INDEPENDENT REGULATION OF Ca^{2+} ENTRY
AND RELEASE FROM INTERNAL STORES
IN ACTIVATED B CELLS

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After receptor ligand interaction, activation of human B lymphocytes is accompanied by a rapid rise in cytoplasmic-free Ca^{2+} ([Ca^{2+}]_{i}) (1). The changes in [Ca^{2+}]_{i} are biphasic, being comprised of a rapid, transient peak, followed by a sustained, but smaller increase. The transient peak appears to be predominantly due to the release from internal stores, whereas the sustained plateau requires entry of extracellular Ca^{2+} across the plasma membrane since, in contrast to the transient peak, the sustained increase is dependent on the availability of external Ca^{2+} (1, 2). It has been suggested that release from internal stores and Ca^{2+} entry across the plasma membrane are coupled. Pivotal in these assumptions is the recognized role of inositol 1,4,5-trisphosphate (IP3) in releasing Ca^{2+} from internal stores (3-5) and its potential role in mediating Ca^{2+} uptake across the plasma membrane directly (6, 7), through changes in [Ca^{2+}]_{i} (8), or through a phosphorylated derivative (9).

The nature of the pathway responsible for transmembrane Ca^{2+} uptake in B cells is not well defined and the role of the initial [Ca^{2+}]_{i} increase in regulating Ca^{2+} entry in lymphocytes remains unclear. Partial dissociation of the [Ca^{2+}]_{i} changes produced by release of stores and entry from the medium can be obtained by loading the cells with Ca^{2+} chelating agents such as 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N' tetraacetate (BAPTA). In B cells loaded with increasing BAPTA concentrations, the transient peak elicited by stimulation with anti-IgM was gradually reduced, while the level of the sustained plateau remained unaltered (10). The latter implied that more Ca^{2+} had to enter the cells to overcome the buffering power of BAPTA and to maintain the plateau levels. In this study we have examined further the differential properties of the two sources of Ca^{2+}. The data indicated that the two pathways are regulated independently and that Ca^{2+} entry across the plasma membrane is regulated by [Ca^{2+}]_{i} in activated human B cells.

Erwin Gelfand is a Scholar of the Raymond and Beverly Sackler Foundation and Sergio Grinstein is recipient of a Medical Research Council Scientist Award. This work was supported in part by grants from the National Institutes of Health (AI-26490), the Medical Research Council, and the National Cancer Institute.
Materials and Methods

Cells. Fresh human tonsil tissue was obtained and the mononuclear cell fraction isolated after Ficoll-Hypaque gradient centrifugation. B lymphocytes were obtained after rosette depletion of contaminating T lymphocytes with neuraminidase-treated sheep erythrocytes (11). Greater than 90% of the cells expressed B cell markers.

Reagents. The acetoxymethyl ester (AM) forms of indo-1 and BAPTA were obtained from Molecular Probes (Junction City, OR). Goat anti-human IgM, (Fab)2, was from Atlantic Antibodies (Scarborough, ME). Ionomycin was from Calbiochem-Behring Corp. (San Diego, CA).

Solutions. RPMI 1640 was obtained from Gibco Laboratories (Grand Island, NY). Stock solutions of indo-1 AM, and ionomycin were made up in DMSO. EGTA and CaCl2 stocks were made up in aqueous solutions. For intracellular calcium measurements, the cells were suspended in buffer containing: 140 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 3 mM KCl, 10 mM glucose, and 10 mM Hepes (pH 7.3). All buffers were adjusted to pH 7.3 and 290 mOsm. Calcium-free buffer was prepared without the addition of 1 mM CaCl2 and with 0.5 mM EGTA.

Cytoplasmic Calcium Determinations. Cells (40 x 10^6 in 1 ml Hepes-buffered RPMI) were incubated with 1 mM indo-1 AM or with 1 μM indo-1 AM plus 10 μM BAPTA AM for 30 min at 37°C as described (12). Cytoplasmic calcium concentrations were monitored fluorimetrically using a 650-40 spectrofluorimeter (Perkin-Elmer Corp., Pomona, CA). Calibration of indo-1 fluorescence vs. [Ca2+] was performed using ionomycin and Mn2+ as described (12). Routinely, the cells were stimulated with 37 μg of goat anti-human IgM.

Results and Discussion

After crosslinking of surface IgM by antibody, there is an elevation of [Ca2+], due in large part to uptake of extracellular Ca2+ and an initial transient peak due primarily to release from internal stores (1, 2, 10). In Fig. 1, human B cells isolated from tonsillar tissue were loaded with indo-1 with or without BAPTA, and levels of [Ca2+] were monitored fluorimetrically after addition of anti-IgM antibody. As shown in Fig. 1 A, ligand binding to BAPTA-loaded cells suspended in Ca2+-containing medium resulted in an increase in [Ca2+]; that was sustained over the period monitored. Stimulation of BAPTA-loaded cells in Ca2+-free medium produced only a negligible increase in [Ca2+]; (not shown), indicating that external Ca2+ is required for the sustained response and that the amount of BAPTA used sufficed to buffer the release of Ca2+ from intracellular stores. When BAPTA-free cells were stimulated in Ca2+-free medium, ligand binding was associated with a transient [Ca2+] response, attributable to mobilization of internal stores (Fig. 1 D).

The properties of the two Ca2+ pathways were compared using BAPTA and by maintaining the external Ca2+ concentration. Depolarization of the cells, by elevation of extracellular K+ (1), greatly reduced the entry of extracellular Ca2+ (Fig. 1 C), whereas the transient response was unaffected (Fig. 1 F). The effect of high K+ was due to depolarization and not to omission of Na+, since normal entry of Ca2+ was recorded when Na+ was replaced by N-methylglucamine positive (NMG+), an impermeant organic cation (Fig. 1 B). These data indicate that Ca2+ entry is inhibited by depolarization, as expected from the reduced force driving a cation through a conductive pathway. However, a direct effect of potential on the sequence of events leading to the permeability increase cannot be ruled out. In the latter case, the potential-sensitive step would be downstream from the ligand-receptor interaction, inasmuch as release of Ca2+ stores was unaffected by depolarization.

The increases in Ca entry in Fig. 1 could be due to an increased influx of Ca2+,
but could also represent decreased efflux. These alternatives were distinguished using Mn\(^{2+}\), an ion that permeates Ca\(^{2+}\)-selective channels in other cell types (13). Unlike Ca\(^{2+}\), which enhances indo-1 fluorescence, Mn\(^{2+}\) quenches the fluorescence of the probe. In Fig. 2 we utilized the rate of quenching of indo-1 fluorescence as a measure of the rate of uptake of Mn\(^{2+}\), used as a Ca\(^{2+}\) analog. When otherwise untreated indo-1-loaded cells are exposed to Mn\(^{2+}\), the fluorescence intensity declines at a constant rate (Fig. 2 A). Sudden chelation of external Mn\(^{2+}\) with EGTA arrests the progression of quenching. Notice that no reversal (increase in fluorescence) was detected, indicating that efflux of Mn\(^{2+}\) is minimal and that the rate of indo-1 fluorescence quenching indeed reflects unidirectional divalent cation entry.

This technique was applied to ligand-activated fluxes. Cells were loaded with indo-1 and BAPTA and suspended in Ca\(^{2+}\)-free (but Mn\(^{2+}\)-containing) medium. Addition of anti-IgM increased the rate of quenching, consistent with an increased rate of divalent cation uptake (Fig. 2 B). As before, EGTA prevented progression of quenching but did not induce reversal. In cells suspended in K\(^+\) medium (Fig. 2 C), the anti-IgM-induced change in the rate of quenching was greatly reduced. These data indicate that the membrane potential-sensitive step, triggered by anti-IgM, is the entry of Ca\(^{2+}\) (Mn\(^{2+}\)) without affecting Ca\(^{2+}\) efflux. This contrasts with the findings in murine B cells, where membrane depolarization, induced by ionomycin, reportedly augments \(^{45}\)Ca\(^{2+}\) efflux (14).

Release of Ca\(^{2+}\) from internal stores and entry across the plasma membrane also behave differently towards artificially imposed changes in [Ca\(^{2+}\)]. This was inves-
tigated by pretreating B cells with varying concentrations of the divalent cation ionophore ionomycin before addition of anti-IgM. As shown in Fig. 3 A, in cells prepulsed with ionomycin (and hence with elevated baseline [Ca\(^{2+}\)])], addition of antibody still produced a further increase in [Ca\(^{2+}\)]. However, whereas the magnitude of the transient increase was relatively unaffected, the sustained phase was greatly reduced as the basal (prestimulation) [Ca\(^{2+}\)] was elevated. The results of several determinations are summarized in Fig. 3 B, which show that when the basal [Ca\(^{2+}\)] is sufficiently elevated (in the presence of ionomycin), the sustained response is practically eliminated. It is interesting to note that virtually complete suppression occurs when the baseline cytosolic Ca\(^{2+}\) concentration is elevated to the level normally attained in stimulated cells (~300–350 nM). This strongly suggests that the pathway for Ca\(^{2+}\) entry is regulated by [Ca\(^{2+}\)]. This conclusion is consistent with the observed constancy of the stimulated [Ca\(^{2+}\)] levels attained in cells loaded with varying amounts of BAPTA (10).

The failure of [Ca\(^{2+}\)] to alter ligand-activated release of Ca\(^{2+}\) from internal stores confirmed that this pathway is regulated independently from the transmembrane Ca\(^{2+}\) flux. For T lymphocytes, IP\(_3\) is the acknowledged mediator of internal store release and may also mediate entry of extracellular Ca\(^{2+}\) (6, 7). If IP\(_3\) is also pivotal in the response of human B cells to ligand binding, then the IP\(_3\)-induced channels in the endoplasmic reticulum behave differently than the channels (10) in the plasma membrane; the latter being sensitive to both membrane potential changes and [Ca\(^{2+}\)]. Alternatively, IP\(_3\) may play no role in the opening of the plasma membrane Ca\(^{2+}\) channels, accounting for the independent regulation of Ca\(^{2+}\) entry and release from internal stores. The present results do not rule out the possibility that external Ca\(^{2+}\) must traverse the endoplasmic reticulum to enter the cytoplasm (15).
However, the sensitivity of $\text{Ca}^{2+}$ uptake to $[\text{Ca}^{2+}]_i$ and to membrane potential may be most simply explained by an independent pathway located elsewhere on the plasma membrane.

**Summary**

Addition of crosslinking antibody to B lymphocytes results in a rapid rise in cytoplasmic-free $\text{Ca}^{2+} ([\text{Ca}^{2+}]_c)$ due to release of $\text{Ca}^{2+}$ from internal stores and uptake of $\text{Ca}^{2+}$ across the plasma membrane. Inositol 1,4,5-trisphosphate is believed to mediate the release of internal $\text{Ca}^{2+}$ stores and has also been proposed to mediate extracellular $\text{Ca}^{2+}$ entry. We have compared the properties of these two pathways for $\text{Ca}^{2+}$ mobilization by dissociating the $[\text{Ca}^{2+}]_c$ changes in ligand-activated human B cells after loading of the cells with the $\text{Ca}^{2+}$ chelator BAPTA. In the present paper we show that: (a) the sustained increase in $[\text{Ca}^{2+}]_c$ is due to increased unidirectional influx of external $[\text{Ca}^{2+}]_c$; (b) entry of extracellular $\text{Ca}^{2+}$, but not release of internal stores, is sensitive to the transmembrane potential; and (c) entry of extracellular $\text{Ca}^{2+}$, but not release of internal stores, is inhibited by increasing $[\text{Ca}^{2+}]_c$. These findings suggest that the permeation pathways mediating the translocation of $\text{Ca}^{2+}$ across the plasma membrane and endoplasmic reticulum membrane are not identical.

Received for publication 14 March 1989.

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