HETEROGENEITY OF LYMPHOCYTE CALCIUM METABOLISM IS CAUSED BY T CELL-SPECIFIC CALCIUM-SENSITIVE POTASSIUM CHANNEL AND SENSITIVITY OF THE CALCIUM ATPase PUMP TO MEMBRANE POTENTIAL

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Crosslinking of lymphocyte antigen receptors increases intracellular ionized calcium ($[Ca^{2+}]_i$) (1-4). Phorbol ester-mediated protein kinase C activation complements TCR crosslinking to cause IL-2 production (5) and cell proliferation (6). The antigen receptor can be bypassed by calcium ionophores and phobol esters that act synergistically to induce proliferation of lymphoid cells (7-9). These observations indicate that an increase in free ionized calcium ($[Ca^{2+}]_i$) and activation of protein kinase C (PKC),[1] which binds and is activated by phorbol esters (10), are essential early components in the activation of lymphoid cells. This physiologic state is normally produced by a biologic cascade: antigen binds to its clonally distributed specific receptor in the cell membrane, engaging and activating a presumptive GTP binding protein which, in turn, stimulates membrane phosphodiesterase. Phosphodiesterase hydrolyses membrane phosphatidyl inositol bis-4,5-diphosphate (PIP_2) producing water soluble inositol tris phosphate (IP_3) and membrane soluble diacyl glycerol (DG) (11). IP_3 causes Ca^{2+} release from the rough endoplasmic reticulum, elevating $[Ca^{2+}]_i$ (12). The combination of increased $[Ca^{2+}]_i$ and DG produces PKC activation (13). IP_3 may contribute to Ca^{2+} influx (14). It is metabolized to inositol (1, 3-5) tetrakisphosphate (IP_4) (15), which also may be involved in the influx of extracellular Ca^{2+} (16, 17).

The cytoplasmic ionic environment, including calcium, is tightly regulated in resting cells. Resting $[Ca^{2+}]_i$ is maintained at 100 mM (4), in contrast to the extracellular Ca^{2+} of 1 mM. Since calcium cations cannot be metabolized, and significantly elevated $[Ca^{2+}]_i$ levels are toxic to cells, energy must be expended to maintain the 4-log calcium concentration gradient across the membrane. Calcium is removed from the cell by Ca^{2+}-H+ ATPase (18-22), present in the membrane of the cell and the endoplasmic (sarcoplasmic) reticulum. A Na^+-Ca^{2+} antiporter is often present in the membrane of nonexcitable cells in addition to the Ca^{2+}-H^+ ATPase (23-25). Since these are the only active Ca^{2+} transporting systems known they are considered responsible for regulation of resting $[Ca^{2+}]_i$.

[1] *Abbreviations used in this paper:* DG, diacyl glycerol; IP_3, inositol tris phosphate; PIP, phosphatidyl inositol bis-4,5-diphosphate; PKC, protein kinase C.

There is a 70-mV gradient in electrical potential (Ψ) across the lymphocyte cell membrane (26–29). Since the cytoplasm is negative with respect to extracellular space, this would force Ca^{2+} into the cell through an electrically open Ca^{2+} channel, augmenting the concentration gradient. Calcium channels in neural and muscle cells are voltage dependent, being triggered by depolarization (30–32). There is no convincing evidence for this type of calcium channel in lymphocytes, however, and the relationship between $[Ca^{2+}]_i$ and Ψ in these cells is not well understood.

Nothing is known about $[Ca^{2+}]_i$ regulation above the resting level of 100 nM. A useful approach to this question is to insert a fixed Ca^{2+} influx into the cell membrane with a calcium ionophore and observe cell responses after various manipulations. We have combined this strategy with the measurement of $[Ca^{2+}]_i$ by indo-1 (4, 33, 34), Ψ by oxonol fluorescence (1, 35), and surface markers by immunofluorescence in the flow cytometer. In this paper, we report that ionomycin induces different $[Ca^{2+}]_i$ levels in peripheral T and B lymphocytes and we explore the basis of this phenomenon.

Materials and Methods

Animals and Cell Preparation. 6–8-wk-old BALB/c mice were obtained from the Small Animal Section, Veterinary Resources Branch, NIH. Spleen and/or mesenteric lymph node cells were removed and single cell suspensions were made in serum free medium containing 1 mg/ml BSA.

Reagents. Indo-1 acetoxymethyl ester (indo-1/AM; membrane permeant), indo-1 pentapotassium salt (membrane impermeable), bis-(1,3-dibutylbarbiturate) trimethine oxonol [di-BA-C_4(3), and bis-(1,3-dibutylbarbiturate) pentamethine oxonol [di-BA-C_4(5)] were purchased from Molecular Probes, Inc. (Junction City, OR). A new pH indicator dye, carboxy SNRF-1 (^{3}H-Benzo[c]xanthene,7-(2',4'-Dicarboxyphenyl)-10-dimethylamino-3-one), was generously supplied by Dr. Richard P. Haugland, Molecular Probes, Inc. (Haugland, R. P., Y. Ishida and T. M. Chused, manuscript in preparation). The calcium ionophores, ionomycin (36) and A23187 (37), nigericin, gramicidin, valinomycin, and monensin were obtained from Calbiochem-Behring Corp. (La Jolla, CA). Adenosine 5'-triphosphate was from Sigma Chemical Co. (St. Louis, MO). Biotinylated anti-Ly-2, avidin-phycoerythrin (PE) and PE-conjugated anti-L3T4 were purchased from Becton-Dickinson Monoclonal Center, Inc. (Mountain View, CA). Other reagents, anti-Thy-1.2 (J1j) (38), anti-B220 (RA_3-3A1/6.1) (39), anti-heat stable antigen (M1/69) (40), anti-Ly-1 (53–7.3), anti-$IA^{b,d,q}IE^{d,k}$ (M5/114) and anti-rat Ig κ chain (MAR-18.5) (41) were prepared from culture supernatants or ascites in our laboratory and, when required, conjugated with biotin or FITC. PE conjugation of anti-rat κ chain (clone MAR-18.5) was performed by Molecular Probes, Inc. Rabbit complement (Low-Tox-M) was obtained from Cedarlane Laboratories, Ltd. (Hornby, Ontario, Canada). Serum-free medium used all experiments was prepared by mixing Iscove's medium and F-12 nutritional mixture (Gibco Laboratories, Grand Island, NY) as described previously (42).

Cell Separation. B cells were prepared by treatment of whole spleen cells with anti-Thy-1.2 (J1j) plus anti-Ly-1 (53–7.3) and T cells by exposure to anti-B220 (RA_3-3A1/6.1), anti-HSA (M1/69), and anti-Ia (M5/114) on ice for 30 min followed by anti-rat κ chain (MAR-18.5) for 30 min. After washing, cells were resuspended with 10 times diluted rabbit complement in medium 199 containing 5% FCS and were incubated for 30 min at 37°C. Ionomycin "sensitive" and "resistant" (see below) peripheral lymphocytes were separated by a modified Percoll gradient method. Whole spleen and/or lymph node cells were incubated with 1 μM ionomycin for 20 minutes at 37°C and placed on a discontinuous ionomycin-Percoll gradient (40, 50, 60, 70, and 80% Percoll containing 1 μM ionomycin). Separation was carried out at 2,000 *g* for 10 min at 20°C. The most dense cells, recovered from beneath the 80% Percoll layer (fraction 7) were greatly enriched in resting T cells (>98% of recovered cells expressed Thy-1 and either L3T4 or Ly-2; B cell contamination was <1%). All fraction 7 cells were

ionomycin sensitive. Most of the B cells and a minor portion of T cells were recovered from the middle layer, between 40 and 60% Percoll (fractions 4 and 5), and all were ionomycin resistant.

Preparation of Plasma Membrane Vesicles. Inside-out plasma membrane vesicles were prepared by a modification of the method of Scully et al. (43). 10^9 cells were suspended in 3 ml of lysis buffer (10 mM Tris HCl, pH 7.4, 2 mM $MgCl_2$, 1 mM $NaHCO_3$, 0.5 mM $CaCl_2$) containing 1 mg/ml of indo-1 pentapotassium salt (membrane impermeable) at 0°C and disrupted in a Dounce homogenizer (10–20 strokes). The whole cell lysate was centrifuged 800 *g* for 10 min to remove nuclear debris. The supernatant was placed on 40% Percoll (diluted with Ca^{2+} and Mg^{2+}-free HBSS, pH 7.4) and spun at 27,000 *g* for 1 h (44, 45). The visible band with a density of ~1.03 (determined by density marker beads; Pharmacia Fine Chemicals, Piscataway, NJ), which contained a relatively high number of inside-out membrane vesicles, was collected.

Measurement of $[Ca^{2+}]_i$, Ψ and pH_i. Single cell measurements of $[Ca^{2+}]_i$, Ψ, and pH_i were carried out indirectly using flow cytometry and specific indicator dyes, indo-1 for $[Ca^{2+}]_i$, di-BA-C_4(3) for Ψ, and SNRF-1 for pH_i. Indo-1 AM, di-BA-C_4(3), di-BA-C_4(5), and SNRF-1 were dissolved in DMSO at 1–2 mM and stored at −20°C. Cells were loaded with 1 μM indo-1 AM, 0.2 μM di-BA-C_4(3), 0.5 μM di-BA-C_4(5), or 3 μM SNRF-1 for 45 min (indo-1 AM and SRNF-1) or 10 min. [di-BA-C_4(3) and di-BA-C_4(5)] at 37°C in serum-free medium. For double loading with indo-1 and di-BA-C_4(5), di-BA-C_4(5) was added to the medium during the final 10 min of indo-1 loading. Loaded cell suspensions were maintained under 5% CO_2 at room temperature until use. Under these conditions, indo-1-loaded cells were stable for at least 3 h. Loaded cells were diluted to 1–2 × 10^6 cells/ml with warm medium and brought to 37°C before flow cytometric analysis. For multicolor analysis combined with measurement of $[Ca^{2+}]_i$ or Ψ, cells loaded with the indicator dye were incubated on ice with individual antibodies for 20–30 min. After two washings, cells were resuspended to serum-free medium, warmed as usual, and analyzed by flow cytometry.

Flow Cytometry. Cells were analyzed with a modified FACS-II (Becton-Dickinson Immunocytometry Systems, Sunnyvale, CA) equipped with an argon ion laser (model 2025; Spectra Physics, Mountain View, CA) and a krypton laser (model 164–01; Spectra Physics). The argon laser was operated at 150 mW in all band UV mode for into-1 excitation, 500 mW at 514 nm for SNRF-1, or 500 mW at 488 nm for di-BA-C_4(3). The krypton ion laser was operated at 40 mW at 482 nm to excite FITC and PE or 170 mW at 567 nm for di-BA-C_4(5). For ratio measurement of indo-1 and SNRF-1, emissions were measured simultaneously at two wavelengths. A 22-nm bandpass filter centered at 485 nm (485/22) and a 25-nm bandpass filter centered at 404 (404/25) were used for indo-1. For SNRF-1, 575/30 and 670/13.5 filters were used. In measuring $[Ca^{2+}]_i$, the ratio of linear fluorescence at 485 nm to that at 404 nm was calculated by the data acquisition program. For pH_i, the ratio of 575–670 nm fluorescence was similarly determined. For multicolor studies, all parameters including ratio were recorded in list mode and reprocessed using a PDP 11/84 computer (Digital Equipment Corp., Maynard, MA) with programs developed in our laboratory. The sample tube and nozzle area were maintained at 37°C, except for membrane vesicle experiments, by an airstream incubator (ASI-400; Nicholson Precision Instruments, Gaithersburg, MD).

Calibration. Calibration of $[Ca^{2+}]_i$ was described previously (4). Calibration of pH_i was carried out as follows: SNRF-1–loaded cells were suspended in pH standard buffers containing 145 mM KCl, 15 mM NaCl, 20 mM K-Hepes, 2.5 μg/ml nigericin, and 10 mM sodium azide for 30 min at room temperature and warmed to 37°C 10 min before analysis. After measurement of SNRF-1 fluorescence, pH of individual samples was measured directly with a pH meter (model 3500; Beckman Instruments Inc., Palo Alto, CA). Calibration was carried out at the end of every experiment.

^{45}Ca Efflux. Purified T or B cells were loaded with 10 μCi/ml ^{45}Ca (New England Nuclear, Boston, MA) for 30 min at 37°C. Loaded cells were washed twice with warm serum-free medium immediately before the experiment using a Beckman Instruments Inc. Microfuge B and were resuspended in fresh medium. Recording of time was begun at the end of the final resuspension. 50-μl aliquots were removed from each tube every 0.5 or 1 min and placed on 200 μl of silicon fluid mixture (84% silicon fluid [Siliconoel DC 550, Serva Feinbiochemica,

New York, NY] plus 16% paraffin oil, Saybolt Viscosity 125/135 [Fisher Scientific Co., Fair Lawn, NJ]) using 400-μl microfuge tubes (Beckman Instruments Inc.) and centrifuged within 20 s. Supernatants were removed from each tube, mixed with 2 ml of Aquasol (NEN Research Products, Boston, MA), and counted in a liquid scintillation counter. All experiments were carried out with duplicate samples. The percentage efflux was calculated by dividing counts of samples by the total count of control tubes. ^{45}Ca was released linearly during the first 5 min of incubation, after which the rate gradually decreased due to the recycling of the radioactive Ca^{2+}. The efflux rate observed during the initial 5 min was used to calculate calcium turnover.

Meaurement of ATP-dependent Ca^{2+} Accumulation into Membrane Vesicles. Aliquots of membrane vesicles from the ionomycin-Percoll gradient were diluted with Ca^{2+}/EGTA buffers containing 130 mM K^+ and 20 mM Na^+ at pH 7.2 and analyzed by the flow cytometer. The instrument was triggered by fluorescence so only inside-out vesicles containing sufficient volume of the originally extracellular indo-1 pentapotassium salt were detected. In addition, since ATP binding sites of Ca-ATPase are present on the inner surface of the plasma membrane, only inside-out vesicles would be expected to accumulate Ca^{2+} upon addition of ATP. All the indo-1-containing vesicles responded homogeneously to ATP, suggesting that they were uniformly inside-out. Low level signals caused by debris were gated out allowing detection of the fluorescence of the limited number of inside-out vesicles recovered. As Ca^{2+} accumulation did not occur without Mg^{2+} ATP (used at 1 mM), this process was regarded as ATP-dependent active transfer of Ca^{2+} through the membrane. A total of 1,500–3,000 vesicles were collected for each point.

Results

Kinetics of $[Ca^{2+}]_i$ Response to Ionomycin. The calcium ionophore, ionomycin, specifically transfers calcium ion across the plasma membrane, elevating $[Ca^{2+}]_i$. The response occurs fairly rapidly, reaching a maximum at 2 min and decreasing slightly over the next 3 min to a steady level (Fig. 1), which is maintained unchanged for at least 8 h (data not shown).

The $[Ca^{2+}]_i$ Response to Inomycin Is Heterogeneous. The $[Ca^{2+}]_i$ distribution in spleen, lymph node and peripheral blood lymphocytes treated with 0.7 μM ionomycin was bimodal (Fig. 2 *a*). Simultaneous measurement of $[Ca^{2+}]_i$ and either T cell-specific Thy-1.2 or B cell-specific B220 surface immunofluorescence in two-color flow cytometric analysis of whole spleen or lymph node cells showed that >80% of the T cells had high $[Ca^{2+}]_i$, whereas $[Ca^{2+}]_i$ was low in all the B cells (Figs. 2, *b* and *c*). Measurement of purified T and B cell preparations confirmed this result. A small but

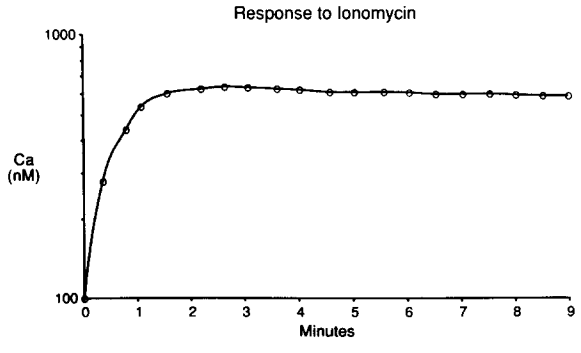

FIGURE 1. Kinetics of ionomycin-dependent $[Ca^{2+}]_i$ elevation. Thymocytes loaded with 1 μM of indo-1/AM at 37°C for 45 min were diluted to 10^6/ml in serum-free medium and analyzed by flow cytometry. Resting $[Ca^{2+}]_i$ was stable at 100 nM. Recording was begun immediately after adding 1 μM ionomycin. $[Ca^{2+}]_i$ values were calculated by the ratio of 485–404 nm linear fluorescence. The mean of 10,000 cells at each time point is shown. Areas surrounding the sample tubing and nozzle were maintained at 37°C. $[Ca^{2+}]_i$ peaked at 530 nM and then declined slightly to a stable level.

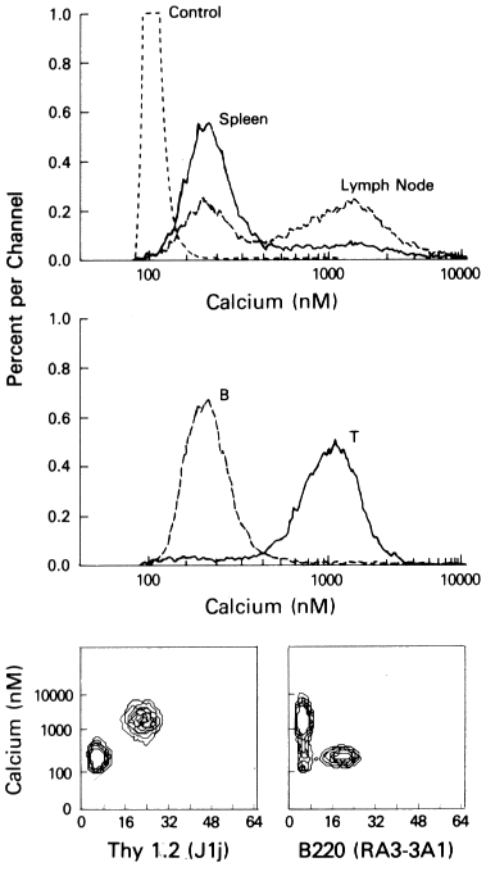

FIGURE 2. $[Ca^{2+}]_i$ response to ionomycin differs between peripheral T and B cells. (*a*) Whole spleen and lymph node cells loaded with 1 μM indo-1/AM were treated with 0.7 μM ionomycin for 15 min before determining $[Ca^{2+}]_i$ by flow cytometry. The $[Ca^{2+}]_i$ distribution of both cell types was bimodal, with a larger fraction of high $[Ca^{2+}]_i$ cells in lymph node. The control $[Ca^{2+}]_i$ of both spleen and lymph node cells was ~100 nM (*top panel*). (*b*) Purified splenic T and B cells were loaded with indo-1/AM and treated with 0.7 μM ionomycin for 15 min. 90% of the T cells were a high $[Ca^{2+}]_i$ peak while all of the B cells formed a low $[Ca^{2+}]_i$ group (*middle panel*). (*c*) Whole spleen cells were first loaded with indo-1/AM at 37°C and then stained with FITC-conjugated anti-Thy-1 or anti-B220 on ice. These cells were rewarmed to 37°C and exposed to 0.7 μM ionomycin. The data were collected in list mode and reprocessed. the Thy-1.2$^+$ T cells were "sensitive" to ionomycin but the B220$^+$ B cells were "resistant" (*bottom panel*). The small fraction of "resistant" T cells are not seen in the lower left panel because they are below the contour levels shown. The indicated $[Ca^{2+}]_i$ is derived from a computer program based on the calibration described previously (4). The data in the upper two panels were recorded at 1,000-channel resolution. Both parameters are shown at 64 channel resolution in the bottom panels.

significant portion of T cells consistently behaved the same as B cells. We designated these responses as ionomycin sensitive (high $[Ca^{2+}]_i$ in response to ionomycin) and ionomycin resistant (low $[Ca^{2+}]_i$). All B cells appear to be ionomycin resistant, while most T cells are ionomycin sensitive.

Effect of Ionomycin Dose. In view of this result, the effect of ionomycin dose on $[Ca^{2+}]_i$ in T and B cells was examined (Fig. 3). At concentrations of ionomycin <0.1 μM, corresponding to ~150 nM $[Ca^{2+}]_i$, $[Ca^{2+}]_i$ was similar in both. Above this level, however, there was a significant difference between the two cell types. $[Ca^{2+}]_i$ increased exponentially with increasing ionomycin in both but with a greater slope in the T cells. For example, at 1 μM ionomycin mean $[Ca^{2+}]_i$ was 2.6 μM in T cells but only 0.3 μM in B cells, nearly a tenfold difference. The range in which T cells were more sensitive to ionomycin than B cells includes the optimal dose for inducing proliferation in this system, 0.5 μM.

A trivial explanation of the difference between T and B cell ionomycin sensitivity would be differential uptake of the ionophore by the two cell types. That this was not the case was demonstrated with a mono-carboxyl calcium ionophore, A23187 (46), which produced heterogenous $[Ca^{2+}]_i$ responses in the same manner as ionomycin (Fig. 4 *a*). Since A23187 itself is fluorescent (unlike ionomycin), we could

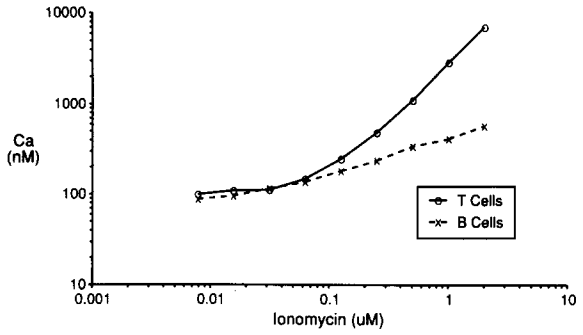

FIGURE 3. Response of T and B cells to ionomycin. T and B cells were isolated by cytotoxic elimination (see Materials and Methods). After indo-1 loading cells were treated with the indicated dose of ionomycin and analyzed by flow cytometry.

measure its uptake. As shown in Fig. 4 *b*, A23187 fluorescence was unimodal, suggesting that there is not a sufficient difference in ionophore uptake to account for the 10-fold difference in $[Ca^{2+}]_i$ between T and B cells. In an additional experiment, A23187 and FITC-conjugated Thy-1 or B220 fluorescence were measured simultaneously. There was no difference in A23187 fluorescence between the T and B cells (data not shown).

Ionomycin Alters the Volume and Density of T but not B Cells. When exposed to hypotonic media T lymphocytes, but not B lymphocytes, exhibit a regulatory volume decrease that requires the presence of extracellular Ca^{2+} (47). For this reason we

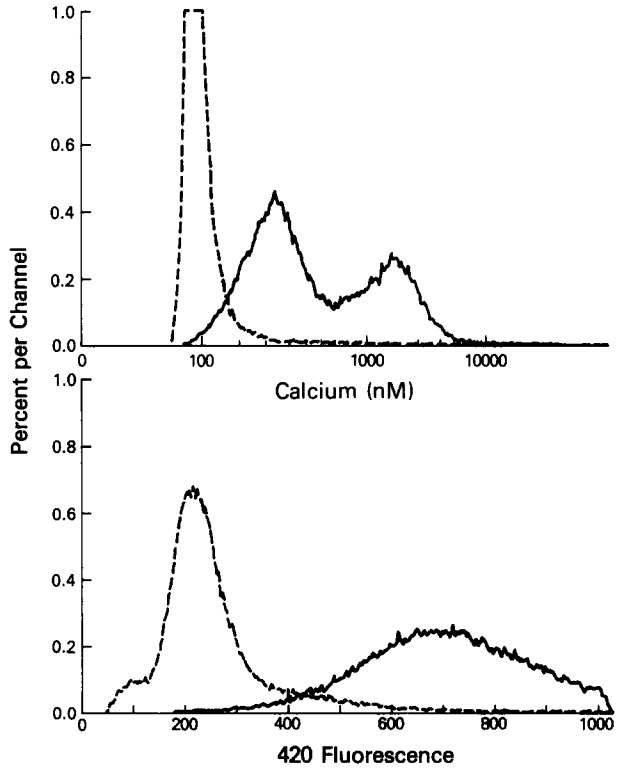

FIGURE 4. The heterogenous ionomycin response of T and B cells is not caused by difference in uptake of ionophore. (*a*) $[Ca^{2+}]_i$ of whole spleen cells exposed to 2 μM A23187 (*solid line*) compared to control (*dashed line*). A bimodal distribution similar to that produced by ionomycin was observed (*top panel*). (*b*) Fluorescence at 420 nm of the same cell suspension without indo-1 loading. 2 μM A23187 (*solid line*) produces a unimodal distribution displaced from the control (*dashed line*). The indo-1 ratio could be determined in the presence of A23187 because its fluorescence is much brighter and measured at somewhat different wavelengths than that of A23187 (*bottom panel*). The data were recorded at 1,000 channel resolution.

compared the T and B cell effects of ionomycin on narrow angle forward light scatter (LS), which is closely related to cell volume (48, 49). As shown in Fig. 5, ionomycin decreased the LS of T cells but had no effect on B cells. This was not a direct effect of ionomycin since it was not observed in calcium-free medium. LS equilibrium was reached in 20 min, in contrast to the 5 min required for $[Ca^{2+}]_i$ equilibrium.

The decrease in T cell volume is presumably due to the selective loss of K^+, passively followed by Cl^- and H_2O, caused by the T cell Ca^{2+}-sensitive K^+ channel (34, 50, and see below). Since this should concentrate intracellular proteins, we reasoned that T cell density would be increased. This was the case, allowing efficient separation of ionomycin-sensitive T cells from other spleen cells by an ionomycin-Percoll gradient (described in Materials and Methods). This technique was used for such purification (see below).

Minimal Effect of Ionomycin on Cytoplasmic pH. Because ionomycin exchanges Ca^{2+} for H^+ its effect on pH_i was determined. The resting pH_i of T cells was 0.1 pH unit higher than that of B cells. pH_i increased slightly during the initial 5 min of exposure to ionomycin but then returned to slightly below the resting level. This indicated that both T and B cells could compensate adequately for the loss of H^+ induced by ionomycin.

^{45}Ca Efflux from T and B Cells. The different T and B cell responses to ionomycin suggested that ^{45}Ca efflux be examined in the two lymphoid populations. In the absence of ionomycin, at resting $[Ca^{2+}]_i$, ^{45}Ca release was similar in T and B cells (Fig. 6 *a*). However, after exposure to 1 μM ionomycin, efflux was more rapid in B than T cells (Fig. 6 *b*), even though under these conditions B cell $[Ca^{2+}]_i$ is much lower than T cell $[Ca^{2+}]_i$ (Fig. 3). This result indicated that the Ca^{2+} pump of B cells is

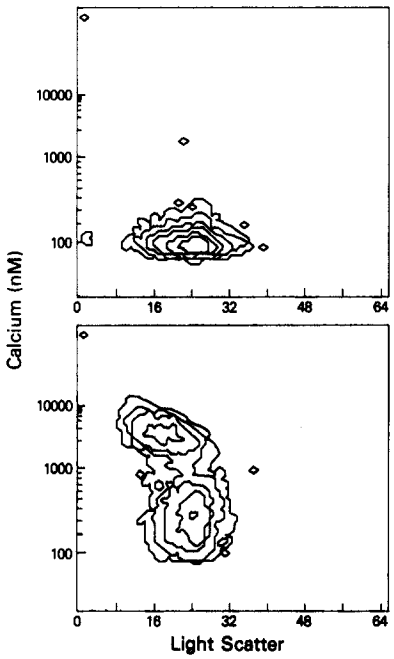

FIGURE 5. Ionomycin decreases the size of ionomycin-sensitive T cells. Contour plots of narrow angle forward light scatter (*x* axis) versus $[Ca^{2+}]_i$ (*y* axis) are shown. (*a*) Resting spleen cells (*top panel*). (*b*) Spleen cells treated with 1 μM ionomycin. The light scatter, which depends on cell size, of the ionomycin-sensitive, high $[Ca^{2+}]_i$ T cells, was reduced (*bottom panel*).

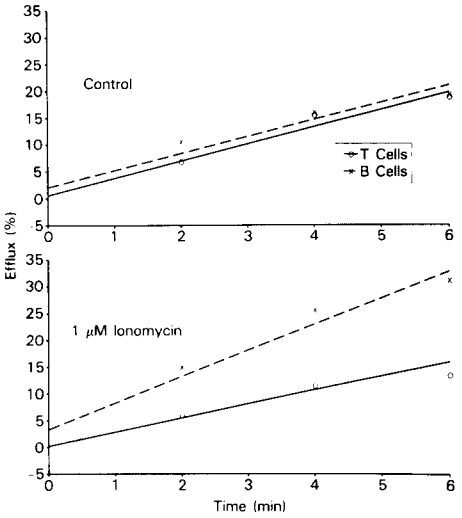

FIGURE 6. ^{45}Ca efflux splenic T and B cells. 10^8 cells/ml in serum free medium were labeled with 10–50 μCi of $^{45}CaCl_2$ for 1 h at 37°C, washed, and resuspended in warm serum-free medium. Aliquots were removed at the times indicated and supernatants were separated by centrifuging the cells through a silicon oil mixture. (*a*) Percent ^{45}Ca efflux of control T and B cells (*top panel*). (*b*) Percent ^{45}Ca efflux of T and B cells treated with 1 μM ionomycin (*bottom panel*). Ionomycin increases the efflux rate in B cells but does not change it significantly in T cells.

more active than that of T cells at elevated $[Ca^{2+}]_i$. It provides an explanation for the difference between T and B cell $[Ca^{2+}]_i$ responses to ionomycin.

ATP-dependent Ca^{2+} Transport Differs in T and B Cell Plasma Membranes. Although a difference in ^{45}Ca efflux between T and B cells in the presence of ionomycin was a consistent finding, the difficulty of such experiments made it important to confirm the result. Inside-out plasma membrane vesicles were prepared separately from T and B cells recovered from an ionomycin-Percoll gradient. They were suspended in graded Ca^{2+}/EGTA buffers and the ATP-dependent accumulation of Ca^{2+} was determined (Fig. 7). Such Ca^{2+} accumulation was completely dependent on ATP. Maximum $[Ca^{2+}]$ occurred 3 min after adding Mg^{2+}-ATP, decreased slightly over the next 5 min, and was then stable for more than 30 min. Ca^{2+} accumulation was significantly higher in B cell vesicles at external $[Ca^{2+}]$ levels above 100 nM, the $[Ca^{2+}]_i$ level of intact resting lymphocytes.

Relationship Between Ψ- and Ionomycin-induced $[Ca^{2+}]_i$ Elevation. Ionomycin hyperpolarized T cells, whereas B cells were more heterogenous and tended to depolarize, as described by Wilson et al. (Fig. 8 and reference 35). At low ionomycin concentrations, corresponding to $[Ca^{2+}]_i$ of 150–200 nM, T cells depolarized slightly, then

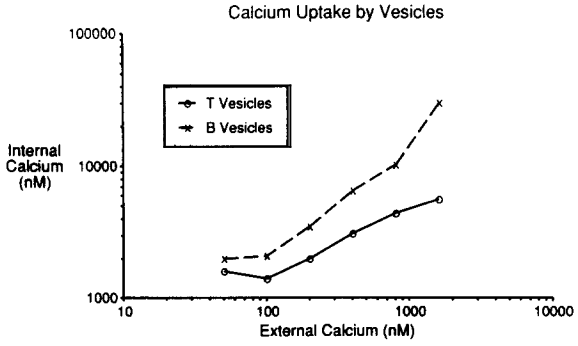

FIGURE 7. B cell membrane vesicles accumulate Ca^{2+} more effectively than T cell vesicles. Inside-out membrane vesicles containing free indo-1 were prepared as described in Materials and Methods. Vesicles were diluted into graded Ca^{2+}/EGTA buffers. $[Ca^{2+}]_i$ was determined 15 min after addition of 1 mM Mg^{2+} ATP to the membrane vesicle suspension. Vesicles from B cells established a steeper Ca^{2+} gradient than those from T cells, with the difference increasing at higher $[Ca^{2+}]_o$ levels.

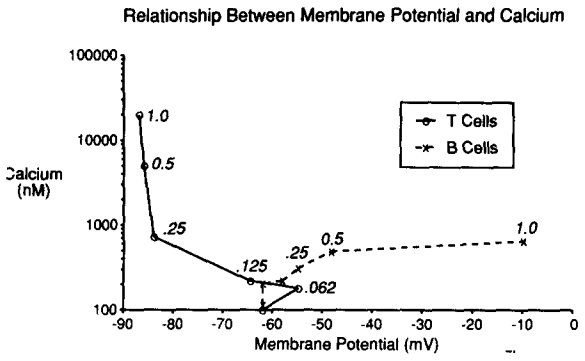

FIGURE 8. Elevated $[Ca^{2+}]_i$ hyperpolarizes T cells but depolarizes B cells. (*a*) Ionomycin-Percoll gradient purified, indo-1 loaded, T and B cells were treated with doubling doses of ionomycin for 15 min at 37°C. Ψ was determined by fluorescence of the oxonol dye, di-BA-C_4 (5). Increasing B cell $[Ca^{2+}]_i$ caused substantial depolarization. As T cell $[Ca^{2+}]_i$ was increased a slight initial depolarization was replaced by hyperpolarization. The concentration of ionomycin (μM) is indicated. Ψ calibration is approximate and B cells were assumed to have the same oxonol fluorescence/membrane potential relationship as T cells.

strongly hyperpolarized as $[Ca^{2+}]_i$ exceeded 200–300 nM. By contrast, B cells depolarized at $[Ca^{2+}]_i$ >200 nM. Calcium was essential for this reaction because its removal from the medium completely eliminated T cell hyperpolarization (data not shown). Wilson et al. demonstrated that quinine, which has relative specificity for Ca^{2+}-sensitive K^+ channels inhibited the ionomycin dependent hyperpolarization of T cells. Thus T cells, but not B cells, demonstrated Ca^{2+}-sensitive K^+ channels that increased Ψ when $[Ca^{2+}]_i$ was elevated.

We then considered the opposite relationship: the effect of Ψ on $[Ca^{2+}]_i$. As the T cell Ca^{2+}-induced hyperpolarization was counteracted by increasing $[K^+]_o$ before exposure to ionomycin, the T cell $[Ca^{2+}]_i$ was dramatically reduced and the difference between T and B cell $[Ca^{2+}]_i$ was greatly decreased (Fig. 9). This observation indicated that hyperpolarization enhanced the T lymphocyte $[Ca^{2+}]_i$ response to ionomycin.

Discussion

Calcium is a primary intracellular messenger in the activation of both T and B lymphocytes. It differs from most other molecules used by cells to transduce signals in that it cannot be metabolized to an inactive form but must be regulated by energy-requiring pumps that extrude it from the cell or sequester it within cell compartments. We have used calcium ionophores, particularly ionomycin, to investigate lym-

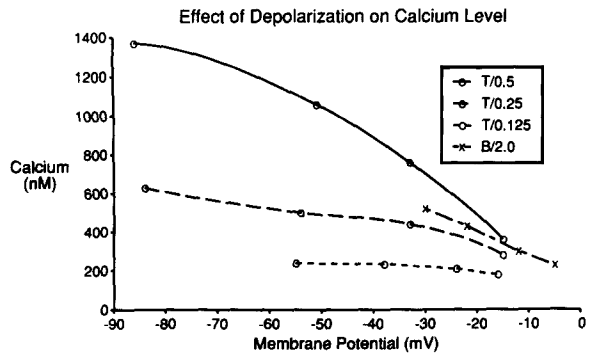

FIGURE 9. Effect of Ψ on ionomycin induced $[Ca^{2+}]_i$ elevation. Ionomycin-Percoll gradient-purified, indo-1 loaded, T and B cells were exposed to ionomycin (0.125 [*short dash*], 0.25 [*long dash*], and 0.5 μM [*solid*] for T cells; 2.0 μM for B cells) in the presence of increasing $[K^+]_o$ (5, 18.7, 37.5, 75 mM). As the cells were depolarized, the $[Ca^{2+}]_i$ level decreased substantially. The effect was more pronounced at higher initial $[Ca^{2+}]_i$. Ψ calibration is approximate.

phocyte calcium management. The data presented show that ionomycin rapidly produces a stable $[Ca^{2+}]_i$ elevation in lymphocytes (Fig. 1). However, there was a bimodal $[Ca^{2+}]_i$ distribution in sleen and lymph node cells treated with ionomycin. This heterogeneity was caused by a difference between T and B cells, most of the former being sensitive to ionomycin and all of the latter resistant (Fig. 2). The difference between T and B cells was apparent at ionomycin doses above 0.1 μM, corresponding to a $[Ca^{2+}]_i$ of 150 nM, and the difference increased to 10-fold at 1.0 μM ionomycin (Fig. 3). Thus, the mechanisms responsible for the T-B heterogeneity are active in the physiological range of $[Ca^{2+}]_i$ elevation. The trivial explanation of differential ionophore uptake by T and B cells was excluded (Fig. 4). Measurement of pH_i showed that the effects of ionomycin were transient and could not account for the subset differences observed.

$[Ca^{2+}]_i$ elevation decreased the size of the ionomycin-sensitive T cells (Fig. 5), increasing the density of these cells sufficiently for them to be separated on a Percoll gradient (Ishida, Y., and T. M. Chused, manuscript in preparation). This technique proved of value for the rapid preparation of ionomycin-sensitive T cells and ionomycin-resistant B cells. It should be noted that this process was completely reversible upon removal of ionomycin. The behavior of cells purified in this manner could not be distinguished from those obtained by the conventional antibody and complement method.

B cell ^{45}Ca efflux increased in the presence of ionomycin while T cell efflux did not (Fig. 6), leading to a lower $[Ca^{2+}]_i$ level in B cells than T cells at the same ionomycin dose. This suggests that the B cell Ca^{2+} pump is more active than the T cell pump at elevated $[Ca^{2+}]_i$. This was confirmed by directly examining the ability of inside-out T and B cell vesicles to develop a Ca^{2+} gradient (Fig. 7). B cell vesicles were two to three times more active than T vesicles. This could represent an intrinsic difference between T and B cell Ca^{2+} pump activity or an effect of Ψ on Ca^{2+} pump activity (see below), since the Na^+-K^+ ATPase would be expected to establish a K^+ gradient in the vesicles.

Simultaneous examination of $[Ca^{2+}]_i$ and Ψ showed diametrically opposite responses in T and B cells (Fig. 8): B cells depolarize with $[Ca^{2+}]_i$ elevation while T cells hyperpolarize. The T cell response depends on a Ca^{2+}-sensitive K^+ channel that is not present in B cells. Activation of this channel increases the K^+ permeability of the membrane, shifting Ψ closer to the K^+ equilbrium potential of −87 mV. The ionic basis of the B cell depolarization is not yet known, but presumably reflects the activity of a Ca^{2+}-sensitive Na^+ and/or Cl^- channel(s). The crucial observation is that Ψ affects $[Ca^{2+}]_i$ (Fig. 9). Depolarizing ionomycin-treated T cells by increasing $[K^+]_o$ markedly reduced their $[Ca^{2+}]_i$. An effect of Ψ on $[Ca^{2+}]_i$ was also apparent in B cells but was more limited, perhaps because they were depolarized by the ionomycin-elevated $[Ca^{2+}]_i$.

There are three possible explanations for an effect of Ψ on ionomycin-induced $[Ca^{2+}]_i$ elevations. An inside negative Ψ would drive Ca^{2+} ions through an electrically open channel. However, the ionomycin-Ca^{2+} complex that traverses the membrane is not charged and thus its motion should be independent of Ψ (51). Further, the force of Ψ on Ca^{2+} moving through an electrically open channel would be <10% of that provided by the 4-log Ca^{2+} concentration gradient across the cell membrane. Thus a significant direct effect of Ψ on ionomycin-mediated Ca^{2+} influx is unlikely.

A second possibility would be an effect of Ψ on a calcium- and voltage-sensitive ion channel. A calcium-sensitive, nonselective (i.e., Ca^{2+}-carrying) cation channel has been described in human neutrophils (52). However, this channel opens more frequently at a positive potential, like typical voltage-sensitive calcium channels, the opposite of the behavior required to explain a greater Ca^{2+} influx at more negative Ψ. An undescribed type of calcium-sensitive calcium channel whose open state was favored by membrane hyperpolarization could account for our observations. None of the currently available data exclude this alternative, although manipulation of Ψ in the absence of calcium ionophores has no effect on $[Ca^{2+}]_i$.

The preferred interpretation is that Ψ influences Ca^{2+} pump activity. Our results suggest there is inhibition of Ca^{2+} extrusion by membrane hyperpolarization and augmentation by depolarization. The failure of T cell Ca^{2+} efflux to increase upon exposure to ionomycin (Fig. 6) is particularly striking. Replacement of sodium with choline does not affect the $[Ca^{2+}]_i$ response to ionomycin (data not shown) indicating that Ψ changes act on the Ca^{2+}-ATPase pump rather than Na^+-Ca^{2+} exchange. The influence of Ψ on Ca^{2+}-ATPase has not been examined previously and its effects on Na^+-Ca^{2+} exchange are equivocal (22). However, the resting Ψ generates a strong, 500,000 V/cm, electrical field in which the Ca^{2+} efflux mechanism normally operates. Thus, alteration of Ψ could affect these structures.

Augmentation of Ca^{2+} efflux by membrane depolarization provides a likely explanation for the observation that lymphocyte membrane depolarization inhibits $[Ca^{2+}]_i$ responses to crosslinking antigen receptor and, therefore, the ensuing cell proliferation (53, 54, and Ishida, Y., and T. M. Chused, unpublished observations). Since T cell hyperpolarization, produced by opening K^+ channels, appears to augment $[Ca^{2+}]_i$ responses, pharmacologic blockade of these channels would be expected to diminish $[Ca^{2+}]_i$ and proliferative responses. This may be the basis of reports that K^+ channel blockers inhibit mitogenesis (55, 56).

Effects on $[Ca^{2+}]_i$ similar to ours have been observed with a rat basophilic leukemia cell line. Depolarization of these cells inhibits both antigen and ionophore induced $[Ca^{2+}]_i$ elevation (57). Upon antigen activation this line depolarizes (58) in the manner of B lymphocytes, suggesting that it does not possess a calcium-sensitive potassium channel. One group working with human T cells did not observe an effect of K^+-mediated depolarization on ionomycin-induced $[Ca^{2+}]_i$ increases (54). While the reason for this is not clear, a contributing factor may be that the experiments were performed in PBS. We find that cell viability and responses are much stronger and more consistent in complete medium.

In conclusion, the increased T cell $[Ca^{2+}]_i$ response to ionomycin appears to be due to an intrinsically less active Ca^{2+} pump (or pumps), which is further inhibited by the membrane hyperpolarization produced by a Ca^{2+}-sensitive K^+ channel. $[Ca^{2+}]_i$ regulation of T cells differs significantly from that of B cells. This may be related to differences in T and B cell differentiation pathways and/or their modes of antigen recognition.

Summary

Calcium management differs in T and B lymphocytes. $[Ca^{2+}]_i$ elevation in response to calcium ionophores is up to 10 times greater in T cells than B cells. There is no difference between them in ionophore uptake. T cells, but not B cells, possess

a calcium-sensitive potassium channel which produces membrane hyperpolarization at $[Ca^{2+}]_i$ above 200 nM. This alters T cell density providing a rapid and easy method of cell separation. In contrast, B cells depolarize when $[Ca^{2+}]_i$ is increased. Isolated B cell membrane vesicle ATP-dependent calcium pump activity is higher than T cell vesicles. Membrane depolarization reduces the $[Ca^{2+}]_i$ response to ionomycin, most dramatically in T cells because they are hyperpolarized by increased $[Ca^{2+}]_i$. The most likely basis of this behavior is an effect of membrane potential on lymphocyte membrane calcium pump activity. This mechanism provides an explanation of the inhibitory effect of membrane depolarization on T lymphocyte responses.

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