Ionomycin activates electrogenic Ca^{2+} influx in rat thymic lymphocytes

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Increases in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) after addition of the carboxylic Ca^{2+} -transporting ionophore ionomycin have been reported to be inhibited by depolarization in mouse T- and B-lymphocytes, a result attributed to effects on the plasma-membrane Ca^{2+} pump. The present experiments were undertaken to test the hypothesis that activation of electrogenic Ca^{2+} uptake, due to release of Ca^{2+} from intracellular pools, underlies the membrane-potential (E_m)-sensitivity of ionomycin-mediated Ca^{2+} uptake in T-lymphocytes. To address this proposal we have compared the characteristics of Ca^{2+} influx induced by ionomycin with those of Ca^{2+} influx activated by release of Ca^{2+} from intracellular stores (store-regulated Ca^{2+} uptake, SRCU). The endosomal Ca^{2+} -ATPase inhibitor thapsigargin was used to

bring about loss of Ca^{2+} from intracellular pools. In support of this hypothesis, we report that: (1) ionomycin releases Ca^{2+} from a thapsigargin-sensitive endosomal pool, a pool previously demonstrated to regulate electrogenic SRCU in rat thymic lymphocytes, (2) Ca^{2+} and Mn^{2+} uptake mediated by low doses of ionomycin are inhibited by compounds previously reported to inhibit SRCU, and (3) in the absence of SRCU, ionomycin-mediated Ca^{2+} uptake displays no sensitivity to E_m . We conclude that activation of electrogenic SRCU can adequately account for the E_m -sensitivity of Ca^{2+} flux previously attributed to alterations in Ca^{2+} -pump activity. Such a mechanism of action may underlie previous reports of electrogenic Ca^{2+} transport mediated by ionomycin in other tissues.

INTRODUCTION

The importance of elevations of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) to the proliferative response of lymphoid tissues is well established (for review see [1]). Accordingly, such elevations in $[\text{Ca}^{2+}]_i$ necessitate the presence of effective mechanisms to regulate cytosolic $[\text{Ca}^{2+}]_i$ in lymphoid tissues. A simple method to investigate the Ca^{2+} -handling capabilities of cells is to insert a fixed Ca^{2+} -influx pathway into the plasma membrane while monitoring the change in $[\text{Ca}^{2+}]_i$. The carboxylic ionophore ionomycin, first reported by Liu and Hermann [2] to transport Ca^{2+} across solvent barriers, has gained widespread acceptance for such a purpose, due to its selectivity for Ca^{2+} over other bi- and univalent ions. Ionomycin has been utilized by Ishuda and Chused [3] to elevate $[\text{Ca}^{2+}]_i$ in investigations of the Ca^{2+} -handling capabilities of mouse B- and T-lymphocytes. The ionomycin-mediated change in $[\text{Ca}^{2+}]_i$ in these cells was markedly dependent on membrane potential (E_m), with hyperpolarization stimulating and depolarization inhibiting the rise in $[\text{Ca}^{2+}]_i$. Interestingly, the rise in $[\text{Ca}^{2+}]_i$ after ionomycin addition was far greater in T-cells than in B-cells. Such a finding is consistent with the effect of E_m on the response, given that the authors have reported that ionomycin hyperpolarizes mouse T-lymphocytes while depolarizing B-lymphocytes [3]. The authors concluded that the differences in mouse T- and B-lymphocyte $[\text{Ca}^{2+}]_i$ responses to ionomycin addition was attributable to effects of E_m on the plasma-membrane Ca^{2+} pump, with depolarization stimulating pump activity. Such a conclusion relies on the following assumptions: (1) ionomycin-mediated transport of Ca^{2+} is electroneutral and (2) ionomycin addition does not activate an E_m -sensitive Ca^{2+} -uptake pathway independent of this compound's ionophoretic effect.

Ionic transport by other Ca^{2+} -transporting carboxylic ionophores such as A23187 has been thought to occur via an electroneutral cationic exchange mechanism involving protons

[4]. A similar counter-ion transport of protons has been envisaged to account for Ca^{2+} transport by ionomycin. Such a proposal is supported by the observations that ionomycin-mediated Ca^{2+} transport is markedly dependent on the extracellular proton concentration, with decreases in proton concentration stimulating Ca^{2+} transport across solvent barriers and artificial lipid barriers [2,5]. Although such results have been interpreted in the biological literature as support for the assumption that Ca^{2+} transport by ionomycin is due to $\text{Ca}^{2+}/\text{H}^+$ counter-transport, it is not possible to exclude the possibility that this effect is a reflection of the pH-sensitivity of Ca^{2+} binding by ionomycin. Fasolato and Pozzan [6] have investigated the inhibitory effect of depolarization on ionomycin- and A23187-mediated Ca^{2+} transport in a variety of cell lines and concluded that these ionophores are capable of transporting Ca^{2+} both electroneutrally and electrogenically. Such a finding could account for the E_m -sensitivity of ionomycin-mediated Ca^{2+} transport reported by Ishuda and Chused in T- and B-lymphocytes [3].

Evidence is accumulating in favour of the contention that an electrogenic Ca^{2+} -influx pathway in the plasmalemma of most mammalian cells is regulated by the Ca^{2+} content of an intracellular Ca^{2+} -storing compartment (store-regulated Ca^{2+} uptake, SRCU). Such a model was originally proposed by Putney [7] to explain agonist-mediated Ca^{2+} influx in epithelial cells and has been extended to include an increasing number of cell types (for review see [8]). Such a mechanism has been reported to underlie the regulation of the plasma-membrane Ca^{2+} permeability in rat thymic lymphocytes [9–12].

The present experiments were designed to investigate the role of SRCU in E_m -sensitive Ca^{2+} transport mediated by ionomycin. To address this question, we have compared the characteristics of Ca^{2+} influx in rat thymic lymphocytes induced by ionomycin with those of Ca^{2+} influx activated by the sesquiterpene lactone thapsigargin, which has been demonstrated to stimulate Ca^{2+} influx in rat thymic lymphocytes via the SRCU pathway [11,12].

Abbreviations used: SRCU, store-regulated Ca^{2+} uptake; E_m , membrane potential; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; AM, acetoxymethyl ester; BHQ, 2,5-di-(*t*-butyl)-1,4-hydroquinone; SKF 96365, 1- β -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl-1*H*-imidazole hydrochloride.

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MATERIALS AND METHODS

Reagents and solutions

The AM (acetoxymethyl ester) derivatives of indo-1 and quin2 were purchased from Molecular Probes (Eugene, OR, U.S.A.). Ionomycin and BSA (Fraction V; fatty acid-, nuclease- and protease-free) were obtained from Calbiochem Behring. The culture medium RPMI 1640 (bicarbonate-free), EGTA and Hepes were purchased from Sigma. 1- β -[3-(4-Methoxyphenyl)propoxy]-4-methoxyphenethyl-1*H*-imidazole hydrochloride (SKF 96365) was generously given by Dr. Janet Merritt while at SmithKline and Beecham Pharmaceuticals (Welwyn, Herts., U.K.). Thapsigargin was purchased from LC Services Corp. (Woburn, MA, U.S.A.). 2,5-Di-(*t*-butyl)-1,4-hydroquinone (BHQ) and anhydrous dimethyl sulphoxide were from Aldrich. NaCl, KCl, CaCl₂, MgCl₂, MnCl₂, D-glucose, NaOH and KOH were purchased from Fisher Scientific. Stock solutions of indo-1 AM, quin2 AM, thapsigargin and BHQ were made up in anhydrous dimethyl sulphoxide. Ionomycin was dissolved in ethanol.

The basic Na⁺ solution contained 140 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM D-glucose and 20 mM Hepes (free acid), plus 1 mg/ml BSA. The solution was titrated to pH 7.25 at 37 °C with NaOH. K⁺ solutions were made by substituting KCl and NaCl and titrating to the required pH with KOH. Ca²⁺-free solution was made by omitting Ca²⁺ and adding 0.5 mM EGTA. When required, Mn²⁺ was added directly to the solution as MnCl₂. The osmolarity of the solution was adjusted to 295–300 mosM before addition of BSA by using an Osmette freezing-point osmometer. 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), treated as previously described [13] and maintained at room temperature at a concentration of (30–50) × 10⁶ 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 37 °C with a Hitachi model F-4000 fluorescence spectrophotometer equipped with a magnetic stirrer. Results are representative of experiments in a minimum of three preparations.

Determination of free cytosolic Ca²⁺ concentration

[Ca²⁺]_i was determined by measuring the fluorescence of indo-1 or quin2 as previously reported [11]. Quin2 was employed in experiments designed to investigate the rate of Ca²⁺ influx from the extracellular medium. Due to its high Ca²⁺ buffering power, changes in [Ca²⁺]_i in quin2-loaded cells are greatly slowed and are due almost exclusively to Ca²⁺ uptake from the extracellular medium. Such conditions provide for easier monitoring of the rate of Ca²⁺ uptake from the extracellular medium. The excitation and emission wavelengths used were 331 nm (3 nm slit width) and 410 nm (10 nm slit width) respectively for indo-1 and 339 nm (3 nm slit width) and 495 nm (10 nm slit width) for quin2. Thymocyte suspensions (25 × 10⁶ cells/ml) were loaded with the required fluorescent indicator by incubation with 2 μM indo-1 AM or 4 μM quin2 AM for 25 min at 37 °C in basic Na⁺ solution devoid of BSA and EGTA. The cells were then sedimented, resuspended in basic Na⁺ solution devoid of BSA and EGTA, counted, and kept at room temperature until required. To

monitor fluorescence, samples containing the required cell number were sedimented, resuspended in Na⁺ solution with or without Ca²⁺, as indicated, and added to the cuvette. Fluorescence was calibrated by using ionomycin and Mn²⁺ as previously described [14]. A dissociation constant of 250 nM for indo-1 [15] and 115 nM for quin2 [16] was used to calculate [Ca²⁺]_i.

Determination of Mn²⁺ influx

Mn²⁺ uptake was monitored as the rate of quenching of indo-1 fluorescence measured at a Ca²⁺-insensitive isosbestic point for the Ca²⁺/indo-1 complex (excitation 345 nm, emission 439 nm). Appropriate wavelengths were determined from wavelength scans of intracellularly trapped indo-1 under conditions of variable [Ca²⁺]_i.

RESULTS

We investigated the effect of *E_m* depolarization on SRCU in rat thymic lymphocytes activated by the addition of 33 nM thapsigargin. Thapsigargin-mediated release of Ca²⁺ from intracellular stores resulted in a marked rise in [Ca²⁺]_i in quin2-loaded rat thymic lymphocytes suspended in Na⁺-containing medium (Figure 1a). Due to the high Ca²⁺-buffering power of quin2, this rise in [Ca²⁺]_i is due almost exclusively to Ca²⁺ uptake from the extracellular medium. Both the rate of change of [Ca²⁺]_i and the magnitude of the steady-state plateau level were markedly inhibited in cells depolarized by exposure to 140 mM K⁺ (Figure 1b). Given that 140 mM K⁺ effectively depolarizes the *E_m* from its normal value of ≈ –60 mV to ≈ –5 mV [17], such an inhibitory effect is consistent with SRCU in rat thymic lymphocytes occurring via an electrogenic mechanism as previously proposed [11].

We have investigated the *E_m*-sensitivity of the changes in [Ca²⁺]_i induced by modest concentrations of ionomycin (Figures 1c and 1d). In comparison with the rate of rise of [Ca²⁺]_i induced by 100 nM ionomycin in cells suspended in normal Na⁺ medium (Figure 1c), the rate of change of [Ca²⁺]_i and steady-state [Ca²⁺]_i were markedly decreased in cells suspended in 140 mM KCl (Figure 1d). Such a result is consistent with ionomycin-mediated electrogenic Ca²⁺ uptake. This may be a result of *E_m* effects on the transport properties of ionomycin. However, subsequent addition of high concentrations of ionomycin (6 μM in the presence of BSA) showed little sensitivity to *E_m*, a finding inconsistent with such a proposal (Figure 1d). Alternatively, it can be proposed that low concentrations of ionomycin activate the electrogenic SRCU pathway by releasing Ca²⁺ from the thapsigargin-sensitive Ca_i²⁺ pool. At low ionophore concentrations, electroneutral Ca²⁺ transport mediated directly by ionomycin is overwhelmed by the larger electrogenic influx component attributable to SRCU.

Experiments were undertaken to investigate directly the role of the thapsigargin-sensitive intracellular Ca²⁺ stores in the Ca²⁺ response to low doses of ionomycin. Experiments were performed to determine if ionomycin released Ca²⁺ from this intracellular compartment. Indo-1-loaded cells, suspended in Ca²⁺-free Na⁺-containing medium, were exposed to sequential additions of 100 nM ionomycin and 30 nM thapsigargin. Indo-1 was employed to minimize exogenous Ca²⁺ buffering, thereby enabling us to monitor rises in [Ca²⁺]_i due to the release of small quantities of Ca²⁺ from the intracellular compartments. In the absence of extracellular Ca²⁺, the magnitude of the transient rise in [Ca²⁺]_i is proportional to the amount of Ca²⁺ released from the stores. The addition of 100 nM ionomycin resulted in a marked increase in [Ca²⁺]_i, followed by recovery to pre-exposure levels (Figure 2a). Subsequent exposure to 30 nM thapsigargin resulted in a

Figure 1 Effect of high K⁺ on elevations in [Ca²⁺]_i induced by thapsigargin and ionomycin in the presence of 1 mM CaCl₂

Where indicated, 30 nM thapsigargin (TG), 100 nM ionomycin or 6 μM ionomycin was added to quin2-loaded cells suspended in Na⁺ (a and c) or high-K⁺ (b and d) media containing 1 mM CaCl₂, and the fluorescence was monitored as described in the Materials and methods section.

Figure 2 Ionomycin releases Ca²⁺ from the thapsigargin-sensitive Ca²⁺ pool

Indo-1-loaded cells were suspended in Na⁺ media devoid of Ca²⁺ and containing 0.5 mM EGTA, and the fluorescence was monitored. (a) Where indicated, 100 nM ionomycin and 30 nM thapsigargin (TG) were added. (b) Where indicated, ethanol (EtOH; solvent control for ionomycin addition) and 30 nM thapsigargin were added.

small transient rise in [Ca²⁺]_i. In contrast, addition of 30 nM thapsigargin to cells suspended in Ca²⁺-free solution for an equivalent amount of time in the absence of ionomycin (solvent control only) resulted in a considerably larger transient increase in [Ca²⁺]_i, indicating that the Ca²⁺ content of the intracellular pool was greater under these conditions (Figure 2b). Such results indicated that exposure to ionomycin resulted in a significant depletion of the thapsigargin-sensitive Ca²⁺ pool. Hence, low doses of ionomycin would be expected to activate the electrogenic SRCU pathway.

The effect of Ca²⁺ release from intracellular stores in rat thymic lymphocytes has been shown to have a graded effect on the plasma-membrane Ca²⁺ permeability, with greater Ca²⁺ release from intracellular stores correlating with greater Ca²⁺ uptake [9,10]. As a result, preferential release of Ca²⁺ from the thapsigargin-sensitive intracellular pool in the presence of Na⁺-containing medium could account for the greater rate of Ca²⁺ uptake and the higher peak increase in [Ca²⁺]_i detected in Na⁺ than in K⁺ medium. To investigate this possibility, Ca²⁺ mobilization from intracellular stores was monitored in high-Na⁺- and high-K⁺-containing solutions. The results of a representative experiment are presented in Figure 3. The transient rise in [Ca²⁺]_i induced by 30 nM thapsigargin in indo-1-loaded

Figure 3 Effect of high K⁺ on release of Ca²⁺ from intracellular stores

Where indicated, 30 nM thapsigargin (THG) or 100 nM ionomycin was added to indo-1-loaded cells suspended in Na⁺ (a and c) or high-K⁺ (b and d) media devoid of Ca²⁺ and containing 0.5 mM EGTA, and the fluorescence was monitored.

cells was independent of E_m , in that identical Ca²⁺ transients were observed in Na⁺ and high-K⁺ medium (Figures 3a and 3b). Similarly, the ability of ionomycin to mobilize Ca²⁺ from intracellular compartments was unaffected by the K⁺ concentration of the medium (Figures 3c and 3d). On the basis of these data, we conclude that the inhibitory effect of high K⁺ observed in Figure 1 cannot be attributed to differences in the amount of Ca²⁺ released from the intracellular pool, and most likely reflects the sensitivity of the plasma-membrane Ca²⁺ influx pathway to E_m .

We have previously demonstrated in rat thymic lymphocytes that SRCU in rat thymic lymphocytes is inhibited by the addition of the substituted imidazole reagent SKF 96365 [11,18]. If Ca²⁺ uptake induced by ionomycin is mediated in part by the release of Ca²⁺ from intracellular Ca²⁺ pools, then the change in [Ca²⁺]_i should be sensitive to this inhibitor. We have investigated this hypothesis, and a representative experiment is illustrated in Figure 4. The addition of 100 nM ionomycin to quin2-loaded thymocytes resulted in a rapid sustained increase in [Ca²⁺]_i to

Figure 4 Effect of SKF 96365 on the ionomycin-mediated rise in $[Ca^{2+}]_i$

(a) Rise in $[Ca^{2+}]_i$ induced by addition of 100 nM ionomycin to quin2-loaded cells suspended in Na^+ medium containing 1 mM $CaCl_2$. (b) Effect of prior addition of 100 μ M SKF 96365 on the rise in Ca^{2+} induced by addition of 100 nM or 6 μ M ionomycin.

Figure 5 Effect of SKF 96365 and BHQ on the rise in $[Ca^{2+}]_i$ induced by 100 nM ionomycin

(a) Rise in $[Ca^{2+}]_i$ induced by addition of 100 nM ionomycin to indo-1-loaded cells suspended in Na^+ medium containing 1 mM $CaCl_2$. (b, c) Effect of addition of 100 μ M SKF 96365 or 100 μ M BHQ on the rise in $[Ca^{2+}]_i$ induced by addition of 100 nM ionomycin. Where indicated, 6 μ M ionomycin was added. Break in trace (c) corresponds to 5 min.

approx. 280 nM (Figure 4a). Pre-treatment of quin2-loaded cells with 100 μ M SKF 96365 results in a slight but measurable increase in $[Ca^{2+}]_i$ as a result of release of Ca^{2+} from an intracellular pool (Figure 4b). Such an effect of SKF 96365 has previously been reported in human platelets, neutrophils and rat hepatocytes [19], and most likely arises from the ability of substituted imidazole reagents to inhibit endomembrane Ca^{2+} -ATPase activity, resulting in net Ca^{2+} loss from the intracellular

Figure 6 Effect of high K^+ on thapsigargin- and ionomycin-mediated Mn^{2+} uptake

(a) Cells were suspended in Na^+ or high- K^+ media containing 1 mM Ca^{2+} and 20 μ M Mn^{2+} ; 30 nM thapsigargin (THG) and 6 μ M ionomycin were added where indicated. (b) Cells were suspended in Na^+ or high- K^+ media containing 1 mM Ca^{2+} and 30 μ M Mn^{2+} ; 100 nM ionomycin and 6 μ M ionomycin were added where indicated.

stores [18]. Subsequent addition of 100 nM ionomycin resulted in only a modest increase in $[Ca^{2+}]_i$ (Figure 4b), consistent with low concentrations of ionomycin mediating its effect through an SKF 96365-sensitive plasma-membrane pathway. Ensuing addition of micromolar concentrations of ionomycin easily overcame this inhibitory effect of SKF 96365, consistent with both SKF 96365-sensitive and -insensitive mechanisms underlying ionomycin-mediated Ca^{2+} transport.

To investigate further the similarity between ionomycin-induced Ca^{2+} uptake and uptake regulated by the Ca^{2+} content of intracellular Ca^{2+} -storing compartments, we have investigated the effect of an alternative potent inhibitor of SRCU. The endomembrane Ca^{2+} -ATPase inhibitor BHQ is an effective inhibitor of the bivalent-cation uptake pathway regulated by the Ca^{2+} content of the intracellular compartment [12]. To investigate further the role of this pathway in ionomycin-mediated Ca^{2+} uptake, we have investigated the effect of BHQ on $[Ca^{2+}]_i$ induced by ionomycin. Addition of 100 μ M BHQ after ionomycin-mediated elevations in $[Ca^{2+}]_i$ resulted in a marked fall in $[Ca^{2+}]_i$ (Figure 5c), a result markedly different from the sustained control response (Figure 5a). This partial recovery of $[Ca^{2+}]_i$ is indistinguishable from that observed after addition of SKF 96365 (Figure 5b). Such a result is consistent with either inhibition of the Ca^{2+} -influx mechanism and subsequent Ca^{2+} extrusion across the plasma membrane or sequestration into intracellular compartments. Given that both BHQ and SKF 96365 inhibit endomembrane Ca^{2+} -ATPase activity [12,18], this fall in $[Ca^{2+}]_i$ most likely reflects extrusion across the plasma membrane. In addition, the radically different chemical structures of BHQ and SKF 96365 support the contention that the inhibitory effect of these agents arises out of their inhibitory effect on SRCU rather than on direct transport of Ca^{2+} by ionomycin. Such a conclusion is supported by the finding that addition of higher concentrations of ionomycin easily overcomes the inhibitory effect of both of these agents (Figures 5b and 5c).

The inhibitory effects of E_m depolarization and SKF 96365 on both thapsigargin- and ionomycin-induced increases in $[Ca^{2+}]_i$ are consistent with the mediation of Ca^{2+} uptake via a common electrogenic pathway. Alternatively, however, it can be suggested that depolarization and SKF 96365 mediate their inhibitory effect on $[Ca^{2+}]_i$ by stimulating Ca^{2+} extrusion across the plasma membrane. To assess this possibility, we have measured the unidirectional uptake of Mn^{2+} , a Ca^{2+} surrogate, into indo-1-

Figure 7 Effect of SKF 96365 on thapsigargin- and ionomycin-mediated Mn²⁺ uptake

(a, b) Cells were suspended in Na⁺ medium containing 1 mM Ca²⁺, 200 μM EGTA and 100 μM Mn²⁺; 30 nM thapsigargin (THG), 100 μM SKF 96365 and 6 μM ionomycin were added where indicated. (c) Cells were suspended in Na⁺ media containing 1 mM Ca²⁺ and 20 μM Mn²⁺ in the absence or presence of 100 μM SKF 96365. Where indicated, 100 nM ionomycin and 6 μM ionomycin were added.

loaded thymic lymphocytes (see the Materials and methods section). Mn²⁺ has successfully been used as a probe for the SRCU pathway in rat thymic lymphocytes [9–11,18].

Consistent with previous observations [12], the addition of 30 nM thapsigargin to cells suspended in Na⁺ media containing 20 μM Mn²⁺ resulted in a marked acceleration of the rate of Mn²⁺ uptake, as measured by the quenching of intracellularly trapped indo-1 (Figure 6a). Addition of 6 μM ionomycin, which can effectively transport Mn²⁺, rapidly abolished the remaining fluorescence. In the presence of high K⁺ (140 mM), the stimulatory effect of thapsigargin was markedly decreased, as illustrated in Figure 6(a). This inhibitory effect of high K⁺ was readily by-passed by the addition of 6 μM ionomycin.

Similarly, low doses of ionomycin (100 nM) also stimulate Mn²⁺ uptake. As depicted in Figure 6(b), the addition of 100 nM ionomycin to cells suspended in Na⁺ media containing 30 μM Mn²⁺ resulted in a significant increase in the rate of Mn²⁺ uptake. This stimulated uptake displayed a similar sensitivity to high K⁺ (140 mM), consistent with a significant electrogenic component of Mn²⁺ transport at low ionomycin concentrations.

Consistent with the inhibitory effect of SKF 96365 on Ca²⁺ uptake previously reported [11], Mn²⁺ uptake stimulated by addition of thapsigargin is also inhibited by this agent. A representative experiment is presented in Figures 7(a) and 7(b). Additionally, pre-exposure of cells to 100 μM SKF 96365 markedly inhibits ionomycin-mediated Mn²⁺ uptake (Figure 7c), consistent with the hypothesis that Mn²⁺ uptake mediated by ionomycin is due in part to activation of the store-regulated plasma-membrane pathway. Of particular significance is the finding that at concentrations of SKF 96365 which virtually abolish Ca²⁺ and Mn²⁺ uptake stimulated by thapsigargin (Figure 7b and ref. [18]), only partial inhibition of ionomycin-mediated uptake occurred. Such data are consistent with the hypothesis that ionomycin-mediated Mn²⁺ uptake occurs by both SKF 96365-sensitive and -insensitive pathways. In agreement with this hypothesis, higher doses of ionomycin (6 μM) easily overcame the inhibitory effect of SKF 96365 (Figure 7c).

In an effort to test directly the hypothesis that the electrogenic component of ionomycin-mediated Ca²⁺ uptake arises from an increase in the contribution of store-regulated plasma-membrane Ca²⁺ permeability, we have monitored the effect of membrane potential on ionomycin-mediated Ca²⁺ uptake under conditions

Figure 8 Effect of high K⁺ on elevations in [Ca²⁺]_i induced by ionomycin in the absence of SRCU

Quin2-loaded cells were suspended in Na⁺ (a) or high-K⁺ media (b) containing 1 mM Ca²⁺, and the fluorescence was monitored. Where indicated, 100 μM SKF 96365, 30 nM thapsigargin (THG) and 1 μM ionomycin were added. (c, d) Quin2-loaded cells were suspended in Na⁺ (c) or high-K⁺ (d) media containing 1 mM Ca²⁺, and the fluorescence was monitored. Where indicated, 1 μM ionomycin was added.

in which SRCU is abolished. Figure 8 shows the results of a representative experiment. Quin2-loaded cells suspended in normal- Na^+ (Figure 8a) or high- K^+ (Figure 8b) solution were exposed to concentrations of SKF 96365 known to block SRCU in thymic lymphocytes (100 μM in the presence of 1 mg/ml BSA [11]). As discussed above, addition of SKF 96365 causes a modest rise in $[\text{Ca}^{2+}]_i$ in quin2-loaded cells. Since SKF 96365 inhibits SRCU, subsequent addition of thapsigargin was without effect in cells suspended in either normal- or high- K^+ solution. Addition of 1 μM ionomycin results in a rapid increase in $[\text{Ca}^{2+}]_i$, the rate and magnitude of this rise being independent of E_m . In the absence of SKF 96365, the rate of Ca^{2+} uptake induced by 1 μM ionomycin displayed a marked sensitivity to E_m , with depolarized cells displaying a markedly slower rate of rise of $[\text{Ca}^{2+}]_i$ (Figures 8c and 8d). These findings support the hypothesis that the electrogenic component of Ca^{2+} uptake induced by this concentration of ionomycin in rat thymic lymphocytes is due to the activation of SRCU, rather than electrogenic transport directly mediated by ionomycin. It should be noted that it was necessary to increase the concentration of ionomycin to 1 μM from 100 nM as used in earlier experiments, to ensure that a measurable rise in $[\text{Ca}^{2+}]_i$ could be observed in the presence of SKF 96365. Given that all experiments were performed in the presence of BSA, the effective concentration of ionomycin is expected to be well below 1 μM , due to binding of the ionophore to this protein. Regardless, changes in $[\text{Ca}^{2+}]_i$ induced by 1 μM ionomycin in the presence of BSA were markedly sensitive to E_m , as evident from the results of Figures 8(c) and 8(d).

DISCUSSION

We have demonstrated that bivalent-cation transport mediated by the carboxylic ionophore ionomycin is sensitive to alterations in E_m . A similar observation has previously been reported by Ishuda and Chused [3] in mouse B- and T-lymphocytes, a result attributed to E_m effects on the plasma-membrane Ca^{2+} pump. The experiments outlined in the present paper were undertaken to test the hypothesis that activation of electrogenic SRCU, due to ionomycin-mediated depletion of Ca_i^{2+} pools, underlies the E_m -sensitivity of Ca^{2+} uptake. In support of this hypothesis, we have found that: (1) ionomycin releases Ca^{2+} from a thapsigargin-sensitive endosomal pool, a pool which has been previously shown to regulate SRCU [11,12], (2) Ca^{2+} and Mn^{2+} uptake mediated by low doses of ionomycin are markedly inhibited by compounds previously reported to inhibit store-regulated Ca^{2+} and Mn^{2+} uptake in these cells, and (3) in the absence of SRCU, ionomycin-mediated Ca^{2+} uptake displays no sensitivity to membrane potential. Taken in concert, these data support the hypothesis that activation of SRCU underlies the electrogenic component of Ca^{2+} transport observed in thymic lymphocytes after ionomycin addition.

Activation of such a pathway could account for the finding that ionomycin-mediated Ca^{2+} uptake is markedly inhibited by depolarization in mouse B- and T-cells [3]. The presence of such a pathway, and the finding that ionomycin produces a marked hyperpolarization in T-cells, while inducing a depolarization in B-cells, would also reconcile the apparent discrepancy between the effects of ionomycin on Ca^{2+} efflux in mouse T- and B-lymphocytes [3]. In these experiments, Ca^{2+} efflux from B-cells was reported to be stimulated by ionomycin addition while modestly inhibited in T-cells exposed to identical concentrations of ionophore. The depolarization detected in B-cells would be expected to facilitate efflux via the SRCU pathway, due to the decrease in the electrical driving force resisting Ca^{2+} efflux

through this pathway. Accordingly, the E_m hyperpolarization reported in T-cells after exposure to ionomycin would be expected to decrease Ca^{2+} efflux via this pathway, consistent with the results reported by Ishuda and Chused [3]. Although the present data do not allow one to rule out the possibility that the plasma-membrane Ca^{2+} pump is measurably stimulated by depolarization, as proposed by those authors [3], recent work in cultured T-cell lines has been unable to observe such an effect [20]. Additionally, we have been unable to detect a E_m -sensitive component of Ca^{2+} efflux in rat thymic lymphocytes (I. Marriott and M. J. Mason, unpublished work). In our view, a more likely source for the reported E_m -sensitivity is electrogenic Ca^{2+} uptake stimulated by release of Ca^{2+} from intracellular stores, a view supported by the detection of SRCU in B- and T-lymphocytes from a variety of sources ([21,22,11,12]; I. Marriott, K. Bost and M. J. Mason, unpublished work).

E_m depolarization has additionally been shown to influence ionomycin-mediated Ca^{2+} uptake in rat basophilic leukaemia cells [23]. The authors have speculated that the inhibitory effect of depolarization on ionophore-mediated Ca^{2+} transport may be a result of Ca^{2+} -induced Ca^{2+} influx. In this scheme, increases in $[\text{Ca}^{2+}]_i$ associated with ionophore-mediated transport give rise to the activation of a non-selective cation channel permeable to Ca^{2+} . Such a channel has been reported to underlie the elevation in $[\text{Ca}^{2+}]_i$ associated with neutrophil stimulation by chemotactic peptides [24]. Although previous reports do not support the existence of such a mechanism in thymic lymphocytes [11], activation of SRCU could adequately account for the observed electrogenic component of ionophore transport in these cells.

Direct electrogenic uptake of Ca^{2+} mediated by ionomycin has been proposed to underlie the E_m -sensitivity of Ca^{2+} transport observed in cells of the neurosecretory cell line PC12, mouse macrophage cell line J774 and human T-cell line Jurkat [6]. Our data in rat thymic lymphocytes would not support such a mechanism, given that we were unable to detect an electrogenic component of ionomycin-mediated Ca^{2+} transport in the absence of SRCU. In our view, SRCU can adequately account for the electrogenic component of ionomycin-mediated Ca^{2+} transport previously reported. The contribution of this electrogenic Ca^{2+} -transport pathway would be expected to decline dramatically with increasing concentrations of ionomycin and the corresponding increase in electroneutral Ca^{2+} transport by the ionophore.

Release of Ca^{2+} from intracellular stores induced by low doses of ionomycin has been proposed to underlie the stimulation of Mn^{2+} uptake in rat thymic lymphocytes [10]. Additionally, thrombin-induced inhibition of ionomycin-mediated elevations in $[\text{Ca}^{2+}]_i$ has been accounted for by activation of SRCU in endothelial cells [25].

Ionomycin has been employed as a means of activating electrogenic Ca^{2+} transport in rat peritoneal mast cells [26]. The inwardly rectifying current/voltage relationship for the whole cell current induced by ionomycin addition is indistinguishable from that activated by the addition of InsP_3 or the depletion of intracellular Ca^{2+} pools induced by addition of Ca^{2+} buffers to the pipette. Since, like these stimuli, ionomycin is known to release Ca^{2+} from intracellular Ca^{2+} pools, it is most likely that pool depletion rather than electrogenic Ca^{2+} transport by ionomycin underlies the increase in Ca^{2+} conductance. Clearly, our findings agree with such a conclusion.

The use of ionomycin in experiments designed to study electrogenic Ca^{2+} -transport pathways relies heavily on the assertion that ionomycin transport is electroneutral. Although in the absence of SRCU we have been unable to detect electrogenic Ca^{2+} uptake attributable to ionomycin in rat thymic lymphocytes,

we cannot rule out the possibility that some minor contribution of electrogenic transport exists. Fasolato and Pozzan [6] have shown that alterations in transmembrane potential of liposomes can alter the rate of Ca²⁺ transport mediated by carboxylic ionophores such as ionomycin. Since SRCU is not expected to be present in liposomes, such results argue in favour of some degree of electrogenic Ca²⁺ transport by these ionophores. Consistent with this component being small, Fasolato and Pozzan [6] were unable to detect any increase in conductance due to the addition of ionomycin or A23187 to black lipid films. However, given the affect of alterations in membrane potential on ionomycin-mediated Ca²⁺ transport in liposomes, we caution against the use of ionomycin as the sole basis for activation of store-regulated Ca²⁺ uptake and measurement of the associated Ca²⁺ current.

In summary, when used at low concentrations, ionomycin promotes Ca²⁺ uptake mainly indirectly, by SRCU. The effects of membrane potential on this flux are largely the result of an effect on translocation through the electrogenic SRCU pathway, and not a reflection of the operation of the ionophore. Nevertheless, small effects of potential on the ionophore cannot be ruled out. Activation of SRCU circumvents the necessity to evoke a stimulatory effect of depolarization on the plasma-membrane Ca²⁺ pump as previously reported [3]. Because of the complex nature of the fluxes triggered by ionomycin, caution must be exercised when interpreting the results of experiments employing ionomycin as a means of elevating [Ca²⁺]_i.

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