CROSSLINKING BY LIGANDS TO SURFACE IMMUNOGLOBULIN TRIGGERS MOBILIZATION OF INTRACELLULAR $^{45}\text{Ca}^{2+}$ IN B LYMPHOCYTES

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ABSTRACT

Detailed studies of steady-state ion fluxes in murine lymphocytes were used to examine for possible ionic changes generated by surface Ig, the antigen receptor of B lymphocytes. When bound by ligands, surface Ig triggered the mobilization and release of $^{45}\text{Ca}^{2+}$ from the cell interior by a transmembrane process requiring crosslinking of the bound receptors. This ionic event was unique for two reasons: (a) it did not occur when other common lymphocyte surface macromolecules were bound with rabbit anti-lymphocyte antibodies; and (b) it was not accompanied by a general perturbation of lymphocyte ionic properties such as a change in $^{42}\text{K}^{+}$ fluxes nor did it depend on the presence of extracellular ions. Capping of surface Ig shares the same time sequence, dose response, requirement for crosslinking, and lack of dependence on extracellular ions. These correlations suggest that mobilization of intracellular $\text{Ca}^{2+}$ may represent an early ionic signal for the contractile activation of lymphocytes that generates capping of surface Ig.

KEY WORDS lymphocytes - ion changes - $\text{Ca}^{2+}$ fluxes - membrane redistribution - capping

Surface immunoglobulin (surface Ig), the antigen receptor of B lymphocytes, promotes several sequential contractile processes when it is bound by ligands: the bound receptors are selectively redistributed to a pole of the cell (capping); a phase of translatory movement is generated; and the capping complexes are cleared from the surface by endocytosis (29, 35, 9). Among these events, capping has received central attention because it may be followed on a molecular and functional level with relative ease. Early studies of the capping process demonstrated a requirement for metabolic energy and sensitivity to cytochalasins (35, 8), suggesting a role for the contractile apparatus. Subsequently, electron microscope and immunocytochemical studies indicated the rapid and dramatic reorganization of the contractile proteins during capping (discussed in reference 4). Recently, preliminary biochemical evidence for the physical association of actin with bound surface Ig has been reported (10). It should be noted, however, that the capacity of surface Ig to modulate contractile activity may be shared by a restricted class of surface macromolecules (4, 29).

Overall, these results imply that surface Ig, when bound by ligands, elicits a biochemical event which activates the contractile activity. It is possible that this biochemical event consists of the mobilization of intracellular $\text{Ca}^{2+}$. In general, the level of free intracellular $\text{Ca}^{2+}$ is well known to
play a crucial role in regulating contractile activity. Although capping can occur in the absence of extracellular Ca$^{2+}$ (29), two experiments suggest that this ion regulates the capping process. First, calcium ionophores inhibit capping or disperse caps of surface Ig in a process requiring external Ca$^{2+}$ and metabolic energy. These agents may act by inducing hypercontraction of microfilaments, an interpretation supported by immunofluorescence (5) and electron microscope (15) studies which reveal the presence of coarse, myosin-containing aggregates or dense accumulations of subplasmalemmal microfilaments. Second, local anesthetics also stop capping or disperse capping surface Ig in an energy-independent fashion (28), perhaps by the capacity of these drugs to competitively inhibit Ca$^{2+}$-dependent processes (30), such as the linkage of the contractile apparatus to surface Ig (5). This view is supported by the partial reversal of drug action by increasing concentrations of extracellular Ca$^{2+}$ and by the drug-induced dispersion of cap-associated myosin.

To test whether Ca$^{2+}$ may have a physiological role in Ig capping, we have directly studied the properties of exchangeable calcium in lymphocytes using $^{45}$Ca$^{2+}$. We also describe two experiments examining the role of other ions in capping: (a) the effect of removal of extracellular ions on capping; (b) the influence of anti-Ig on the fluxes of $^{45}$K$^+$. Both these experiments suggest that release of $^{45}$Ca$^{2+}$ may be the original ionic response triggered by ligands to surface Ig.

**MATERIALS AND METHODS**

**Cells**

All cells were obtained from 2- to 3-mo-old A/St mice (West Seneca, Buffalo, N. Y.) and purified by Ficoll-Hypaque centrifugation. Except where indicated, unfractionated splenic lymphocytes were used. Preparations rich in splenic T cells were obtained by passing whole spleen cells through a nylon-wool column (17) followed by Ficoll-Hypaque centrifugation to remove erythrocytes. All cell suspensions contained >95% viable erythrocytes as judged by trypan-blue exclusion.

In one experiment the spleen cells were depleted of T lymphocytes by treatment with 1:10 dilution of anti-Thy-1.2 for 1 h at 4°C. Cells were then rinsed and resuspended in complement (rabbit serum previously adsorbed with T spleen cells/ml serum) and incubated for 45 min at 37°C. Dead cells were removed by Ficoll-Hypaque centrifugation. Before treatment, 60% spleen cells were T lymphocytes as judged by negative staining with fluorescein-conjugated anti-Ig, after treatment, the fraction of T lymphocytes was 10%.

**Media**

A modified Hanks's balanced salt solution (hereafter designated as "medium") was used in all experiments: 137 mM NaCl, 5.2 mM KCl, 0.8 mM MgCl$_2$, 6 mM d-glucose, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) buffer (Sigma Chemical Co., St. Louis, Mo.), 0.5 mg/ml bovine serum albumin (Sigma), at pH 7.2. Divalent anions were excluded from this medium to permit experiments with LaCl$_3$, their absence did not reduce cell viability even after 6 h in culture. In some experiments, NaCl was replaced by equimolar KCl or choline chloride (Sigma), and bovine serum albumin was deleted.

**Reagents**

LaCl$_3$ was obtained from Pfaltz and Bauer, Stamford, Conn. New England Nuclear, Boston, Mass., was the supplier for $^{45}$CaCl$_2$ (20 Ci/g) and $^{45}$KCl (5 Ci/g) and typically used at concentrations of 2 µCi/ml and 40 µCi/ml, respectively.

**Antisera**

(a) Non-immune rabbit IgG (RGG): RGG was prepared from serum of non-immune rabbits using DE-52 ion exchange chromatography (Pharmacia Fine Chemicals, Uppsala, Sweden).

(b) Anti-Ig: the polyspecific rabbit anti-mouse immunoglobulin antibody was the same preparation used in past studies (27); the IgG fraction was purified by ion exchange chromatography. Fab'-anti-Ig was prepared from the same material by pepsin digestion and Sephadex G-100 chromatography. In one case, anti-Ig activity was removed by exhaustive adsorption with Sepharose 4B-coupled mouse Ig. After adsorption, no antibody activity could be detected by Ouchterlony against mouse serum or against purified myeloma Ig (IgM, IgG1, IgG2a, IgG2b).

(c) Anti-IgM: rabbit anti-mouse IgM was prepared and characterized previously (4). It was specific for mu heavy chain and had been exhaustively absorbed with mouse IgG to remove any possible anti-Fab specificities. The IgG fraction was prepared by ion exchange chromatography.

(d) Anti-lymphocyte antibodies: rabbit anti-lymphocyte antibody was prepared by repeated immunization with mouse lymphocytes (36). An IgG fraction was prepared by sequential precipitation with 50, 40, and 35% ammonium sulfate.
(e) Anti-rabbit IgG: goat-anti-rabbit IgG antibodies were prepared by immunization with rabbit IgG and were characterized previously (4). The IgG fraction was prepared by ion exchange chromatography.

(f) Non-immune goat IgG: non-immune goat IgG was prepared from serum of non-immune animals (a gift of L. K. Gordon, Sidney Farber Cancer Institute, Boston, Mass.) by sequential precipitation with 50 and 40% ammonium sulfate.

All antibodies were dialyzed against the medium and spun at 12,500 for 15 min immediately before use.

Experimental Design

The purpose of these experiments was to determine whether antibodies to surface Ig trigger a change in the amount of cell-associated calcium. To test for Ca$^{2+}$ influx, the experimental approach was to use Ca$^{2+}$ as a tracer isotope. Cells first were suspended at 1 x 10$^6$/ml in siliconized flasks and preequilibrated at 37°C on a rotary shaker at 90 rpm for 60 min. No effect of cell concentration was seen between 1 x 10$^6$/ml and 1.5 x 10$^6$/ml; however, preequilibration was necessary to obtain accurate measurements of the cell response, presumably by allowing cells to achieve a steady-state distribution of intracellular ions (20). So that the final change in cell concentration would be <5%, Ca$^{2+}$ and other reagents were added as concentrated solutions in a small volume. Except where noted, all experiments were performed at 37°C.

For all three manipulations described below, the amount of cell-associated label was determined by a tracer isotope. Cells first were suspended at 1 x 10$^6$/ml in siliconized flasks and preequilibrated at 37°C on a rotary shaker at 90 rpm for 60 min. No effect of cell concentration was seen between 1 x 10$^6$/ml and 1.5 x 10$^6$/ml; however, preequilibration was necessary to obtain accurate measurements of the cell response, presumably by allowing cells to achieve a steady-state distribution of intracellular ions (20). So that the final change in cell concentration would be <5%, Ca$^{2+}$ and other reagents were added as concentrated solutions in a small volume. Except where noted, all experiments were performed at 37°C.

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inferentially the stimulation of ion influx) did not appear necessary for triggering the capping process.

**Anti-Ig Reduces the Size of the Exchangeable Ca²⁺ Pool**

The effect of bound surface Ig on unidirectional "Ca²⁺ exchange was studied by measuring the rates of "Ca²⁺ influx and efflux in the absence or presence of anti-Ig. The rate of "Ca²⁺ uptake was determined by preequilibrating cells in medium containing various concentrations of extracellular Ca²⁺, then adding "Ca²⁺ (2 μCi/ml) and withdrawing aliquots of the cell suspension at different times for measurement of cell-associated "Ca²⁺ (Fig. 2). Uptake of "Ca²⁺ followed first-order kinetics with a half-time of ~36 min and was essentially complete within 150 min (Fig. 2, inset); this rate was only slightly affected by external Ca²⁺ concentration. The initial amount of "Ca²⁺ associated with cell pellets represents the small volume of extracellular medium trapped by the cells [see Materials and Methods].) No effect of anti-Ig could be measured on the rate of "Ca²⁺ uptake regardless of external Ca²⁺ concentration, suggesting that anti-Ig did not obviously alter cell permeability to Ca²⁺.

The rate of "Ca²⁺ efflux was tested in cells equilibrated in "Ca²⁺ for 150 min, rinsed, and placed in medium without label to measure the rate of "Ca²⁺ release (Fig. 3). The rate of "Ca²⁺...
FIGURE 3 Efflux of $^{45}$Ca$^{2+}$: Lymphocytes were equilibrated for 150 min with $^{45}$Ca$^{2+}$ in medium containing: (a) no added Ca$^{2+}$; (b) 0.2 mM Ca$^{2+}$; or (c) 2.0 mM Ca$^{2+}$. Cells were rinsed, placed in 37°C medium lacking Ca$^{2+}$ ($t = 0$ min), after which anti-Ig (200 µg/ml), normal rabbit IgG (200 µL/ml), or equal volume of medium was added ($t = 2$ min); and samples were withdrawn at indicated times to determine amounts of cell-associated $^{45}$Ca$^{2+}$. Medium: - - - normal rabbit IgG; △-△ anti-Ig: O- - O. Insets: Unidirectional rate constants were determined as in Fig. 2.

release closely resembled first-order kinetics predicted by closed two-compartmental analysis with a half-time of ~45 min (Fig. 3, insets). The appearance of $^{45}$Ca$^{2+}$ in the supernates paralleled exactly its disappearance from cell pellets. The rate of efflux was similar over a wide range of extracellular Ca$^{2+}$ concentrations, suggesting that the rate-limiting step in $^{45}$Ca$^{2+}$ efflux was the same regardless of the concentration gradient across the membrane. When anti-Ig was added at the beginning of the efflux period, an abrupt release of 20-30% of cell-associated $^{45}$Ca$^{2+}$ occurred with 2 min (earliest time tested). The relative $^{45}$Ca$^{2+}$ release induced by anti-Ig was not dependent on the concentration of extracellular Ca$^{2+}$, indicating that the magnitude of the Ca$^{2+}$ gradient was not an important feature of the response.

The apparent release of $^{45}$Ca$^{2+}$ induced by anti-Ig was further tested by measuring the effect of anti-Ig on the relative size of the exchangeable Ca$^{2+}$ pool. Cells were equilibrated for 150 min with $^{45}$Ca$^{2+}$, then treated with anti-Ig while still in the presence of $^{45}$Ca$^{2+}$ and sampled at various times to determine the amount of cell-associated $^{45}$Ca$^{2+}$ (Fig. 4). Anti-Ig induced a rapid release of cell-associated $^{45}$Ca$^{2+}$ which was quantitatively similar to that measured during $^{45}$Ca$^{2+}$ efflux; the release occurred within 5 min (earliest time tested) and persisted for >3 h (longer than the time required for $^{45}$Ca$^{2+}$ equilibration). The same relative amount of $^{45}$Ca$^{2+}$ was released by cells regardless of extracellular Ca$^{2+}$ concentration, again suggesting that the primary effect of anti-Ig was mobilization of cell-associated $^{45}$Ca$^{2+}$ rather than a change in cell permeability to Ca$^{2+}$.

We next determined whether anti-Ig induced the release of $^{45}$Ca$^{2+}$ by binding to surface Ig of B cells. We first compared the responsiveness of fractionated lymphocyte populations depleted or enriched for the subpopulation bearing surface Ig. Spleenic T lymphocytes or thymocytes, two subpopulations which lack surface Ig, did not respond to anti-Ig, even though unseparated splenic lymphocytes from the same animals gave a typical $^{45}$Ca$^{2+}$ release (Fig. 5). Conversely, splenic B lymphocytes (the subpopulation which expresses surface Ig) obtained by depletion of T cells using anti-Thy-1.2 plus complement retained their responsiveness to anti-Ig (Table 1). The requirement for antibody activity to surface Ig was demonstrated by use of Fab'-anti-Ig in which the effect of anti-Ig was dose dependent and was lost upon removal of anti-Ig activity (Fig. 6). Because Fab'-anti-Ig does not bind to the B-cell Fc receptor, we also concluded that the effects of the antibody did not involve direct interaction with the Fc receptor. Fig. 6 also shows that purified anti-IgM antibodies (which recognize one of the isotypes of surface Ig) have results similar to those
found with polyspecific anti-Ig.

In a related experiment, antilymphocyte antibodies did not elicit "Ca²⁺ release even at a concentration 20 times greater than that required to induce capping (Fig. 6). Because these antibodies recognize a wide variety of lymphocyte surface macromolecules, the result indicates that the capacity to induce "Ca²⁺ release is not a general property of bound surface macromolecules.

**Requirement for Crosslinking of Surface Ig**

In what configuration does bound surface Ig promote "Ca²⁺ release: as individual molecules or as crosslinked complexes? To test these two possibilities, we examined the effects of monovalent Fab'-anti-Ig on cells previously equilibrated with "Ca²⁺ (Fig. 7). The monovalent ligand did not induce "Ca²⁺ release in these cells even at 200 μg/ml—a 100-fold molar excess when compared to the lowest optimal concentration of divalent Fab²⁻-anti-Ig. Next, we tested whether bound surface Ig, when crosslinked with antibody against the ligand, could be induced to trigger "Ca²⁺ release. This experiment was done by treating cells with the rabbit Fab'-anti-Ig, rinsing them to remove unbound ligand, and following the rate of "Ca²⁺ efflux in the presence of native goat anti-rabbit IgG antibodies (Fig. 8). Clearly, the anti-rabbit IgG promoted rapid "Ca²⁺ release in cells treated with Fab-anti-Ig, while it had no effect on un-

**TABLE I**

| Treatment       | "Ca²⁺" at Equilibrium | "Ca²⁺" after Medium, 10 Min | "Ca²⁺" after Anti-Ig, 10 Min |
|-----------------|------------------------|-----------------------------|----------------------------|
| Medium          | 15,733 ± 100           | 15,334 ± 100                | 12,400 ± 100                |
| Complement      | 12,805 ± 300           | 12,927 ± 300                | 8,780 ± 300                 |
| Anti-Thy 1.1 plus complement | 11,846 ± 200 | 11,903 ± 100 | 8,037 ± 200 |

After treatment and recovery of viable cells (see Materials and Methods), lymphocytes were resuspended at 10⁷/ml, preequilibrated in medium without Ca²⁺ (~0.01 mM), and incubated with "Ca²⁺ (4 μCi/ml) for 100 and 80 min. Medium or anti-Ig (200 μg/ml) was added, and then aliquots were sampled at various times. The results at 10 min were representative of the values in each group between 5 and 60 min after addition of reagents.
Effect of antibody specificity on Ca release: Lymphocytes were equilibrated with $^{45}$Ca$^{2+}$ as described in Fig. 5, then treated for 20 min with various antibodies in the indicated concentrations. Fab'-anti-Ig: $\circ\circ\circ\circ$; Fab'-anti-Ig absorbed with mouse Ig-Sepharose: $\bullet\bullet\bullet\bullet$; anti-IgM: $\bigtriangleup\bigtriangleup\bigtriangleup\bigtriangleup$; rabbit anti-lymphocyte antibodies: $\Delta\Delta\Delta\Delta$. In other experiments, comparable results were found after incubation for 10 or 60 min. Because some of the antibodies were tested in separate experiments, results are shown as %cpm/pellet relative to medium alone (100%).

Effect of monovalent anti-Ig on $^{45}$Ca$^{2+}$ release: Lymphocytes were equilibrated with $^{45}$Ca$^{2+}$ (4 $\mu$Ci/ml) as described in Fig. 5, then treated with Fab'-anti-Ig for the indicated times. The legend is included in the figure.

Binding of Surface Ig Causes $^{45}$Ca$^{2+}$ Mobilization from Cell Interior

Preliminary localization of the $^{45}$Ca$^{2+}$ released by anti-Ig was determined by experiments with La$^{3+}$. La$^{3+}$ is a nonpenetrating ion which, at millimolar concentrations, has two effects on cell-associated Ca$^{2+}$: (a) it displaces Ca$^{2+}$ bound to the external surface of the cell; and (b) it prevents exchange of Ca$^{2+}$ across the plasma membrane (19). By exposing cells to La$^{3+}$ and anti-Ig in different sequences, the manner of $^{45}$Ca$^{2+}$ release by anti-Ig was compared to the known effects of La$^{3+}$ on cell-associated $^{45}$Ca$^{2+}$. In Fig. 9, La$^{3+}$ alone (closed squares) displaced $\sim$50% of the cell-associated $^{45}$Ca$^{2+}$ within 1–2 min, after which little further displacement occurred. This displaced $^{45}$Ca$^{2+}$ was taken to represent the amount of $^{45}$Ca$^{2+}$ associated with the external cell surface (13). A 5-minute exposure to anti-Ig alone caused the release of $\sim$25% of the cell-associated $^{45}$Ca$^{2+}$, a level which persisted for $>2$ h (data not shown).

Two experimental manipulations were done to compare the $^{45}$Ca$^{2+}$ release produced by these agents. First, cells were exposed to La$^{3+}$ for various
Location of "Ca" released by anti-Ig: Lymphocytes were equilibrated with "Ca" as described in Fig. 5, then treated with anti-Ig (200 μg/ml) and La" (1 mM) in two different protocols: (a) anti-Ig → La" treatment with anti-Ig for 5 min followed by treatment with La" beginning at the time indicated by the arrow; (b) La" → anti-Ig, treatment with La" (beginning at 0 min) for various times followed by treatment with anti-Ig for 5 min. Medium alone: 0-----0; anti-Ig alone: 0--O; La" alone: /---0; anti-Ig La" → anti-Ig: E----0. Note that anti-Ig induced "Ca" release only when present before La"; also, treatment with anti-Ig followed by La" caused greater "Ca" release than that produced by La" alone.

Anti-Ig Does Not Affect "K" Exchange

The previous results indicated the striking effect of bound surface Ig on internal cell "Ca". To determine whether this effect was unique for Ca" or part of a more general perturbation of the ionic properties of the B cell, we performed similar measurements on the rates of exchange of "K" and 22Na". With "K", the rates of influx (Fig. 10a) and efflux (Fig. 10c), as expected, followed first-order kinetics; the value of the rate constant for influx greatly exceeded that for efflux, presumably because of the presence of a large transmembrane K" gradient (in human blood lymphocytes, the concentration of intracellular K" was estimated to be 150 mM, compared to an extracellular K" concentration of 5 mM [16, 21]). Under these conditions, anti-Ig did not affect the rates of influx and efflux for "K" nor did it alter the equilibrium level of cell-associated "K" (Fig. 10b). In preliminary experiments, the level of 22Na" uptake was so low that no significant measurements could be made, and we were unable to include this ion in our studies. In related experiments, anti-Ig-induced "Ca" release was tested in the presence of media in which NaCl had been replaced with equimolar KCl or choline chloride (Table III). Although these media grossly altered the normal transmembrane gradients of Na" and K", they did not block the stimulated "Ca" release. Thus, "Ca" release was not dependent on normal fluxes of Na" and K".

DISCUSSION

Ion Transport in Unstimulated Lymphocytes

We report in this paper detailed studies of steady-state ion fluxes in murine lymphocytes and the use of this method for examining the ionic signals that may be generated by surface Ig, the antigen receptor of B lymphocytes. Both "Ca" and "K" rapidly equilibrate across the lymphocyte plasma membrane when followed using the silicon oil centrifugation technique. The rate constants for "Ca" influx and efflux were ~0.020 min" and 0.015 min", respectively. Although this type of analysis has not been used in previously published studies on lymphocyte ion fluxes, we calculated similar values for "Ca" influx (0.02-0.03 min") from data reported by Whitney and Sutherland (37) on human blood lymphocytes. As in our study, the steady-state rate of calcium ex-
FIGURE 10 Effect of anti-Ig on $^{42}\text{K}^+$ exchange: (a) Uptake of $^{42}\text{K}^+$: Lymphocytes were pre-equilibrated for 60 min in medium supplemented with 1.2 mM Ca$^{2+}$, then treated simultaneously with $^{42}\text{K}^+$ (40 pC/ml) and anti-Ig (or equal volume of medium), and sampled at the indicated times to determine the amount of cell-associated $^{42}\text{K}^+$. (b) Equilibrium of $^{42}\text{K}^+$: Lymphocytes were treated with $^{42}\text{K}^+$ as described in (a), equilibrated for 200 min, then treated with anti-Ig or equal volume of medium sampled at the indicated times. (c) Efflux of $^{42}\text{K}^+$: Lymphocytes were equilibrated for 200 min with $^{42}\text{K}^+$, then rinsed, placed in fresh medium containing anti-Ig or equal volume of medium, and sampled at the indicated times. Medium: ■, anti-Ig; ○. Insets: Unidirectional rate constants were determined by plotting values for $^{42}\text{K}^+$ uptake or efflux according to the relationship described in Materials and Methods. The rate constants were representative of two different experiments (range in values between experiments was <0.003 min$^{-1}$).

change appeared to be relatively constant over a 1,000-fold range of extracellular calcium concentration, suggesting that the rate-limiting step was not dependent on the transmembrane ion gradient. Several other groups have reported cursory studies of $^{46}\text{Ca}^{2+}$ fluxes in human blood lymphocytes, murine spleen cells, and thymocytes (1, 11, 12, 14, 23). However, the magnitude and rate of ion exchange varied widely, probably reflecting species differences, the relative purity of the lymphocyte population from contaminating monocytes (a cell type which transports Ca$^{2+}$ much more rapidly than lymphocytes [3, 20]), and the different methods used for measuring cell-associated Ca$^{2+}$.

The rate constants for $^{42}\text{K}^+$ influx and efflux were $\sim$0.027 and 0.010 min$^{-1}$, respectively; the large difference in unidirectional rates probably reflect the high concentration of K$^+$ in the lymphocytes, together with a relatively high cell permeability to K$^+$ (16, 21). We are not aware of studies on the kinetics of K$^+$ exchange in murine lymphocytes; among the available studies on human blood lymphocytes, we calculated values ranging between 0.005 and 0.01 min$^{-1}$ for both influx and efflux (26, 33). The slow rate of $^{42}\text{K}^+$ influx reported in these studies was surprising, but comparison with other studies is hampered by the differences in methods and species already enumerated. The particular sensitivity of ion transport studies to experimental artifacts has been analyzed by some of these authors (14, 33).

Bound Surface Ig Mobilizes Intracellular Ca$^{2+}$

Lymphocytes released 20-30% of their exchangeable calcium pool within 2 min after treatment with anti-Ig, and the reduced size of the exchangeable pool persisted after stimulation for >2 h. The exclusive role of surface Ig in mediating this response was demonstrated by the: (a) dependence on anti-Ig concentration; (b) effectiveness of Fab'-anti-Ig or anti-IgM antibodies (thus excluding the requirement for the conventional Fc receptor); (c) ineffectiveness of anti-Ig adsorbed with IgG-Sepharose to remove anti-Ig specificities; and (d) restriction of the response to B cells, the lymphocyte subpopulation which bears the surface Ig molecule.

Two other features were identified concerning the steps which lead from binding of surface Ig to the release of $^{46}\text{Ca}^{2+}$: (a) binding alone (by rabbit monovalent Fab'-anti-Ig) did not trigger $^{46}\text{Ca}^{2+}$ release, but crosslinking the bound monovalent antibody by anti-rabbit IgG antibodies restored the response. These results demonstrated that anti-Ig most likely did not cause $^{46}\text{Ca}^{2+}$ release by
directly displacing $^{45}$Ca$^{2+}$ from the surface Ig molecule but instead elicited $^{45}$Ca$^{2+}$ release after a signal involving receptor crosslinking, an event which also promotes surface Ig redistribution. A role for receptor crosslinking has also been demonstrated in Ca$^{2+}$-dependent mast cell degranulation triggered by the IgE receptor (31) and the accelerated glucose metabolism of adipocytes elicited by the insulin receptor (18).

(b) Experiments with La$^{3+}$ demonstrated that surface Ig triggers release of $^{45}$Ca$^{2+}$ from the cell interior, not from the external surface of the plasma membrane. This result implies that bound surface Ig initiates a transmembrane signal which causes the mobilization of $^{45}$Ca$^{2+}$ from an internal store, thereby raising the concentration of free cytoplasmic Ca$^{2+}$. The nature of this signal and the precise location of the Ca$^{2+}$ store are unknown. However, preliminary experiments with chlorotetracycline, a lipophilic dye with fluorescence characteristics that reflect the amount of membrane-associated Ca$^{2+}$ (6, 7), suggest that a rapid release of membrane-associated Ca$^{2+}$ occurs within 15 s after exposure to anti-Ig (unpublished). It is interesting to note that La$^{3+}$ (1 mM) does not block Ig capping (unpublished observation). The difference between the effect of La$^{3+}$ on Ig capping and anti-Ig-induced $^{45}$Ca$^{2+}$ release may reflect the capacity of cells to internally sequester free cytoplasmic Ca$^{2+}$ into mitochondria or other membrane-bound sites (2).

Other studies of the ionic responses triggered by ligands to lymphocyte surface macromolecules have been limited to lectins with mitogenic activity, such as concanavalin A and phytohemagglutinin. In certain conditions, these agents accelerate Ca$^{2+}$ influx (37, 1) and K$^{+}$ fluxes (26, 32); in addition, phytohemagglutinin increases lymphocyte permeability to other types of small molecules (24, 25) perhaps, in part, by promoting the formation of gap junctions (22). Thus, the effects of lectins on lymphocyte ionic properties are quite distinct from the effects of anti-Ig.

Steady-state ion fluxes may be complicated by subtle variations in experimental technique which alter the equilibrium state. Two discrepant results may be examples of this difficulty. In one case, treatment of splenic lymphocytes with anti-Thy-1.2 and complement enriched for lymphocytes bearing surface Ig but did not proportionately enrich for responsiveness of the lymphocytes to anti-Ig (Table I). This discrepancy may have been related to the reduced equilibrium level of $^{45}$Ca$^{2+}$ per cell also observed when cells were treated with complement. The reduced $^{45}$Ca$^{2+}$ uptake may have indicated cell damage among the surviving lymphocytes which also attenuated their responsiveness to anti-Ig. In another example, anti-Ig appeared to reduce the equilibrium level of $^{45}$Ca$^{2+}$ in efflux (Fig. 3) and equilibrium (Fig. 4) experiments but not in influx experiments (Fig. 2). We suspected that this discrepancy might have been related to incomplete preequilibration time or poor nutritional state of the cells. However, the effect of anti-Ig on $^{45}$Ca$^{2+}$ uptake was not altered by varying the preequilibration time or by use of medium supplemented with amino acids, divalent anions, and vitamins (data not shown). Therefore, the apparent lack of effect on equilibrium $^{45}$Ca$^{2+}$ levels under these conditions may have reflected complex processes such as a simultaneous change in both exchangeable and nonexchangeable Ca$^{2+}$ pools that could not be resolved by our techniques.

Is Mobilization of Ca$^{2+}$ the Event Which Mediates Capping and Locomotion?

The close association of Ca$^{2+}$ mobilization and surface Ig capping is demonstrated by the same time course, dose dependence on anti-Ig, require-
ment for crosslinking of surface Ig, and lack of requirement for extracellular ions. Furthermore, anti-lymphocyte antibodies which induce capping by a process apparently unrelated to microfilament activation do not produce detectable changes in Ca\textsuperscript{2+} fluxes. (As described previously, this second form of capping is slow and not accompanied by stimulation of translatory motion [4]). These correlations suggest that mobilization of intracellular calcium may represent an early, physiological, ionic signal required for the contractile activation of lymphocytes resulting in capping of surface Ig.

We believe that this ionic process may be divided into certain distinct steps: (a) Crosslinking of surface Ig triggers local mobilization of Ca\textsuperscript{2+} from an underlying Ca\textsuperscript{2+} store. (b) Resultant elevation of free cytoplasmic Ca\textsuperscript{2+} concentration segmentally activates the associated microfilament network, producing asymmetric contraction. (c) Ca\textsuperscript{2+} transport across the plasma membrane (and resequestration within the cell) restores the original cytoplasmic Ca\textsuperscript{2+} level, while producing a net redistribution to a pole of the cell, i.e., capping.

Proof of this scheme will await clarification of the transmembrane events leading to Ca\textsuperscript{2+} mobilization and the identification of the calcium-sensitive contractile unit. The central role of calcium in lymphocyte contractility is supported by the experiments with Ca\textsuperscript{2+} ionophores, mentioned previously, in which direct elevation of the level of intracellular calcium promotes dramatic activation of the contractile apparatus.

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