

Na⁺/Ca²⁺ Exchange-mediated Calcium Entry in Human Lymphocytes

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Abstract

Regulation of cytosolic Ca²⁺ and cytosolic Na⁺ is critical for lymphocyte cation homeostasis and function. To examine the influence of cytosolic Na⁺ on Ca²⁺ regulation in human peripheral blood lymphocytes, Ca²⁺ entry and cytosolic Ca²⁺ (measured with fura-2) were monitored in cells in which cytosolic Na⁺ was increased and/or the Na⁺ gradient was decreased by reduction of external Na⁺ concentration. Ouabain-treated cells (0.1 mM for 30 min at 37°C), suspended in Na⁺-free medium, showed a 30–65% increase in Ca²⁺ uptake compared to cells in 140 mM Na⁺ medium. Enhanced Ca²⁺ influx was entirely dependent on ouabain pretreatment and reversal of the Na⁺ gradient. Na pump inhibition or Na ionophore addition and subsequent exposure to Na⁺-free medium resulted in a sustained elevation of cytosolic Ca²⁺. As preincubation of cells in Ca²⁺-free medium further enhanced the ouabain-dependent increase in cytosolic Ca²⁺, the effects of the microsomal Ca²⁺-ATPase inhibitor thapsigargin on Ca²⁺ influx and cytosolic Ca²⁺ were studied. Thapsigargin stimulated Ca²⁺ entry following ouabain pretreatment and reversal of the Na⁺ gradient; the effects of thapsigargin were retained in the presence of LaCl₃, a potent inhibitor of store-dependent calcium influx pathways. These results show lymphocytes demonstrate Na⁺/Ca²⁺ exchange activity and suggest the Na⁺/Ca²⁺ exchanger modulates cytosolic Ca²⁺ following intracellular Ca²⁺ store depletion. (*J. Clin. Invest.* 1994. 94:2002–2008.) **Key words:** human T lymphocytes • thapsigargin • Ca²⁺ uptake • store-dependent Ca²⁺ influx • intracellular Ca²⁺ stores

Introduction

The presence of the Na⁺/Ca²⁺ exchanger has been suggested from experiments with rabbit lymphocyte vesicles (1) and human peripheral T lymphocytes and Jurkat T cells treated with Na⁺/Ca²⁺ exchange inhibitors (2, 3). Indeed, modulation of cytosolic Ca²⁺ by Na⁺/Ca²⁺ exchange mechanisms has been suggested as an important component of lymphocyte activation and/or proliferation responses (2). In contrast to these observations, Donnadieu and co-workers (4, 5) found no evidence of Na⁺/

Ca²⁺ exchange activity in T cell clones or the Jurkat T cell. To examine this issue further, we have employed a sensitive assay to detect Na⁺/Ca²⁺ exchange activity in human lymphocytes, i.e., cytosolic Na⁺-dependent Ca²⁺ influx.

Identifying Na⁺/Ca²⁺ exchange activity in lymphocytes would increase our understanding of functional alterations in cation transport in several pathological conditions. White blood cells from subjects exhibiting essential hypertension (6–9) and diabetes mellitus (10, 11) have been studied to gain insight into cellular Na⁺ and Ca²⁺ transport abnormalities. However, in order for lymphocytes to serve as a model for cellular Na⁺ and Ca²⁺ homeostasis, they should express the cation transport mechanisms found in the target cells known to be affected in these disorders. Examples of target cells identified in hypertension and diabetes include the vascular smooth muscle and renal epithelial cells. These cells express plasma membrane Na⁺-K⁺-ATPase, Na⁺-K⁺-Cl⁻ cotransport, Na⁺ H⁺ antiport, Ca²⁺-ATPase, and Na⁺/Ca²⁺ exchange activities, and investigators have detected abnormal function or regulation of one or more of these transport systems in hypertension and diabetes (12–17). Inasmuch as lymphocytes have been shown to express all but Na⁺/Ca²⁺ exchange activity, identifying this transport system would justify the use of this cell as a model for pathological alterations in Na⁺ and Ca²⁺ transport.

Recently, we observed that Ca²⁺ efflux in human lymphocytes was stimulated by the presence of external Na⁺ (18). Both the rate and threshold cytosolic [Ca²⁺] for Ca²⁺ extrusion were increased in Na⁺-containing medium, consistent with the presence of a Na⁺/Ca²⁺ exchange mechanism in these cells. To obtain conclusive evidence for Na⁺/Ca²⁺ exchange in lymphocytes, we have examined the effects of elevated cytosolic [Na⁺] (produced by Na⁺ pump inhibition or treatment with the Na⁺ ionophore monensin) on Ca²⁺ influx.

Methods

Lymphocyte preparation. Unless noted, all reagents and drugs were from Sigma Chemical Co., St Louis, MO. Peripheral blood lymphocytes were obtained from healthy male volunteers by density-gradient centrifugation (Isoprep; Robbins Sci. Corp., Sunnyvale, CA). To limit monocyte contamination, carbonyl iron was used as described (18). Cells were suspended in Na⁺-medium consisting of (in mM): 140 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 20 Hepes, 10 glucose and 0.1% BSA (pH 7.4). Approval and informed consents were obtained from all subjects; the protocol was approved by the Institutional Review Board of UMDNJ-New Jersey Medical School. Phenotypic analysis of three lymphocyte preparations with dual fluorescence Ab labeling (19) on a FACScan® (Becton Dickinson Immunocytometry Systems, Mountain View, CA) indicated (mean ± SE) 92 ± 2% lymphocytes and 9 ± 3% monocytes. CD2⁺ T cells comprised 88 ± 3% of all lymphocytes and B cells identified by the CD20 marker comprised 5 ± 2% of all cells. Cell viability was > 95% as judged by trypan blue exclusion. Lymphocyte specific activators, including 3 μg/ml anti-CD3 (OKT3; Ortho Diagnostics, Raritan, NJ) and 50 μg/ml concanavalin A (type IV), increased cytosolic Ca²⁺ in preparations monitored with the Ca²⁺-sensitive fluorescence dye fura-2.

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Ca²⁺ influx measurements. Lymphocytes were preincubated with or without 0.1 mM ouabain in Na⁺-medium for 30 min. To initiate Ca²⁺ uptake, aliquots (5 μ l) of cells (1 \times 10⁸/ml) were diluted 20-fold into Na⁺ medium or Na⁺-free medium (*N*-methyl-D-glucamine adjusted to pH 7.4 with HCl [NMDG]¹ or LiCl substituted isosmotically for NaCl) containing 10 μ Ci/ml ⁴⁵CaCl₂ (final extracellular [Na⁺]=6.7 mM in Na⁺-free medium). Ouabain pretreatment was used to increase cytosolic Na⁺ through inhibition of the Na⁺-K⁺-ATPase; in these and other ouabain pretreatment experiments, 0.1 mM ouabain was also included in the assay buffer. After 5-s–1-h incubation at 37°C, Ca²⁺ uptake was stopped by addition of 5 ml ice-cold Hepes medium (40 mM Hepes, pH 7.0, 100 mM MgCl₂, and 10 mM LaCl₃); extracellular radioactivity was removed by rapid filtration of cells on 0.45 μ m filters (Millipore, Bedford, MA) with two additional washes of LaCl₃-containing Hepes-medium as described (20). Each data point represents the mean of *n* experiments derived from triplicate or quadruplicate measurements. For Ca²⁺ influx experiments, background counts (medium without cells) and 0 time Ca²⁺ uptake values were subtracted from all experimental time points (except Fig. 1, *inset*, where only background counts were subtracted).

Fluorescence measurements. Lymphocytes were incubated for 30 min at 37°C with 2 μ M acetoxymethyl ester form of fura-2 (fura-2 AM) (Molecular Probes, Inc., Eugene, OR) in Na⁺ medium or Na⁺ medium minus CaCl₂ containing 0.3 mM EGTA (EGTA-Na⁺ medium), with or without 0.1 mM ouabain as indicated. Cells were centrifuged for 5 s to remove external fura-2, resuspended in 50 μ l of nominally Ca²⁺-free Na⁺ medium (medium with CaCl₂ omitted), and injected into cuvettes containing 3 ml nominally Ca²⁺-free Na⁺ medium or Ca²⁺-free, Na⁺-free medium (isosmotic LiCl substitution for NaCl; the final extracellular [Na⁺] was 2.3 mM, due to the 60-fold dilution of cells in Na⁺-free medium). Fluorescence was monitored in stirred cells at 37°C in a Fluorolog II spectrofluorometer (SPEx Industries, Edison, NJ) after addition of CaCl₂ to bring extracellular Ca²⁺ to 1 mM. Excitation and emission wavelengths were 340–380 nm and 505 nm, respectively. Calibration of cytosolic [Ca²⁺] (21) was achieved by exposing lymphocytes to 100 μ M digitonin in Na⁺ medium (R_{max}) followed by adding 15 mM EGTA (pH 8.0, R_{min}). Fura-2 leakage at the completion of the experiment was assessed by adding 0.5 mM MnCl₂; as in previous studies, there was only a small (< 5%) decrease in the fluorescence spectra indicating minimal dye leakage (data not shown). Autofluorescence (determined in the presence of 0.5 mM MnCl₂ and 100 μ M digitonin at the end of each experiment) was subtracted from fluorescence spectra prior to cytosolic [Ca²⁺] calculations.

Mn²⁺ can serve as a Ca²⁺ surrogate to study Ca²⁺ entry in fura-2-loaded cells (18, 22, 23). In these experiments, Mn²⁺ entering the cell binds to fura-2 and quenches its fluorescence; the quenching is monitored at the isosbestic wavelength (360 nm) of fura-2 such that changes in cytosolic Ca²⁺ do not affect the emission fluorescence. Mn²⁺ uptake was performed in fura-2-loaded cells exposed to Na⁺ or Na⁺-free medium containing 30 nM thapsigargin (LC Services, Woburn, MA), 0.5 mM MnCl₂ and 0.1–3 μ M LaCl₃. Fluorescence was monitored at excitation and emission wavelengths of 360 and 505 nm, respectively, and results were normalized using 360 nm values obtained immediately before addition of MnCl₂.

Cytosolic Na⁺ was measured in lymphocytes incubated with 5 μ M sodium-binding benzofuran isophthalate acetoxymethyl ester (SBFI-AM) and 20% (wt/vol) Pluronic acid F-127 (Molecular Probes) in Na⁺ medium for 60 min at 37°C, with or without 0.1 mM ouabain for the last 30 min. Fluorescence was measured at excitation and emission wavelengths 340–385 nm, and 505 nm, respectively, and calibration was performed by the gramicidin method (24). Autofluorescence was determined in unloaded cells and subtracted from fluorescence readings.

Statistical analysis. Repeated measures analysis of variance (ANOVA) was performed on an IBM compatible PC with a Statistical Analysis Systems (SAS) package, using the general linear model procedure (PROC GLM, Version 6.0; SAS Institute, Cary, NC). As Ca²⁺ uptake measurements were performed in triplicate or quadruplicate, a repeated measures (i.e., unbalanced) design using PROC GLM was utilized for this analysis (further details are given in reference 25). Students' *t* test was performed using Sigma Plot, version 4.0 (Jandel Scientific Software, San Rafael, CA). A *P* value < 0.05 was considered statistically significant. Data are reported as mean values \pm SE.

Results

Effect of reversing the Na⁺ gradient on Ca²⁺ influx in lymphocytes. The Na⁺/Ca²⁺ exchanger is a carrier system that mediates the transport of Ca²⁺ across the membrane in direct exchange for Na⁺ (reviewed in reference 26). In most cells expressing Na⁺/Ca²⁺ exchange activity, the physiological role of this system is to pump Ca²⁺ out of the cell ("forward" mode Na⁺/Ca²⁺ exchange); however, under conditions where cytosolic Na⁺ is increased, the exchanger can carry out the net influx of Ca²⁺ ("reverse mode" Na⁺/Ca²⁺ exchange). To determine whether Na⁺/Ca²⁺ exchange activity is present in lymphocytes, we employed ⁴⁵Ca²⁺ to study the nonsteady state accumulation of isotope by reverse mode Na⁺/Ca²⁺ exchange. We first calculated Ca²⁺ uptake in cells pretreated with ouabain (to inhibit the Na⁺ pump and raise cytosolic Na⁺) and suspended in Na⁺ or Na⁺-free media. These conditions were imposed to decrease or reverse the inwardly directed Na⁺ gradient that inhibits Ca²⁺ entry via the Na⁺/Ca²⁺ exchanger. Lymphocytes exposed to 0.1 mM ouabain in Na⁺ medium for 30 min demonstrated increased cytosolic [Na⁺] (42.9 \pm 2.4 vs 20.5 \pm 2.7 mM for untreated cells, *n* = 5). The time course of Ca²⁺ influx is shown in Fig. 1. Ca²⁺ influx was significantly increased (30–65%) in cells in Na⁺-free medium vs. cells in Na⁺ medium, consistent with the presence of a Na⁺/Ca²⁺ exchange mechanism mediating Ca²⁺ entry. As shown in the inset to Fig. 1, Ca²⁺ influx increased linearly during the first 20–30 s in Na⁺-containing and Na⁺-free media. In general, the transient increase in Ca²⁺ uptake was followed by a lower, steady-state amount of Ca²⁺ influx. This decreased Ca²⁺ uptake was observed in cells in Na⁺-free medium and to a lesser extent in cells in Na⁺ medium. The difference may reflect loss of cytosolic Na⁺ from the cells; ouabain-treated cells suspended in Na⁺-free medium underwent a 35–40% decrease in cytosolic [Na⁺] during a 10-min period (data not shown). The data in Fig. 2 show that the enhanced uptake of Ca²⁺ in the Na⁺-free medium was not observed if ouabain was omitted from the preincubation medium. Thus, both an increase in cytosolic [Na⁺] and reversal of the Na⁺ gradient are necessary to demonstrate enhanced Ca²⁺ influx in lymphocytes.

Na⁺/Ca²⁺ exchange-mediated increases in Ca²⁺ entry and cytosolic Ca²⁺ in Na⁺-loaded lymphocytes. Fig. 3, *A* and *B* show the cytosolic Ca²⁺ profiles in cells that had been preincubated 30 min in Na⁺ medium with (Fig. 3 *B*) or without (Fig. 3 *A*) 0.1 mM ouabain. Two traces are shown in each panel, corresponding to cells that were added to the cuvette containing either Na⁺ medium (*solid line*) or Na⁺-free medium (*dashed line*). Similar to the ⁴⁵Ca²⁺ findings, ouabain-treated cells showed an increase in cytosolic Ca²⁺ in the Na⁺-free medium compared to 140 mM Na⁺ medium, whereas no differences between cells in the two media were observed in the absence of ouabain treatment. Fig. 3, *C* and *D* show similar experiments

1. **Abbreviations used in this paper:** fura-2 AM, acetoxymethyl ester form of fura-2; NMDG, *N*-methyl-D-glucamine adjusted to pH 7.4 with HCl; SBFI-AM, sodium-binding benzofuran isophthalate acetoxymethyl ester.

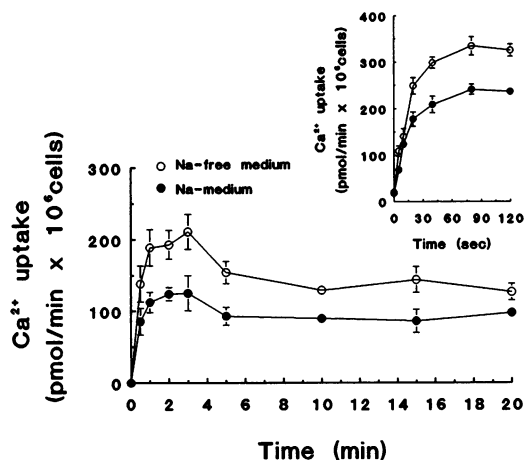


Figure 1. Effect of extracellular Na^+ on Ca^{2+} uptake by ouabain-treated lymphocytes. Lymphocytes were pretreated for 30 min at 37°C in Na^+ medium containing 0.1 mM ouabain and Ca^{2+} uptake assayed at indicated times in the presence of Na^+ -containing (closed circles) or Na^+ -free medium (open circles, NMDG substituted for NaCl). Ca^{2+} uptake at 0.5 and 1 h was unchanged from 20 min values (data not shown). Symbols represent means \pm SE for five experiments. Where error bars are not shown, the SE was smaller than the symbol. ANOVA (repeated measures, PROC GLM) indicated significant differences ($P = 0.0125$) in Ca^{2+} uptake values for cells in Na^+ -containing vs Na^+ -free medium. *Inset*, example of Ca^{2+} influx in the presence of Na^+ or absence of Na^+ from one subject. For this experiment, only the background counts were subtracted from data points. Linear regressions for the first four data points (i.e., 0, 5, 10, and 20 s) were: Na^+ -medium; $y = 8.0(x) + 26.7$, $r = 0.98$, and for Na^+ -free medium; $y = 10.9(x) + 33.7$, $r = 0.99$. Symbols represent means \pm SE for quadruplicate measurements.

using cells preincubated with or without ouabain in EGTA- Na^+ -medium. The EGTA treatment decreases intracellular Ca^{2+} stores (Decreases in intracellular store size were estimated in fura-2-loaded cells resuspended in EGTA- Na^+ medium treated with $5 \mu\text{M}$ ionomycin and 100 nM thapsigargin. The peak of the cytosolic Ca^{2+} transient [reflecting the amount of Ca^{2+} in intracellular stores] was reduced $73.4 \pm 4.2\%$ [plus ouabain] and $67.8 \pm 4.4\%$ [minus ouabain] in cells incubated for 0.5 h in EGTA- Na^+ medium compared to cells incubated in Na^+ medium [$n = 4$ experiments].) and activates extracellular Ca^{2+} entry (see below), resulting in an initial time-dependent increase in cytosolic Ca^{2+} for all preincubation conditions. Ouabain-treated cells showed a larger and more sustained increase in cytosolic $[\text{Ca}^{2+}]$ in Na^+ -free medium than in Na^+ medium,

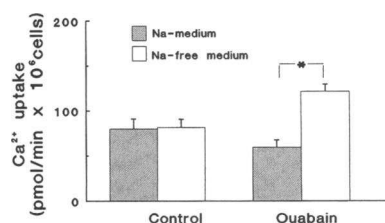


Figure 2. Effect of ouabain on Na^+ -dependent Ca^{2+} uptake. Lymphocytes were preincubated for 30 min at 37°C in Na^+ medium with or without 0.1 mM ouabain and resuspended in Na^+ - or Na^+ -free medium (LiCl substituted for NaCl) containing $^{45}\text{Ca}^{2+}$. Ca^{2+} uptake was measured after 60 s; each experiment was performed in quadruplicate and averaged. Data represent mean values of five (control) and seven (ouabain) experiments. $*P = 0.0002$ (paired t test).

substituted for NaCl) containing $^{45}\text{Ca}^{2+}$. Ca^{2+} uptake was measured after 60 s; each experiment was performed in quadruplicate and averaged. Data represent mean values of five (control) and seven (ouabain) experiments. $*P = 0.0002$ (paired t test).

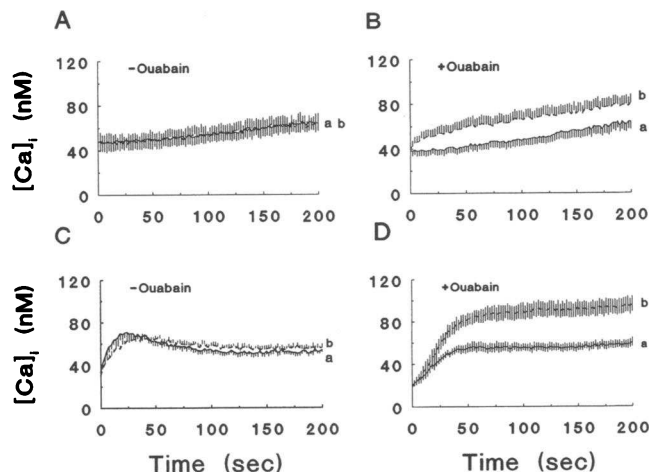


Figure 3. Effect of ouabain and extracellular Ca^{2+} depletion on the cytosolic Ca^{2+} profile in cells reexposed to Ca in Na^+ -containing and Na^+ -free medium. Lymphocytes were incubated for 30 min with fura-2 AM at 37°C in 1 mM Ca -containing media (A and B) or Ca -free medium (0.3 mM EGTA Na^+ medium (C and D), with or without ouabain as indicated. For experiments, cells were resuspended in nominally Ca^{2+} -free Na^+ medium and injected into cuvettes containing 3 ml of nominally Ca^{2+} -free a) Na^+ medium (solid lines), or b) or Na^+ -free medium (LiCl substituted for NaCl; broken lines). Just before fluorescence monitoring, CaCl_2 was added (final extracellular $[\text{Ca}^{2+}] = 1 \text{ mM}$) to the cuvette. Results are from five (A and B), eight (C), and ten (D) experiments; in these and subsequent figures, vertical bars denote SE.

whereas the changes in cytosolic $[\text{Ca}^{2+}]$ in ouabain-free cells were independent of extracellular Na^+ .

Na^+ -dependent Ca^{2+} entry was also studied in ouabain-free cells using alternative procedures to raise cytosolic Na^+ (Fig. 4). In the first procedure, cytosolic $[\text{Na}^+]$ was increased by incubating lymphocytes in K^+ -free medium at 4°C to inhibit the Na^+ pump. Fig. 4 A shows the cytosolic Ca^{2+} profile of these cells suspended in Na^+ -free medium (trace c), 40 mM (trace b) and 140 mM (trace a) Na^+ medium containing 1 mM Ca^{2+} . Following a rapid increase in Ca^{2+} , a sustained level of cytosolic $[\text{Ca}^{2+}]$ is attained that is inversely related to the extracellular $[\text{Na}^+]$. The initial increase in cytosolic Ca^{2+} probably relates to Ca^{2+} entry through $\text{Na}^+/\text{Ca}^{2+}$ exchange and store-dependent Ca^{2+} entry mechanisms, whereas the sustained phase reflects modulation of cytosolic Ca^{2+} by forward mode $\text{Na}^+/\text{Ca}^{2+}$ exchange. For the second maneuver, Na^+ -dependent Ca^{2+} entry in the presence of an active Na^+ pump was examined using monensin, a Na^+ ionophore (Fig. 4 B). Pretreatment of lymphocytes for 2 min with $10 \mu\text{M}$ monensin in Na^+ medium followed by exposure to 40-mM Na^+ medium (trace b) resulted in an approximate doubling in the cytosolic $[\text{Ca}^{2+}]$ compared to ionophore-free cells (trace a). These data suggest Ca^{2+} influx is enhanced by increased cytosolic Na^+ and can be produced independent of ouabain treatment or Na^+ pump inhibition. The results support the conclusion of the Ca^{2+} flux studies and indicate that lymphocytes contain a $\text{Na}^+/\text{Ca}^{2+}$ exchanger which modulates both Ca^{2+} entry and cytosolic Ca^{2+} .

Thapsigargin enhances Na^+ -dependent Ca entry in ouabain-treated lymphocytes. Recently, specific inhibitors of the sarco(endo)plasmic reticulum Ca ATPase (SERCA), including thapsigargin (27), have been used to inhibit Ca^{2+} uptake by the endoplasmic reticulum, deplete intracellular stores of Ca^{2+} and

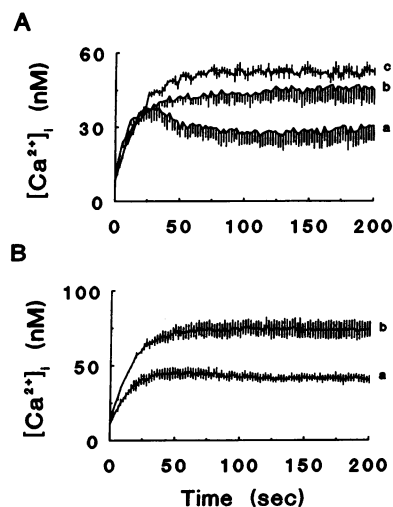


Figure 4. Demonstration of Na^+ -dependent increases in cytosolic Ca^{2+} in the absence of ouabain. (A) Effect of Na^+ pump inhibition and extracellular Na^+ replacement on the cytosolic Ca^{2+} profile. Lymphocytes were loaded with fura-2 AM in nominally Ca^{2+} -free, K^+ -free, Na^+ medium for 30 min (15 min at 37° , followed by 15 min at $4^\circ C$). Cells were resuspended in nominally Ca^{2+} -free Na^+ medium and subjected to (a) 140 mM Na^+ medium, (b) 40 mM Na^+ medium or (c) Na^+ -free medium. $CaCl_2$ was added (final extracellular $[Ca^{2+}] = 1$ mM) to cells at 0 s. Results are from three experiments. (B) Effect of monensin pretreatment and extracellular Na^+ on cytosolic Ca^{2+} profile. Fura-2 loaded lymphocytes were treated with vehicle (ethanol, 0.1% final volume) or 10 μ M monensin in Na^+ medium for 2 min, resuspended in 50 μ l of nominally Ca^{2+} -free, Na^+ medium and respectively subjected to (a) 40 mM Na^+ -medium plus 0.1% ethanol, or (b) 40 mM Na^+ medium plus 10 μ M monensin. $CaCl_2$ (final extracellular $[Ca^{2+}] = 1$ mM) was added to cells at 0 s. Results are from four experiments. In one preparation in which cytosolic Na^+ was measured with SBFI, 10 μ M monensin addition acutely (within 10 s) increased cytosolic Na^+ from 17 to 70 mM.

activate extracellular Ca^{2+} entry (28). The intracellular signal-mediating Ca^{2+} entry is unknown (29, 30), but is identical to that produced in cells incubated in Ca^{2+} -free buffer (29). Ca^{2+} entry mediated by this process has been referred to as "capacitative Ca^{2+} entry" (31) or "store-dependent Ca^{2+} influx" (32). As thapsigargin addition results in a sustained increase in cytosolic Ca^{2+} in lymphocytes in Ca^{2+} -containing media (18, and see Fig. 6 B), we examined thapsigargin-induced Ca^{2+} entry in ouabain-treated or control cells suspended in Na^+ or Na^+ -free medium. Cells were incubated with fura-2 AM in Ca^{2+} -containing medium, centrifuged, and resuspended in nominally Ca^{2+} -free buffer. In Fig. 5, traces c and d show that addition of 30 nM thapsigargin and Ca^{2+} (extracellular $[Ca^{2+}] = 1$ mM) produced similar cytosolic Ca^{2+} responses in ouabain-free cells in both Na -containing and Na^+ -free medium. The cytosolic Ca^{2+}

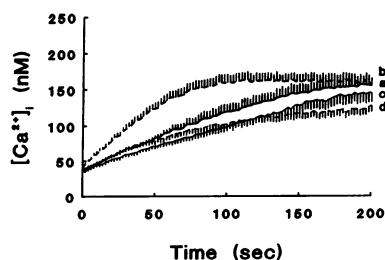


Figure 5. Effect of ouabain pretreatment and extracellular Na^+ on thapsigargin-induced cytosolic Ca^{2+} profiles. Lymphocytes were incubated during fura-2 loading period with or without ouabain in Na^+ medium, resuspended in 50 μ l of nominally Ca^{2+} -free Na^+ medium and injected into cuvettes containing nominally Ca^{2+} -free (a) Na^+ -medium plus ouabain (solid line), (b) Na^+ -free medium plus ouabain (broken line), (c) Na^+ medium (solid line), or (d) Na^+ -free medium (broken line). Prior to fluorescence monitoring, 30 nM thapsigargin and $CaCl_2$ (final extracellular $[Ca^{2+}] = 1$ mM) were added to the cuvette. Results are from 10 experiments.

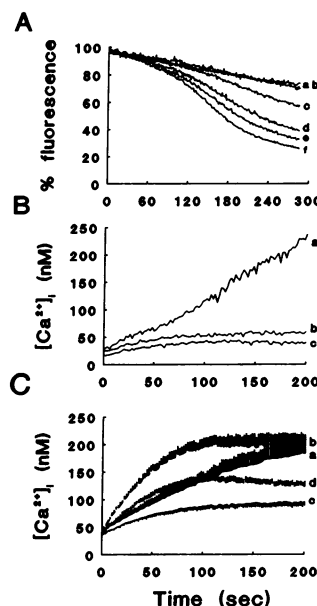


Figure 6. Thapsigargin enhances cytosolic Ca^{2+} independent of store-dependent Ca^{2+} entry. (A) Inhibition of thapsigargin-evoked Mn^{2+} uptake by $LaCl_3$. Lymphocytes were exposed to 30 nM thapsigargin and/or $LaCl_3$ in Na^+ medium and monitored at 360 nm as described in Methods. The decline in percent fluorescence is due to Mn^{2+} entry and quenching of cytosolic fura-2; an increase in the rate of decline results from enhanced Mn^{2+} uptake from the extracellular medium. Traces: (a) vehicle (0.1% DMSO), (b) thapsigargin plus 3 μ M La^{3+} , (c) thapsigargin plus 1 μ M La^{3+} , (d) thapsigargin plus 0.3 μ M La^{3+} , (e) thapsigargin plus 0.1 μ M La^{3+} , (f) thapsigargin alone. Initial 360 nm fluorescence values prior to addition of Mn^{2+} were $1.41 \pm 0.04 \times 10^6$ counts per s ($n = 6$). Results are representative of three experiments. (B) Inhibition of thapsigargin-evoked Ca^{2+} entry by $LaCl_3$. Lymphocytes were resuspended in nominally Ca^{2+} -free Na^+ medium and subjected to 30 nM thapsigargin and (a) 1 mM Ca^{2+} , (b) 1 mM Ca^{2+} plus 3 μ M La^{3+} , or (c) 0.3 mM $EGTA$. Increases in cytosolic $[Ca^{2+}]$ in response to thapsigargin (measured as basal cytosolic $[Ca^{2+}]$ at 0 s subtracted from peak cytosolic $[Ca^{2+}]$ achieved at ~ 100 s) were 42.5 ± 7.4 nM and 35.5 ± 5.3 nM for cells exposed to $LaCl_3$ (trace b) and $EGTA$ (trace c), respectively. Results are representative of six experiments. (C) Effect of ouabain pretreatment and extracellular Na^+ on thapsigargin induced cytosolic Ca^{2+} profile in the presence of $LaCl_3$. Lymphocytes were pretreated with ouabain in Na^+ -medium, resuspended in nominally Ca^{2+} -free medium and injected into cuvettes containing ouabain, 30 nM thapsigargin and (a) Na^+ medium (solid line), (b) Na^+ -free medium (broken line), (c) Na^+ medium plus 3 μ M La^{3+} (solid line), or (d) Na^+ -free medium plus 3 μ M La^{3+} (broken line). Immediately before the experiment, $CaCl_2$ (final extracellular $[Ca^{2+}] = 1$ mM) was added to the cuvette. Results are from six experiments.

profile for ouabain-treated cells suspended in Na^+ medium (trace a) was only slightly elevated compared to ouabain-free cells. Thapsigargin-induced increase in cytosolic Ca^{2+} was accelerated when ouabain-treated cells were assayed in Na^+ -free medium (trace b). Thus, increased Ca^{2+} entry in thapsigargin-stimulated, ouabain-treated cells is sensitive to extracellular Na^+ , suggesting that the Na^+/Ca^{2+} exchanger contributes to thapsigargin-induced Ca^{2+} entry under these conditions.

To determine the extent of Ca^{2+} uptake by Na^+/Ca^{2+} exchange in thapsigargin-treated cells, we utilized $LaCl_3$ to differentiate Ca^{2+} entry by store-dependent Ca^{2+} influx and Na^+/Ca^{2+} exchange pathways. Ca^{2+} entry by the store-dependent pathway is easily monitored by assaying Mn^{2+} uptake (18, 22, 23). In addition, in the presence of 1 mM Ca^{2+} , low concentrations of $LaCl_3$ (1–3 μ M) inhibit store-dependent pathways without affecting Na^+/Ca^{2+} exchange activity (33). We thus used $LaCl_3$ to distinguish thapsigargin-stimulated Ca^{2+} (and Mn^{2+}) entry by store-dependent Ca^{2+} influx pathways and Ca^{2+} uptake via the Na^+/Ca^{2+} exchanger. In Fig. 6 A, thapsigargin-stimulated Mn^{2+} uptake and its inhibition by La^{3+} are shown. Mn^{2+} entry was sensitive to La^{3+} inhibition; at 3 μ M La^{3+} , Mn^{2+} quenching

of fura-2 fluorescence was not different from control (thapsigargin-free) Mn^{2+} uptake. The profile of La^{3+} inhibition of Mn^{2+} uptake in lymphocytes suspended in Na^+ -free medium was unchanged (data not shown). Moreover, La^{3+} inhibited extracellular Ca^{2+} entry in thapsigargin-stimulated, ouabain-free lymphocytes (Fig. 6 B). Thapsigargin-induced increases in cytosolic Ca^{2+} in cells in La^{3+} -containing medium (trace *b*) were similar to cells suspended in EGTA- Na^+ medium (trace *c*); both responses were significantly lower than the thapsigargin-induced increase in cytosolic Ca^{2+} in cells suspended in Ca^{2+} -containing, La^{3+} -free medium (trace *a*). Thus, $3 \mu\text{M}$ La^{3+} inhibits thapsigargin-stimulated Ca^{2+} uptake in the presence of extracellular Ca^{2+} . Finally, the effect of La^{3+} on thapsigargin-stimulated increases in cytosolic Ca^{2+} in ouabain-treated cells is shown in Fig. 6 C. La^{3+} reduced, but did not abolish, the increase in cytosolic Ca^{2+} in both Na^+ -containing (see traces *a* and *c*) and Na^+ -free medium (see traces *b* and *d*); inhibition by La^{3+} was more pronounced in Na^+ medium than in Na^+ -free medium. Thapsigargin increased the rate of Ca^{2+} entry and cytosolic Ca^{2+} by 100–150% in Na^+ -free medium, even in the absence of Ca^{2+} entry via store-dependent Ca influx pathways (see traces *c* and *d*). The results indicate that in human lymphocytes, thapsigargin enhances Ca^{2+} entry through a La^{3+} -insensitive pathway that is stimulated by elevated cytosolic Na^+ and reduced extracellular $[\text{Na}^+]$ (i.e., the $\text{Na}^+/\text{Ca}^{2+}$ exchanger).

Discussion

This study examined Na^+ -dependent Ca^{2+} entry in human peripheral blood lymphocytes. The main finding is that $\text{Na}^+/\text{Ca}^{2+}$ exchange activity is present in lymphocytes, as both isotopic and fluorescence assays showed enhanced Ca^{2+} entry following maneuvers that increased cytosolic Na^+ and decreased extracellular $[\text{Na}^+]$. In addition, this work has developed the concept that Ca^{2+} entry and increased cytosolic Ca^{2+} are enhanced by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger following intracellular Ca^{2+} store depletion. These findings have important implications for Ca^{2+} homeostasis and cation transport in lymphocytes.

Wacholtz et al. (2, 3) have proposed that in T lymphocytes, the $\text{Na}^+/\text{Ca}^{2+}$ exchanger mediates increased Ca^{2+} entry, resulting in interleukin 2 (IL-2) synthesis and IL-2 receptor expression following cell activation. Their findings were based on the effects of inhibitors of $\text{Na}^+/\text{Ca}^{2+}$ exchange such as bepridil and amiloride analogs. These inhibitors, however, are not specific antagonists of $\text{Na}^+/\text{Ca}^{2+}$ exchange and can have multiple effects, particularly when exposed to cells over a prolonged period, as in the experiments of Wacholtz et al. (2, 3). The findings of these investigators were further challenged by Donnadieu and co-workers (4, 5), who were unable to detect $\text{Na}^+/\text{Ca}^{2+}$ exchange activity in T cell clones and Jurkat T cells. We observed increased Ca^{2+} uptake and cytosolic Ca^{2+} only after cytosolic $[\text{Na}^+]$ was approximately doubled (by ouabain pretreatment) and cells were exposed to Na^+ -free medium. These experimental conditions may explain the inability of some investigators to demonstrate $\text{Na}^+/\text{Ca}^{2+}$ exchange-mediated Ca^{2+} entry in T lymphocytes or in Jurkat T cells (4, 5). In this regard, we were also unable to detect exchange activity in Jurkat T cells (34) using experimental protocols similar to those described in this report. These results suggest that expression and/or activity of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger differ between resting and transfected lymphocytes.

Potential of Ca^{2+} influx in lymphocytes by $\text{Na}^+/\text{Ca}^{2+}$

exchange mechanisms following ouabain-pretreatment is similar to cardiac, skin, lung (35), and aortic tissues (36), and renal epithelial cells (37) and Chinese hamster ovary cells stably expressing the cardiac $\text{Na}^+/\text{Ca}^{2+}$ exchanger (38). Assuming initial exchange-dependent Ca^{2+} uptakes of $106 \text{ pmol}/[\text{min} \times 10^6 \text{ cells}]$ (extrapolated from 30-s values, Fig. 1) and a protein concentration of $30 \mu\text{g}/10^6 \text{ cells}$, $\text{Na}^+/\text{Ca}^{2+}$ exchange activity in this peripheral lymphocyte preparation is $\sim 3.5 \text{ nmol}/[\text{min} \times \text{mg}]$. Although this value probably underestimates actual transport activity, it is similar to skin and lung (1.4 and $2.6 \text{ nmol}/[\text{min} \times \text{mg}]$, respectively, 35), but 3–30-fold lower than cardiac ($100 \text{ nmol}/[\text{min} \times \text{mg}]$, 35), aortic ($22 \text{ nmol}/[\text{min} \times \text{mg}]$, 36), epithelial cells ($11.3 \text{ nmol}/[\text{min} \times \text{mg}]$, 37) and transfected Chinese hamster ovary cells ($70 \text{ nmol}/[\text{min} \times \text{mg}]$, 38). The decreased activity of this system may reflect low amounts of exchange protein (Preliminary attempts to identify the lymphocyte $\text{Na}^+/\text{Ca}^{2+}$ exchanger by Western analysis were unsuccessful [M. Condrescu and J. Reeves, unpublished observations]. Although there may be several explanations for this result, we note that attempts to demonstrate the cardiac $\text{Na}^+/\text{Ca}^{2+}$ exchanger in transfected CHO cells (38) were also unfruitful unless immunoprecipitation with ^{35}S -labeled proteins was performed.) or altered kinetic or regulatory properties of the lymphocyte $\text{Na}^+/\text{Ca}^{2+}$ exchanger.

Lymphocytes demonstrated a transient increase in Ca^{2+} uptake that was more pronounced in Na^+ -free than Na^+ -containing medium. The most likely explanation for the transience of this effect is the time-dependent loss of cytosolic Na^+ in Na^+ -free medium, reducing the outwardly directed Na^+ gradient and inhibiting Ca^{2+} influx. In addition, other factors influence regulatory and kinetic aspects of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. For example, NaCl replacement with NMDG (see Fig. 1) results in Na^+/H^+ antiport inhibition and cell acidification, which could reduce $\text{Na}^+/\text{Ca}^{2+}$ exchange mediated Ca^{2+} uptake (39, 40). However, when Ca^{2+} influx was measured in Li^+ -substituted solutions to avoid cell acidification (Li^+ is countertransported in exchange for intracellular H^+), Ca^{2+} uptake in this solution was not enhanced, indicating acute Na^+/H^+ antiport inhibition has minimal effects on Ca^{2+} influx via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. An additional factor is the presence of MgCl_2 , which could decrease Ca^{2+} entry, as Mg^{2+} is a competitive inhibitor of $\text{Na}^+/\text{Ca}^{2+}$ exchange (41, 42). Finally, in addition to forward mode $\text{Na}^+/\text{Ca}^{2+}$ exchange, the plasma membrane Ca^{2+} pump participates in the extrusion of cytosolic Ca^{2+} (18, 43). Accelerated Ca^{2+} pump activity at higher Ca^{2+} levels would tend to diminish cellular Ca^{2+} accumulation.

Enhanced Ca^{2+} uptake was observed in ouabain-treated cells in both NMDG $^+$ - and Li^+ -substituted media. These results suggest $\text{Na}^+/\text{Ca}^{2+}$ exchange-mediated Ca^{2+} influx in lymphocytes is independent of the substituted, extracellular cation. These experiments do not address effects of extracellular cation substitution on kinetic parameters of the $\text{Na}^+/\text{Ca}^{2+}$ exchange, which could affect estimations of V_{max} .

Cytosolic Ca^{2+} experiments in fura-2-loaded cells supported the results of Ca^{2+} uptake studies, and shared the requirement for ouabain pretreatment and decreased extracellular Na^+ to distinguish $\text{Na}^+/\text{Ca}^{2+}$ exchange-dependent Ca^{2+} entry. In some cells, low extracellular $[\text{Na}^+]$ results in inositol trisphosphate generation, Ca^{2+} mobilization from intracellular stores and increases in cytosolic Ca^{2+} (44). A similar mechanism could explain the increase in cytosolic Ca^{2+} in ouabain-treated cells suspended in Na^+ -free medium (Fig. 3 B). However, ouabain-

free cells in Na^+ -free medium did not respond with a cytosolic Ca^{2+} transient. Furthermore, lymphocytes preincubated in ouabain and EGTA- Na^+ medium demonstrated enhanced cytosolic Ca^{2+} responses in Na^+ -free medium. As this treatment decreases intracellular Ca^{2+} stores, increases in cytosolic Ca^{2+} in Na^+ -free medium result from extracellular Ca^{2+} influx. This finding was further explored by treating lymphocytes with thapsigargin to inhibit SERCA- Ca^{2+} ATPase activity and activate store-dependent Ca^{2+} influx mechanisms. Ouabain pretreatment enhanced the rate and magnitude of Ca^{2+} entry in thapsigargin-treated lymphocytes. One possible explanation for this result is that the elevated cytosolic Na^+ inhibits Ca^{2+} efflux mediated by the exchanger, so that higher cytosolic $[\text{Ca}^{2+}]$ are attained during Ca^{2+} entry via the store-dependent Ca^{2+} influx pathway in ouabain-treated cells. This interpretation emphasizes the importance of $\text{Na}^+/\text{Ca}^{2+}$ exchange as a Ca^{2+} efflux mechanism in lymphocytes. Another explanation is that intracellular store depletion enhances Ca^{2+} entry not only through store-dependent Ca^{2+} channels (45), but also through the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. Even when La^{3+} was present to block the Ca^{2+} influx pathway (see Fig. 6 C), ouabain pretreatment and suspension of cells in Na^+ -free medium enhanced the cytosolic Ca^{2+} profile. A similar observation was made in a recent study examining the effects of thapsigargin on Ca^{2+} uptake in transfected Chinese hamster ovary cells expressing the cardiac $\text{Na}^+/\text{Ca}^{2+}$ exchanger (33).

The primary role of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in lymphocytes is most likely cytosolic Ca^{2+} extrusion (18), for example, during Ca^{2+} -spiking events (45–47) or periods of elevated cytosolic $[\text{Ca}^{2+}]$ following cellular activation. However, as discussed above, a role for $\text{Na}^+/\text{Ca}^{2+}$ exchange-mediated Ca^{2+} entry during lymphocyte activation should also be considered. Dolmetsch and Lewis (45) recently reported low concentrations of thapsigargin (e.g., 10–40 nM) induced cytosolic Ca^{2+} oscillations in T lymphocytes that were dependent on partial emptying of intracellular Ca^{2+} stores and extracellular Ca^{2+} influx. Treatment of lymphocytes with thapsigargin and phorbol myristate acetate in vitro increased IL-2 production (a Ca^{2+} -dependent phenomenon) and expression of IL-2 receptors (48), indicating thapsigargin can stimulate cell proliferation. Breittmayer and colleagues (49) reported that microsomal Ca^{2+} -ATPase inhibition was closely associated with intracellular store emptying, inhibition of phosphatidylinositol synthesis and increased $[\text{H}^3]\text{thymidine}$ incorporation in human peripheral T cells. As T cell antigens and lymphocytic mitogens mobilize Ca^{2+} from intracellular stores and activate Ca^{2+} influx pathways (50, 51), the $\text{Na}^+/\text{Ca}^{2+}$ exchanger may play a role in generating a cytosolic Ca^{2+} signal sufficient for inducing cell proliferation.

Finally, lymphocytes are used by many investigators to study physiological (e.g., ion transport and volume regulation) and pathophysiological (6–11, 52, 53) phenomena. Oshima et al. (8) suggested a potential role for $\text{Na}^+/\text{Ca}^{2+}$ exchange mediating increased cytosolic $[\text{Ca}^{2+}]$ in lymphocytes isolated from essential hypertensive subjects. In light of recent findings suggesting that the $\text{Na}^+-\text{K}^+-\text{ATPase}$ may be inhibited by endogenous ouabain (or a factor exhibiting ouabain-like activity) in some hypertensive subjects (16), increased cytosolic $[\text{Na}^+]$ may indeed have a causal role in the regulation of cytosolic Ca^{2+} and/or Ca^{2+} content of intracellular stores. The evidence presented here affirms the exchanger is present in human lymphocytes, and suggests that this cell preparation may be useful for studying $\text{Na}^+/\text{Ca}^{2+}$ exchange-related alterations in cytosolic Ca^{2+} and Na^+ in essential hypertension and other diseases.

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