake of Ca^{2+} from the extracellular medium occurs via an electrogenic pathway inasmuch as it is exquisitely sensitive to alterations in the transmembrane potential [7, 9]. A number of mechanisms have been proposed to account for the activation of the electrogenic plasma membrane Ca^{2+} influx pathway in cul-

tured T-cell lines including direct activation of a Ca^{2+} channel by IP_3 [16] or elevations in $[Ca^{2+}]_i$ [28, 29], activation of voltage-dependent Ca^{2+} channels [4, 5] and activation of Ca^{2+} channels via a direct receptor/channel interaction [31]. Results from our laboratory have recently demonstrated that an electrogenic plasma membrane Ca^{2+} influx pathway can be activated in rat thymic lymphocytes by a mechanism independent of: (i) IP_3 formation, (ii) increases in $[Ca^{2+}]_i$ and (iii) plasma membrane depolarization. These explanations therefore fall short of explaining the mechanism underlying the activation of the plasma membrane influx pathway in thymic lymphocytes [22, 24, 25].

In contrast, a significant body of evidence is accumulating in support of the contention that the plasma membrane Ca^{2+} permeability of lymphoid tissues is regulated in large part by the Ca^{2+} content of the IP_3-sensitive pool (*store-regulated Ca^{2+} uptake; SRCU*) [3, 8, 22, 24, 27, 33]. The coupling between the plasma membrane and the IP_3-sensitive endomembrane pool is important not only in mediating the $[Ca^{2+}]_i$ effects of mitogenic stimuli and other agonists but seemingly also in the control of resting $[Ca^{2+}]_i$ homeostasis [22, 24, 27].

To date, neither direct nor indirect electrophysiological measurement of Ca^{2+} currents attributed to SRCU has been reported in lymphocytes. Using alterations in membrane potential as a measure of changes in the plasma membrane conductance properties, experiments were undertaken to isolate membrane potential changes associated with the activation of SRCU in rat thymic lymphocytes.

Materials and Methods

Reagents and Solutions

The acetoxymethyl ester (AM) derivatives of indo-1 and 1,2-*bis*-(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA) and *bis*(1,3-diethylthiobarbituric acid)trimethine oxonol (*bis*-oxonol) were purchased from Molecular Probes (Eugene, OR). Ionomycin, thapsigargin and HEPES were obtained from Calbiochem (San Diego, CA). The culture medium RPMI 1640 (bicarbonate-free), gramicidin, cyclopiazonic acid and ethyleneglycol-*bis*-(β-aminoethyl)-*N,N,N′,N′*-tetraacetic acid (EGTA) were purchased from Sigma (St. Louis, MO). Charybdotoxin was purchased from Peninsula Laboratories (Belmont, CA). N-methyl-D-glucamine (NMG), $NiCl_2$ hexahydrate, and anhydrous dimethyl sulfoxide were purchased from Aldrich (Milwaukee, WI). NaCl, KCl, $CaCl_2$, $MgCl_2$, $MnCl_2$, D-glucose, NaOH and KOH were purchased from Fisher Scientific (Houston, TX). Stock solutions of indo-1-AM, BAPTA-AM, thapsigargin, cyclopiazonic acid and gramicidin were made up in anhydrous dimethyl sulfoxide. Ionomycin was dissolved in ethanol.

The basic solution used in the fluorescence experiments contained (mM) 140 NMGCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose, and 20 HEPES-free acid. NaCl solution was made by replacing NMGCl with NaCl. Solutions were titrated to pH 7.3 at 35°C with NMG or NaOH as required. Nominally Ca^{2+}-free solution was made by omitting Ca^{2+}. When required, Ca^{2+}, Mn^{2+}, Ba^{2+}, Sr^{2+} and Ni^{2+} were added directly to the solution as chloride salts. All solutions and stocks were stored at −20°C.

Cell Isolation and Manipulations

Thymic lymphocytes were isolated from 140–200 g male Wistar rats (Charles River Breeding Laboratories) as previously described [10]. The cells were counted using a Model ZM Coulter Counter (Coulter Electronics, Hialeah, FL) and maintained at room temperature at a concentration of 25–45 × 10^6 cells/ml in bicarbonate-free RPMI 1640 culture medium buffered to pH 7.4 with 20 mM HEPES.

Fluorescence Determinations

All experiments were performed at 35–37°C using a Photon Technology International fluorescence spectrophotometer (Delta Scan) equipped with a magnetic stirrer and temperature control. The cells were counted immediately after the last manipulation, prior to addition to the cuvette, to ensure that the appropriate cell number was added. All results presented are representative of experiments in a minimum of three preparations.

Determination of Free Cytosolic Calcium Concentration

$[Ca^{2+}]_i$ was determined by measuring the fluorescence of indo-1 as previously reported [24]. The excitation and emission wavelengths used were 331 nm (3 nm slit width) and 405 nm (10 nm slit width), respectively. Thymocyte suspensions (25 × 10^6 cells/ml) were loaded with indo-1 by incubation with a 2 μM concentration of the AM precursor for 25 min at 37°C in basic NaCl solution. When required, cells were simultaneously loaded with BAPTA by addition of 20 μM BAPTA-AM to the incubation medium. The cells were then sedimented, resuspended in basic solution and kept at room temperature until required. To monitor fluorescence, aliquots containing the required cell number were sedimented, resuspended in the appropriate medium plus or minus Ca^{2+}, as indicated, and added to the cuvette. Fluorescence was calibrated using ionomycin and Mn^{2+} as previously described using a dissociation constant of 250 nM [13].

Determination of Membrane Potential (E_m)

Determinations of E_m were made by measuring the fluorescence of the negatively charged dye *bis*-oxonol [32] at excitation and emission wavelengths of 540 nM (3 mm slit width) and 580 nM (10 nm slit width), respectively. Aliquots containing the appropriate cell number were sedimented, resuspended in the indicated medium and added to the cuvette at a final concentration of 1 × 10^6 cells/ml. *Bis*-oxonol was then added to a final concentration of 0.15 μM and the fluorescence was monitored. External calibration was made by adding gramicidin to cells suspended in media containing varying ratios of Na^+ and NMG^+. A calibration curve was constructed as previously described [11], assuming comparable gramicidin-induced Na^+ and K^+ conductances and/or that intra- and extracellular concentrations of alkali cations are identical at equilibrium.

Results

We have previously reported that release of Ca^{2+} from intracellular pools in rat thymic lymphocytes, stimulat-

ed by the addition of the endosomal Ca^{2+}-ATPase inhibitor thapsigargin, is accompanied by a marked membrane potential-sensitive uptake of Ca^{2+} from the extracellular medium (SRCU) [22, 24, 25]. We monitored the effect on E_m of SRCU stimulated by thapsigargin addition in normal thymic lymphocytes in Na^+-free medium. Na^+-free solutions were utilized to eliminate the effect of Na^+ conductance on the interpretation of the results. A representative experiment is presented in Fig. 1*A*. Addition of 33 nM thapsigargin resulted in a pronounced hyperpolarization which was exquisitely sensitive to charybdotoxin, a known inhibitor of Ca^{2+}-gated K^+ channels in these cells [12, 20, 21]. Given that this experiment was performed in Na^+-free medium, subsequent addition of gramicidin (40 nM), a monovalent cation-forming channel antibiotic, resulted in a pronounced hyperpolarization to a value approaching the K^+ equilibrium potential. Additionally, the thapsigargin-mediated hyperpolarization was slowly reversed by the addition of 5 mM Ni^{2+} (Fig. 1*B*). In parallel measurements using indo-1, addition of Ni^{2+} following thapsigargin-mediated elevations in $[Ca^{2+}]_i$ resulted in recovery of Ca^{2+} to near basal levels (*results not shown*). This is consistent with inhibition of Ca^{2+} uptake and subsequent extrusion of Ca^{2+} via the plasma membrane Ca^{2+} pump. Such a fall in $[Ca^{2+}]_i$ would be expected to inactivate a Ca^{2+}-gated K^+ channel, resulting in a return to basal E_m. The recovery to basal levels of E_m following addition of Ni^{2+} is overcome by addition of gramicidin, precluding an artifactual influence of Ni^{2+} on the fluorescence properties of *bis*-oxonol. Taken in concert, these data support the proposal that Ca^{2+} uptake mediated by thapsigargin-stimulated release of Ca^{2+} from intracellular pools results in the activation of Ca^{2+}-gated K^+ channels.

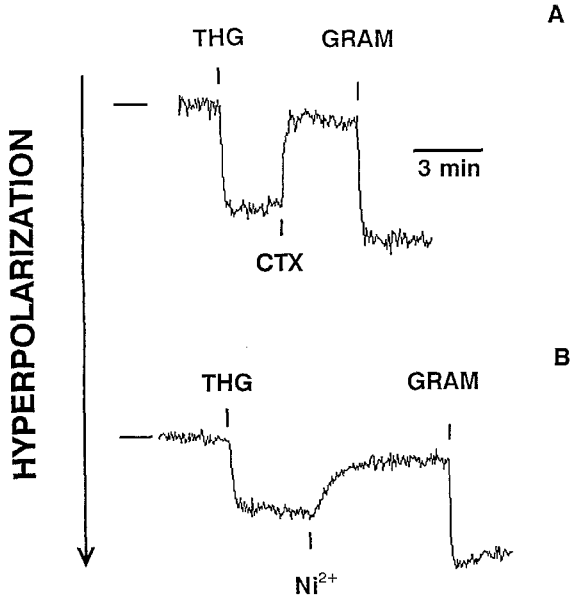

Fig. 1. Effect of thapsigargin on E_m in normal rat thymic lymphocytes. Non-BAPTA-loaded cells were suspended in NMG medium containing 5 mM Ca^{2+}, and the fluorescence of *bis*-oxonol was monitored and calibrated as outlined under Materials and Methods. (*A*) Where indicated 33 nM thapsigargin (*THG*), 50 nM charybdotoxin (*CTX*) or 40 nM gramicidin (*GRAM*) was added. (*B*) Where indicated, 5 mM Ni^{2+} (added a $NiCl_2$) or 40 nM gramicidin (*GRAM*) was added.

To investigate the E_m changes directly attributable to electrogenic influx of Ca^{2+} from the extracellular medium, it was necessary to accentuate the Ca^{2+} current while delaying or abolishing the onset of Ca^{2+}-gated K^+ channel activity described above. To accomplish this, the Ca^{2+}-buffering power of the cells was increased by loading with the Ca^{2+} chelator BAPTA. This procedure effectively increases the amount of Ca^{2+} entering the cell while concomitantly delaying the onset of the Ca^{2+}-gated K^+ current. Such a technique has previously been used for investigations of the E_m changes associated with receptor-linked events in human B-lymphocytes [19]. To further amplify the Ca^{2+} current, extracellular Ca^{2+} (Ca^{2+}_o) was increased to 7 mM. These experiments were performed in Na^+-free medium. It should be noted, however, that the presence of Na^+ had no effect on the results, consistent with a minimal involvement of Na^+ in the response. A representative experiment is presented in Fig. 2*A*. Addition of 33 nM thapsigargin induced a marked depolarization of approximately 30 mV. This depolarization was followed by a pronounced repolarization and subsequent hyperpolarization to a value approximating the K^+ equilibrium potential (−100 mV) as depicted in Fig. 2*A*. Addition of 40 nM gramicidin resulted in a further small hyperpolarization.

Origin of the Depolarization

Experiments were performed to determine the ionic basis of the depolarization phase of the response and the underlying mechanism. In support of the hypothesis that the depolarization phase is a direct result of an increase in the plasma membrane Ca^{2+} conductance, we have found the magnitude of the depolarization to be dependent upon the extracellular concentration of Ca^{2+} within the range of 1 to 7 mM. A representative experiment is presented in Fig. 2*A-C*. Activation of this Ca^{2+}-dependent depolarization could be secondary to release of Ca^{2+} from intracellular stores or alternatively a result of a direct effect of thapsigargin on the Ca^{2+} permeability of the plasma membrane. To distinguish between these possibilities, we investigated the effect on E_m of alternative compounds known to release Ca^{2+} from intracellular pools. Ionomycin has been shown to release Ca^{2+} from intracellular stores in rat thymic lymphocytes [23]. Low doses of ionomycin were used to minimize

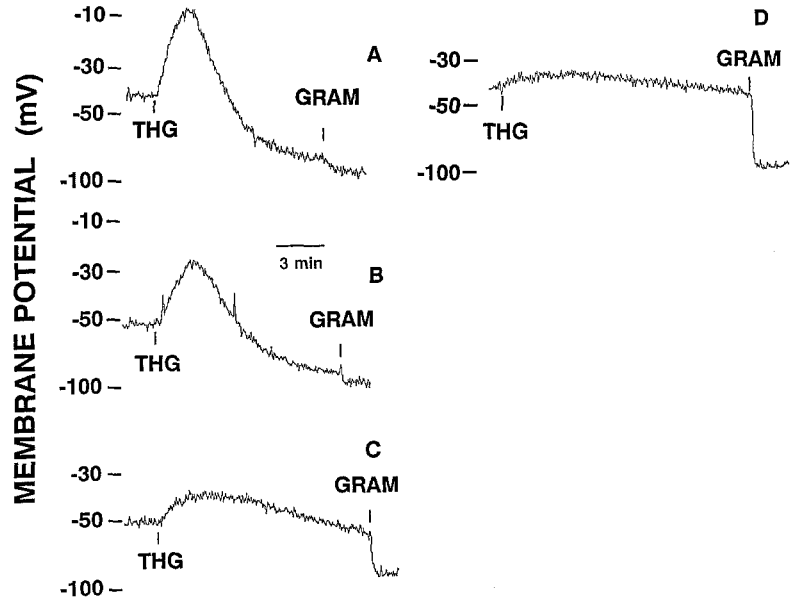

Fig. 2. Effect of external $[Ca^{2+}]$ on thapsigargin-mediated changes in E_m in BAPTA-loaded rat thymic lymphocytes. The fluorescence of *bis*-oxonol was monitored in BAPTA-loaded cells suspended in NMG medium containing 7 mM (*A*), 4 mM (*B*) or 1 mM (*C*) extracellular Ca^{2+}. Where indicated, 33 nM thapsigargin (*THG*) or 40 nM gramicidin (*GRAM*) was added. Divalent cation concentration was maintained at 8 mM by the addition of extra Mg^{2+}. (*D*) Effect of Ni^{2+} on thapsigargin-mediated changes in E_m in BAPTA-loaded cells. Cells were exposed to 7 mM Ca^{2+} in the presence of 5 mM Ni^{2+}, and the fluorescence of *bis*-oxonol was monitored. Where indicated, 33 nM thapsigargin (*THG*) or 40 nM gramicidin (*GRAM*) was added. All experiments were performed with cell aliquots taken from the same BAPTA-loading procedure.

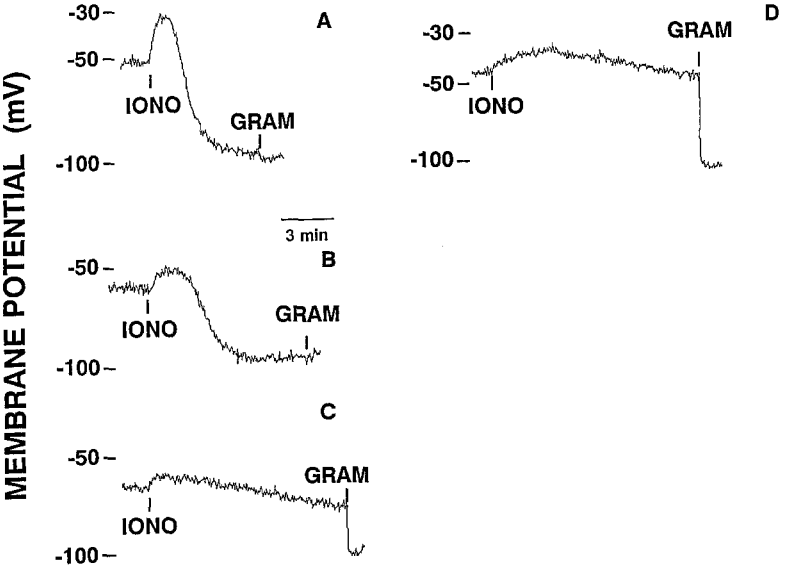

Fig. 3. Effect of external $[Ca^{2+}]$ on ionomycin-mediated changes in E_m in BAPTA-loaded rat thymic lymphocytes. The fluorescence of *bis*-oxonol was monitored in BAPTA-loaded cells suspended in NMG medium containing 7 mM (*A*), 4 mM (*B*) or 1 mM (*C*) extracellular Ca^{2+}. Where indicated, 20 nM ionomycin (*IONO*) or 40 nM gramicidin (*GRAM*) was added. Divalent cation concentration was maintained at 8 mM by the addition of extra Mg^{2+}. (*D*) Effect of Ni^{2+} on ionomycin-mediated changes in E_m in BAPTA-loaded cells. Cells were exposed to 7 mM Ca^{2+} in the presence of 5 mM Ni^{2+}, and the fluorescence of *bis*-oxonol was monitored. Where indicated, 20 nM ionomycin (*IONO*) or 40 nM gramicidin (*GRAM*) was added. All experiments were performed with cell aliquots taken from the same BAPTA-loading procedure.

the concomitant rise in $[Ca^{2+}]_i$ attributable to electroneutral transport of Ca^{2+} by the ionophore. Changes in E_m indistinguishable from those activated by thapsigargin were observed following the addition of 20 nM ionomycin. Additionally, the E_m depolarization activated by this concentration of ionomycin was dependent upon the concentration of extracellular Ca^{2+} (Fig. 3).

Another inhibitor of endosomal Ca^{2+}-ATPase activity, cyclopiazonic acid, has been shown to release Ca^{2+} from the thapsigargin-sensitive pool of rat thymic lymphocytes [22, 25]. Cyclopiazonic acid (5 μM) was found to activate extracellular Ca^{2+}-dependent E_m changes indistinguishable from those activated by thapsigargin or ionomycin (*results not shown*).

Experiments were undertaken to investigate the effect of extracellular Ni^{2+} on the E_m response observed following release of Ca^{2+} from intracellular stores in BAPTA-loaded lymphocytes. Ni^{2+} (5 mM) effectively blocked the depolarization induced by thapsigargin (Fig. 2*A* and *D*) and ionomycin (Fig. 3*A* and *D*). Ni^{2+} (5 mM) also abolished the depolarization induced by cyclopiazonic acid (*results not shown*). In further support of the hypothesis that an increase in Ca^{2+} conductance underlies the depolarization phase, 5 mM Ni^{2+} inhibited the

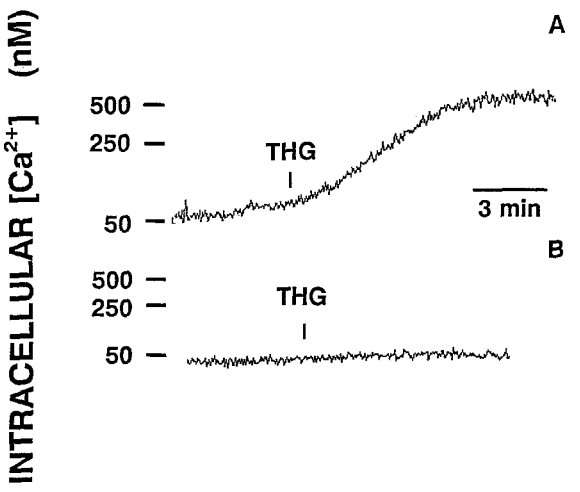

Fig. 4. Changes in $[Ca^{2+}]_i$ induced by thapsigargin in BAPTA-loaded cells. Cells loaded with indo-1 and BAPTA were suspended in NMG medium containing 4 mM Ca^{2+} in the presence (*B*) or absence (*A*) of 5 mM Ni^{2+}, and the fluorescence of indo-1 was monitored. Where indicated, 33 nM thapsigargin (*THG*) was added. These are paired experiments performed with cell aliquots from the same BAPTA-loading procedure.

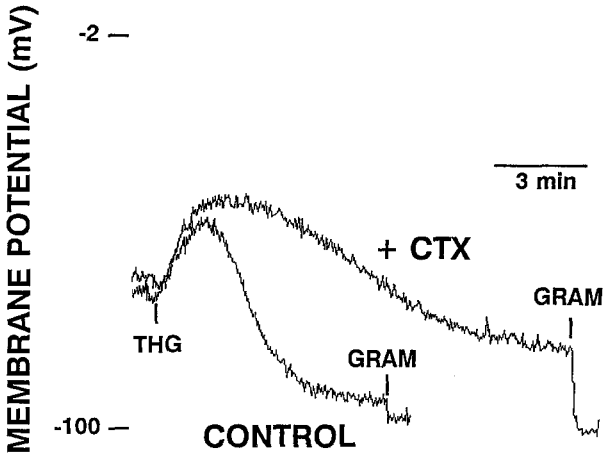

Fig. 5. Effect of charybdotoxin on thapsigargin-mediated changes in E_m in BAPTA-loaded cells. BAPTA-loaded cells were suspended in NMG medium containing 5 mM Ca^{2+} in the presence (top trace) or absence (bottom trace) of 150 nM charybdotoxin (*CTX*), and the fluorescence of *bis*-oxonol was monitored. Thapsigargin (*THG*) (33 nM) and 40 nM gramicidin (*GRAM*) were added where indicated. Paired experiments were performed with cell aliquots from the same BAPTA-loading procedure.

thapsigargin-mediated slow rise in $[Ca^{2+}]_i$ detected in BAPTA-loaded cells (Fig. 4). Extracellular Ni^{2+} also abolished the slow rise in $[Ca^{2+}]_i$ induced by low doses of ionomycin and cyclopiazonic acid (*results not shown*).

Origin of the Hyperpolarization

Experiments were undertaken to investigate the origin of the secondary repolarization and subsequent hyperpolarization phases of the E_m response mediated by release of Ca^{2+} from intracellular stores. Such E_m changes may be a result of activation of Ca^{2+}-gated K^+ channels, secondary to the slow rise in $[Ca^{2+}]_i$ triggered by release of stored Ca^{2+} (Fig. 4). Consistent with the dependence of the observed hyperpolarization on elevations in $[Ca^{2+}]_i$, we have found that in addition to abolishing both Ca^{2+} uptake and the Ca^{2+}-dependent depolarization phase, prior exposure to 5 mM Ni^{2+} also abolishes the secondary hyperpolarization phase (Figs. 2 and 3). Furthermore, prior incubation with 150 nM charybdotoxin, a potent inhibitor of Ca^{2+}-gated K^+ channels in rat thymic lymphocytes [12, 20, 21], markedly slowed the rate of repolarization and inhibited the magnitude of the subsequent hyperpolarization (Fig. 5).

Permeability to Other Divalent Ions

The SRCU pathway has previously been shown to transport Mn^{2+} as judged by Mn^{2+} quenching of intracellularly trapped fluorescent Ca^{2+} indicators such as indo-1 and fura2 [1, 22, 25, 27]. While such experiments have provided qualitative information regarding the state of activation of this pathway, they have not provided information regarding the relative Mn^{2+} permeability in rat thymic lymphocytes. To address this question, we have monitored E_m changes during addition of Ca^{2+} or Mn^{2+} to BAPTA-loaded cells suspended in Na^+-containing medium devoid of these divalents. To ensure thorough activation of the SRCU pathway prior to divalent addition, cell suspensions were pretreated with thapsigargin. Under these conditions, the magnitude of the E_m depolarization, monitored following the re-addition of 5 mM Ca^{2+} or Mn^{2+}, provides an index of the relative permeability of these divalents. To ensure that the magnitude of the depolarization is not affected by the activation of Ca^{2+}-gated K^+ channel activity, we performed all experiments in the presence of 150 nM charybdotoxin. In all experiments, the response to Ca^{2+} or Mn^{2+} addition was tested in the same batch of cells to control for differences between cell preparations and any differences in the effectiveness of the BAPTA-loading procedure. Addition of 5 mM Ca^{2+} resulted in a depolarization of 30 mV from a resting E_m of −60 mV (Fig. 6). In comparison, addition of 5 mM Mn^{2+} to cells taken from the same batch resulted in a depolarization of 24 mV from a resting potential of −60 mV. The results of this series of experiments are summarized in the Table. On average, addition of 5 mM Ca^{2+} depolarized the cells approximately 25 mV com-

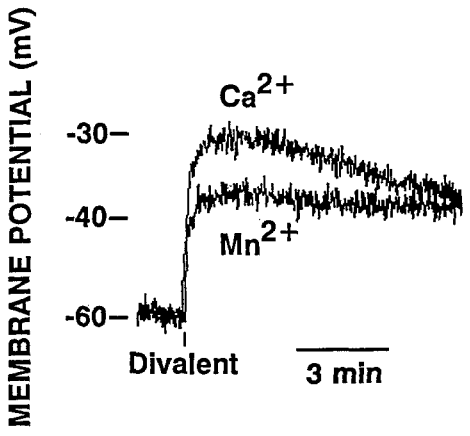

Fig. 6. Effect of the reintroduction of Ca^{2+} or Mn^{2+} on E_m of thapsigargin-treated cells. BAPTA-loaded thymic lymphocytes were suspended in nominally Ca^{2+}-free Na^{2+} medium in the presence of 33 nM thapsigargin and 150 nM charybdotoxin, and the *bis*-oxonol fluorescence was monitored. Ca^{2+} (4 mM) (top trace) or Mn^{2+} (bottom trace) was added as indicated. These are paired experiments performed with cell aliquots from the same BAPTA-loading procedure.

pared with 18 mV for Mn^{2+} addition ($P < 0.05$, $n = 9$). On the basis of these data, we conclude that the pathway is highly permeable to Mn^{2+}.

Consistent with the hypothesis that the Ca^{2+} and Mn^{2+}-mediated depolarization is a result of electrogenic divalent cation uptake, we have found that this depolarization is abolished by pre-exposure to 5 mM Ni^{2+} (Fig. 7). In further support of the hypothesis, we have found that 5 mM Ni^{2+} totally abolished the slow rise in $[Ca^{2+}]_i$ monitored in parallel experiments in which cells were treated identically (Fig. 8).

Experiments identical to those presented in Fig. 6 were performed using low doses of ionomycin as the stimulus to release Ca^{2+} from intracellular stores. The results of these experiments are summarized in the Table. In the presence of 20 nM ionomycin, re-addition of 5 mM Ca^{2+} or Mn^{2+} resulted in a mean depolarization of 22 and 18 mV, respectively ($P < 0.05$, $n = 7$), consistent with significant Mn^{2+} permeability.

As evident in Fig. 7, the addition of 5 mM Ni^{2+} resulted in only a very modest apparent depolarization while virtually abolishing the marked depolarization induced by addition of Ca^{2+} or Mn^{2+}. This small apparent depolarization induced by Ni^{2+} was not always evident, with equal numbers of experiments displaying no change in fluorescence or a modest decline in this parameter. Such results precluded the possibility that the marked depolarization observed following Ca^{2+} or Mn^{2+} addition in the absence of Ni^{2+} was due to artifactual increases in fluorescence due to alterations in surface charge. Alterations in surface charge might be expected as a result of the re-addition of high concentrations of divalent ions to the medium. Surface charge effects were avoided by performing these experiments in Na^+-containing medium. As noted above, the E_m response induced by thapsigargin addition was not influenced by the presence or absence of extracellular Na^+.

Additionally, we have investigated the permeability of this divalent permeant pathway for both Ba^{2+} and Sr^{2+}. To allow for direct comparisons relative to Ca^{2+} and Mn^{2+}, the depolarization detected following the addition of 5 mM divalent to thapsigargin-treated cells was compared only under conditions in which all four divalents were administered to cells obtained from the same BAPTA-loading procedure. Increases in *bis*-oxonol fluorescence following Ba^{2+} or Sr^{2+} addition were found to be small, approximating those sometimes observed in response to the addition of 5 mM Ni^{2+} alone (*see above*). As a result, it is difficult to distinguish between possible surface charge effects induced by re-addition of the divalent and alterations in fluorescence attributable to a valid depolarization. However, it is clear from these experiments that the permeability of the SR-CU pathway for Ba^{2+} and Sr^{2+} is much lower than for Ca^{2+} and Mn^{2+}, if it displays any permeability at all. On the basis of these experiments, we conclude that the pathway conducts divalents with the following relative permeability sequence: $Ca^{2+} > Mn^{2+} >> Ba^{2+}$ and Sr^{2+}.

Discussion

Evidence has accumulated in support of the hypothesis that the plasma membrane Ca^{2+} permeability of lymphoid tissue is regulated in large part by the Ca^{2+} content of an intracellular Ca^{2+} pool (SRCU) [3, 22, 24, 27, 33]. While it is generally accepted that SRCU occurs via an electrogenic pathway in that it is exquisitely sensitive to depolarization, direct measurements of the current have remained elusive, indicative of its low conductance properties. In the present experiments, we have exploited the high input resistance characteristic of primary rat thymic lymphocytes to indirectly monitor small alterations in transmembrane current as large changes in E_m. To effectively monitor alterations in transmembrane current attributable to alterations in SR-CU current, we loaded rat thymic lymphocytes with the divalent cation chelator, BAPTA. This procedure effectively slows the rate of rise of $[Ca^{2+}]_i$ and hence the activation of the dominant Ca^{2+}-gated K^+ conductance present in these cells [12, 20, 21].

Under these conditions, and in the presence of extracellular Ca^{2+}, a biphasic change in E_m could be detected in response to release of intracellular Ca^{2+} stores following addition of thapsigargin, cyclopiazonic acid, or low concentrations of ionomycin. All three of these agents release Ca^{2+} from a common intracellular pool. In response to these agents, an initial depolarization

Table. Effects of divalent cation reintroduction on E_m in BAPTA-loaded thymic lymphocytes exposed to thapsigargin or ionomycin

	Membrane potential (mV)					
	Ca^{2+}			Mn^{2+}		
	Resting	Final	Δ	Resting	Final	Δ
Thapsigargin						
$n = 9$	−52.4 ± 9.7	−27.8 ± 6.9	24.6 ± 4.9	−52.2 ± 8.0	−34.2 ± 6.8	18.0[a] ± 3.6
Ionomycin						
$n = 7$	−53.1 ± 8.7	−31.6 ± 7.8	21.6 ± 5.4	−56.4 ± 11.3	−38.6 ± 7.0	17.9[b] ± 5.0

BAPTA-loaded cells were suspended in nominally Ca^{2+}-free Na^+ medium in the presence of 150 nM charybdotoxin and either 33 nM thapsigargin or 20 nM ionomycin. The E_m changes elicited by addition of 4 mM Ca^{2+} or Mn^{2+} are summarized. Resting E_m prior to Ca^{2+} or Mn^{2+} addition was not significantly different for either condition. [a] and [b]E_m change induced by reintroduction of Mn^{2+} was significantly different from that induced by reintroduction of Ca^{2+} (paired t-test, $t < 0.05$).

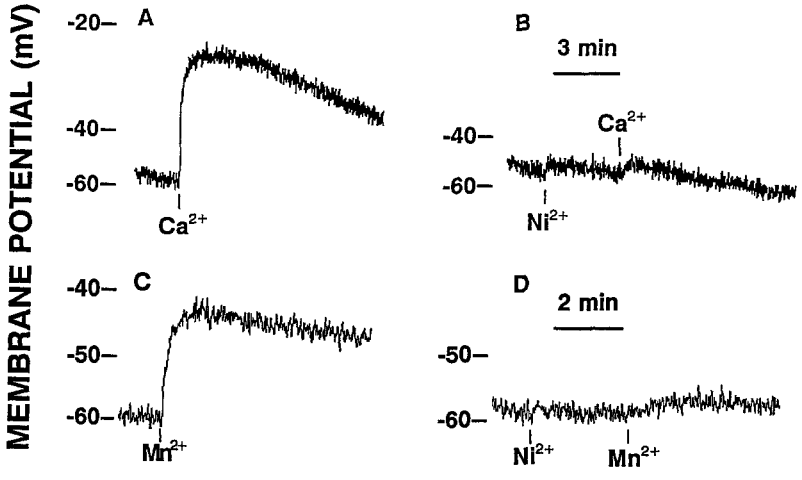

Fig. 7. Effect of Ni^{2+} on E_m changes induced by the reintroduction of Ca^{2+} or Mn^{2+} to thapsigargin-treated cells. BAPTA-loaded cells were suspended in nominally Ca^{2+}-free Na^+ medium in the presence of 33 nM thapsigargin and 150 nM charybdotoxin, and the fluorescence of *bis*-oxonol was monitored. Where indicated, 5 mM Ca^{2+}, Mn^{2+} or Ni^{2+} was added. (*A* and *B*) and (*C* and *D*) are paired experiments performed with cell aliquots from the same BAPTA-loading procedure.

followed by a secondary repolarization and subsequent hyperpolarization was detected. The depolarization phase is consistent with a marked increase in Ca^{2+} conductance following release of Ca^{2+} from intracellular stores inasmuch as: (i) the magnitude of the change is dependent upon the $[Ca^{2+}]_o$ and correlates with elevations in $[Ca^{2+}]_i$ measured fluorimetrically; (ii) the depolarization is blocked by the inorganic Ca^{2+} channel blocker Ni^{2+}, consistent with the ability of Ni^{2+} to inhibit the rise in $[Ca^{2+}]_i$ measured fluorimetrically; and (iii) detection of the depolarization is dependent upon BAPTA loading. While these data are consistent with an increase in Ca^{2+} conductance, possible alterations in other transmembrane conductances must be considered.

Both Na^+ and Cl^- have equilibrium potentials more positive than resting potential in thymic lymphocytes. As such, increases in the conductance of either of these ions would be expected to depolarize the cell. An increase in Na^+ conductance is easily ruled out on the basis that the depolarization was observed in Na^+-free solutions. Additionally, direct comparison of the response in the presence and absence of Na^+ yields no discernable differences (*results not shown*). The finding that BAPTA loading facilitates the detection of the depolarization would argue against a role for Ca^{2+}-gated Cl^- conductance in the observed depolarization. The presence of this chelator would not be expected to facilitate the detection of a conductance which is dependent upon a rise in $[Ca^{2+}]_i$ for activation. However, this procedure effectively increases the amount of Ca^{2+} entering the cell, a condition which would facilitate a longer lasting and possibly larger E_m change induced by such Ca^{2+} influx should it occur via an electrogenic pathway.

The BAPTA-loading technique has previously been used by MacDougal and coworkers [19] for investigations of Ca^{2+}-dependent E_m changes associated with receptor-linked events in human B-lymphocytes. The

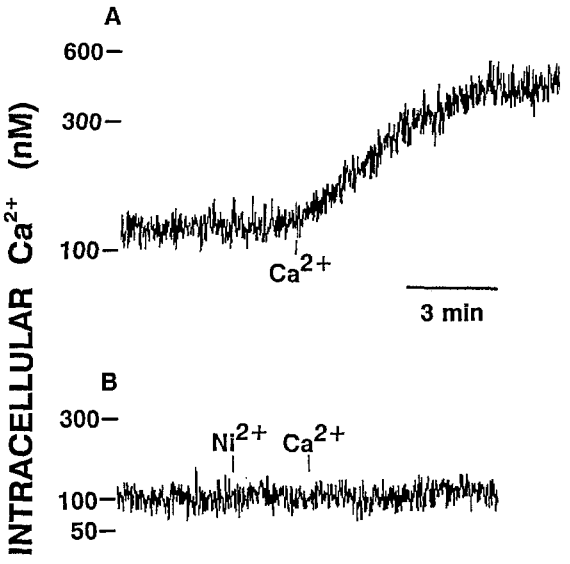

Fig. 8. Effect of Ni^{2+} on the rise in intracellular $[Ca^{2+}]_i$ following reintroduction of Ca^{2+} to thapsigargin-treated cells. Cells loaded with indo-1 and BAPTA were suspended in nominally Ca^{2+}-free Na^+ medium in the presence of 33 nM thapsigargin, and the fluorescence of indo-1 was monitored. Where indicated, 4 mM Ca^{2+} or 5 mM Ni^{2+} was added. These are paired experiments performed with cell aliquots from the same BAPTA-loading procedure.

E_m depolarization detected by these investigators following cross-linking B-cell surface receptors has been ascribed to electrogenic Ca^{2+} uptake. However, the mechanism underlying the activation of this receptor-linked pathway in B-cells was not investigated. Such electrogenic Ca^{2+} uptake may have its origin in the inositol 1,4,5-trisphosphate (IP_3) mediated release of Ca^{2+} from intracellular stores, a documented response initiated by cross-linking B-cell surface receptors [2].

The origin of the subsequent hyperpolarizing component demonstrated in the present experiments is attributable to the activation of a K^+ conductance. This is based on the following observations: (i) K^+ is the only ion with an equilibrium potential more negative than resting potential in these cells and (ii) the repolarizing and hyperpolarizing phases are significantly inhibited by the potent K^+ channel blocker charybdotoxin. The ability of 5 mM Ni^{2+} to inhibit the slow rise in $[Ca^{2+}]_i$ and the secondary hyperpolarizing phase are consistent with a role of Ca^{2+}-gated K^+ channels in the observed hyperpolarization. It is important to note, however, that the concentration of charybdotoxin required to effectively inhibit the secondary repolarization and hyperpolarization phases of this response (150 nM) was considerably higher than that previously shown to abolish Ca^{2+}-gated K^+ channel activity in non-BAPTA-loaded thymic lymphocytes ([12, 20, 21] and Fig. 1). While 50 nM charybdotoxin displayed some inhibitor effect (*results not shown*), 150 nM was much more effective in inhibiting this phase. The activation of voltage-gated K^+ channels secondary to the initial depolarization could account for such a response. Consistent with this notion, the hyperpolarizing phase was inhibited by 5 mM Ni^{2+} possibly due to the Ni^{2+}-dependent block of the triggering depolarizing phase of the response. This may be indicative of the involvement of both Ca^{2+}-gated and a class of K^+ channels displaying less sensitivity to charybdotoxin.

While increases in K^+ channel activity could account for the secondary repolarization and subsequent hyperpolarization in the absence of charybdotoxin, in the presence of 150 nM toxin, E_m stabilized at values only slightly more negative than resting E_m as judged by the marked hyperpolarization following gramicidin addition (Fig. 5). This result may be indicative of inactivation of the Ca^{2+} current rather than activation of charybdotoxin-insensitive K^+ current. Two mechanisms can be proposed to account for such an apparent inactivation. A decrease in inward current may occur due to a decrease in the driving force as a result of elevations in $[Ca^{2+}]_i$ under the membrane. Alternatively, the pathway may be directly inactivated by elevated levels of $[Ca^{2+}]_i$. Further investigations are required to address the origin of the secondary repolarization and modest hyperpolarization in the presence of charybdotoxin.

We have previously reported that IP_3-mediated release of Ca^{2+} from intracellular stores underlies the Ca^{2+} influx response to the mitogenic plant lectin concanavalin-A in rat thymic lymphocytes [24]. Additionally, this is accompanied by a modest depolarization in BAPTA-loaded cells suspended in 1 mM extracellular Ca^{2+} [21]. On the basis of the present investigation, such a depolarization would be consistent with an increase in Ca^{2+} conductance due to IP_3-mediated release of Ca^{2+} from intracellular stores. Activation of a highly selective Ca^{2+} current has been reported by Lewis and Cahalan [18] in the cultured T-cell line Jurkat following addition of the mitogen phytohaemagglutinin (PHA). Addition of this IP_3-generating plant lectin resulted in a whole-cell current of < 1 to 10 pA after a delay of 100–300 seconds. This increase in current was associated with a slow rise in $[Ca^{2+}]_i$. Activation of the current often occurred spontaneously, suggesting an additional activation mechanism independent of the presence of PHA. Elevations in $[Ca^{2+}]_i$ were found to inhibit the pathway, a regulatory response which may account for the slow secondary repolarization phase observed in the presence of charybdotoxin in the present experiments (*see above*).

Gardner and coworkers [17] have described single channel and whole-cell currents activated by PHA in cultured T-cell lines. When recorded in cell-attached mode in isotonic $BaCl_2$, this channel has a conductance

of approximately 7 pS (whole-cell currents of approximately −30 pA at −60 mV), is not detectably voltage sensitive and displays brief channel openings which occur in bursts. A role for IP_3 in the activation of this poorly selective cation channel was concluded from experiments demonstrating the activation of the channel in excised patches by the addition of IP_3 to the bathing medium [16]. More recently, these observations in Jurkat cells have been supported by the results of experiments using rapid elevations in cytosolic IP_3 in intact cells [26]. Although IP_3 has been implicated in the activation of this current, the mechanism underlying this activation remains controversial. Two mechanisms can be proposed to account for the activation of a Ca^{2+} current by this compound. First, IP_3 may mediate its effect via its ability to release Ca^{2+} from intracellular stores. Secondly, IP_3 may directly bind to IP_3 receptors in the plasma membrane and activate a Ca^{2+} channel directly. In support of the second hypothesis, the existence of plasma membrane IP_3 receptors in both Jurkat and rat thymic lymphocytes has been reported [15]. Our data, however, support a role for Ca^{2+} release from intracellular stores in the activation of the divalent cation conductance in rat thymic lymphocytes.

Experiments in mast cells have provided evidence for the regulation of a plasma membrane Ca^{2+} current by the Ca^{2+} content of the IP_3-sensitive intracellular store [14]. Elevations in IP_3 or addition of the Ca^{2+} transporting ionophore ionomycin both activated an identical Ca^{2+} current in support of activation via release of Ca^{2+} from intracellular stores. Although we propose a similar mechanism underlying regulation of plasma membrane Ca^{2+} conductance in thymic lymphocytes, two significant differences between the findings in mast cells and rat thymic lymphocytes exist. First, in mast cells, thapsigargin was not an effective activator of the Ca^{2+} current [14]. Second, the pathway activated by release of mast cell intracellular Ca^{2+} stores was not significantly permeable to Mn^{2+} [14]. Such a lack of Mn^{2+} permeability is a characteristic shared by the IP_3-activated current reported in Jurkat cells [26].

The data derived from the experiments outlined in this investigation provide the first measurement and characterization of E_m changes attributable to increases in the plasma membrane Ca^{2+} conductance following release of Ca^{2+} from intracellular stores in primary lymphoid tissues. While depletion of intracellular Ca^{2+} stores results in the activation of plasma membrane Ca^{2+} conductance in rat thymic lymphocytes, its relationship to previously reported mechanisms in cultured lymphoid tissues and other cell types is presently unclear. Direct characterization of this Ca^{2+} current with patch-clamp techniques is required to clarify this point. Additionally, such techniques will aid investigations into the mechanism underlying regulation of this current by the Ca^{2+} content of the intracellular Ca^{2+} stores.

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References

1. Alvarez, J., Montero, M., Garcia-Sancho, J. 1991. Cytochrome P-450 may link intracellular Ca^{2+} stores with plasma membrane Ca^{2+} influx. *Biochem J.* **274:**193–197.
2. Bijsterbosch, M.K., Rigley, K.P., Klaus, G.G.B. 1986. Cross-linking of surface immunoglobulins on B lymphocytes induces both intracellular Ca^{2+} release and Ca^{2+} influx: analysis with indo-1. *Biochem. Biophys. Res. Commun.* **137:**500–506
3. Chow, S.C., Jondal, M. 1990. Ca^{2+} entry in T cells is activated by emptying the inositol 1,4,5-trisphosphate sensitive Ca^{2+} pool. *Cell Calcium* **11:**641–646
4. Densmore, J.J., Szabo, G., Gray, L. 1991. A voltage-gated calcium channel is linked to the antigen receptor in Jurkat T lymphocytes. *FEBS Lett.* **312:**161–164
5. Dupuis, G., Heroux, J., Payet, M.D. 1989. Characterization of Ca^{2+} and K^+ currents in the human Jurkat T-cell line: Effects of phytohaemagglutinin. *J. Physiol.* **412:**135–154
6. Freedman, M., Raff, C.M., Gomperts, B. 1975. Induction of increased calcium uptake in mouse T lymphocytes by concanavalin A and its modulation by cyclic nucleotides. *Nature* **255:**378–382
7. Gelfand, E.W., Cheung, R.K., Grinstein, S. 1984. Role of membrane potential in the regulation of lectin-induced calcium uptake. *J. Cell Physiol.* **121:**533–539
8. Gouy, H., Cefai, D., Brogger Christensen, S., Debre, P., Bismuth, G. 1990. Ca^{2+} influx in human T lymphocytes is induced independently of inositol phosphate production by mobilization of intracellular Ca^{2+} stores. A study with the Ca^{2+} endoplasmic reticulum-ATPase inhibitor thapsigargin. *Eur. J. Immunol.* **20:**2269–2275
9. Grinstein, S., Dixon, S.J. 1989. Ion transport, membrane potential, and cytoplasmic pH in lymphocytes: Changes during activation. *Physiol. Rev.* **69:**417–481
10. Grinstein, S., Cohen, S., Rothstein, A. 1984. Cytoplasmic pH regulation in thymic lymphocytes by an amiloride-sensitive Na^+/H^+ antiport. *J. Gen. Physiol.* **83:**341–370
11. Grinstein, S., Goetz, J.D., Rothstein, A. 1984. $^{22}Na^+$ fluxes through thymic lymphocytes: I. Na^+/Na^+ and Na^+/H^+ exchange through an amiloride-sensitive pathway. *J. Gen. Physiol.* **84:**565–584
12. Grinstein, S., Smith, J.D. 1989. Ca^{2+} induces charybdotoxin-sensitive membrane potential changes in rat lymphocytes. *Am. J. Physiol.* **257:**C197–C206
13. Grynkiewicz, G., Poenie, M., Tsien, R.Y. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescent properties. *J. Biol. Chem.* **260:**3440–3450
14. Hoth, M., Penner, R. 1991. Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature* **355:**353–355
15. Khan, A.A., Steiner, J.P., Snyder, S.H. 1992. Plasma membrane inositol 1,4,5-trisphosphate receptor of lymphocytes: Selective enrichment in sialic acid and unique binding specificity. *Proc. Natl. Acad. Sci. USA* **89:**2849–2853

16. Kuno, M., Gardner, P. 1987. Ion channels activated by inositol 1,4,5-trisphosphate in plasma membrane of human T-lymphocytes. *Nature* **326:**301–304
17. Kuno, M., Goronzy, J., Weyand, C.M., Gardner, P. 1986. Single-channel and whole cell recordings of mitogen-regulated inward currents in human cloned helper T lymphocytes. *Nature* **323:**269–273
18. Lewis, R.S., Cahalan, M.D. 1989. Mitogen-induced oscillations of cytosolic Ca^{2+} and transmembrane Ca^{2+} current in human leukemic T cells. *Cell Regul.* **1:**99–112
19. MacDougal, S., Grinstein, S., Gelfand, E.W. 1988. Activation of Ca^{2+}-dependent K^+ channels in human B-lymphocytes by anti-immunoglobulin. *J. Clin. Invest.* **81:**449–454
20. Mahaut-Smith, M.P., Schlicter, L.C. 1989. Ca^{2+}-activated K^+ channels in lymphocytes. *Pfluegers Arch.* **414(Suppl. 1):**S164
21. Mahaut-Smith, M.P., Mason M.J. 1991. Single channel recordings of Ca^{2+}-activated K^+ channels in rat lymphocytes: Activation by concanavalin A. *J. Physiol.* **439:**513–528
22. Mason, M.J., Garcia-Rodriguez, C., Grinstein, S. 1991. Effect of endomembrane Ca^{2+}-ATPase inhibitors on the Ca^{2+}-permeability of the plasma membrane: Comparison of the effects of thapsigargin, 2,5-di(*tert*-butyl)1,4-hydroquinone and cyclopiazonic acid in rat thymic lymphocytes. *J. Biol. Chem.* **266:**20856–20862
23. Mason, M.J., Grinstein, S. 1990. Effect of cytoplasmic acidification on the membrane potential of T-lymphocytes: Role of trace metals. *J. Membrane Biol.* **116:**139–148
24. Mason, M.J., Mahaut-Smith, M.P., Grinstein, S. 1991. Role of intracellular Ca^{2+} in the regulation of the plasma membrane Ca^{2+} permeability of rat thymic lymphocytes. *J. Biol. Chem.* **266:**10872–10879
25. Mason, M.J., Mayer, B., Hymel, L.J. 1993. Inhibition of Ca^{2+} transport pathways in rat thymic lymphocytes by econazole, miconazole and SKF 96365. *Am. J. Physiol.* **33:**C654–C662
26. McDonald, T.V., Premack, B.A., Gardner, P. 1993. Flash photolysis of caged inositol 1,4,5-trisphosphate activates plasma membrane calcium current in human T-cells. *J. Biol. Chem.* **268:**3889–3896
27. Montero, M., Alvarez, J., Garcia-Sancho, J. 1990. Uptake of Ca^{2+} and refilling of intracellular Ca^{2+} stores in Ehrlich-ascites tumour cells and in rat thymocytes. *Biochem. J.* **271:**535–540
28. Ng, J., Fredholm, B.B., Jondal, M., Andersson, T. 1990. Regulation of receptor-mediated calcium influx across the plasma membrane in a human leukemic T-cell line: Evidence of its dependence on an initial calcium mobilization from intracellular stores. *Biochim. Biophys. Acta* **971:**207–214
29. Ng, J., Gustavsson, J., Jondal, M., Andersson, T. 1988. Regulation of calcium influx across the plasma membrane of the T-leukemic cell line JURKAT: Dependence on a rise in cytosolic free calcium can be dissociated from formation of inositol phosphate. *Biochim. Biophys. Acta* **1053:**97–105
30. Oettgen, H.C., Terhorst, C., Cantley, L.C., Rossoff, P.M. 1985. Stimulation of the T3-T cell receptor complex induces a membrane-potential-sensitive calcium influx. *Cell* **40:**583–590
31. Pecht, I., Corcia, A., Trisorio Liuzzi, M.P., Alcover, A., Reinherz, E.L. 1987. Ion channels activated by specific Ti or T3 antibodies in plasma membranes of human T cells. *EMBO J.* **6:**1935–1939
32. Rink, T.J., Montecucco, C., Hesketh, T.R., Tsien, R.Y. 1980. Lymphocyte membrane potential assessed with fluorescent probes. *Biochim. Biophys. Acta* **595:**65–70
33. Scharff, O., Foder, B., Thastrup, O., Hofmann, B., Moller, J., Ryder, L.P., Jacobsen, K.D., Langhoff, E., Dickmeiss, E., Christensen, S.B., Skinhoj, P., Svejgaard, A. 1988. Effect of thapsigargin on cytoplasmic Ca^{2+} and proliferation of human lymphocytes in relation to AIDS. *Biochim. Biophys. Acta* **972:**257–264
34. Taylor, M.J., Metcalfe, J.C., Hesketh, T.R., Moore, J.P. 1984. Mitogens increase phosphorylation of phosphoinositides in thymocytes. *Nature* **312:**462–465
35. Tsien, R.Y., Pozzan, T., Rink, T.J. 1982. T-cell mitogens cause early changes in cytoplasmic free Ca^{2+} and membrane potential in lymphocytes. *Nature* **295:**68–70

Note Added in Proof

While this manuscript was under review, a report by Zweifach and Lewis (1993, *Proc. Natl. Acad. Sci. USA* **90:**6295–6299) using patch-clamp techniques was published, demonstrating thapsigargin-mediated activation of a Ca^{2+} current in cultured T-lymphocytes.