

CROSSLINKAGE OF B LYMPHOCYTE SURFACE  
IMMUNOGLOBULIN BY ANTI-Ig OR ANTIGEN INDUCES  
PROLONGED OSCILLATION OF INTRACELLULAR  
IONIZED CALCIUM

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B lymphocyte antigen receptor crosslinkage initiates the degradation of phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) (1), with the resultant formation of two intracellular messengers, diacylglycerol (2) and calcium-releasing inositol polyphosphates (IP<sub>3</sub>) (3). The relative contribution and interactions of these hydrolysis products to B cell activation and differentiation is a subject of intense interest. Because [Ca<sup>2+</sup>]<sub>i</sub> elevation itself activates a number of intracellular pathways, we have been interested in characterizing the magnitude and duration of calcium signals engendered by antigen receptor cross-linkage. Elucidation of the mechanism(s) of [Ca<sup>2+</sup>]<sub>i</sub> elevation should also provide information pertinent to the process of differentiation. These questions are best approached at the single cell level. In flow cytometry experiments with the calcium indicator dye indo-1, we have shown that [Ca<sup>2+</sup>]<sub>i</sub> transients of sufficient (micromolar) amplitude to activate Ca<sup>2+</sup>-dependent processes are observed in the majority of small B cells, even if suspended in Ca<sup>2+</sup>-free medium, when high concentrations of anti-Ig are used to effect an initial Ca<sup>2+</sup> spike. Of perhaps more relevance to cell cycle transitions, [Ca<sup>2+</sup>]<sub>i</sub> remains elevated for hours in cells cocultured with activating concentrations of anti-Ig. This persistent elevation calculated as mean values is nominal (1.5–3-fold above base-line 100 nM), but marked heterogeneity is apparent, such that micromolar free calcium levels are recorded for some cells (4). Using a technique (5) for continuous recording and imaging of [Ca<sup>2+</sup>]<sub>i</sub> in single cells, we now report that sustained [Ca<sup>2+</sup>]<sub>i</sub> elevation is resolvable into repetitive oscillations characterized by the cycling of [Ca<sup>2+</sup>]<sub>i</sub> between resting level troughs and transient peak levels in the micromolar range. The same rhythmic pattern is observable in purified hapten-specific B cells stimulated with antigen. Removal of extracel-

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lular ionized calcium does not immediately terminate oscillations regardless of the length of prior stimulation. These observations indicate that  $[Ca^{2+}]_i$  elevation measured in bulk cell suspensions or in individual cells at only one time point (flow cytometry) severely underestimates  $[Ca^{2+}]_i$  transients in B cells and that  $Ca^{2+}$  release from intracellular pools is sufficient to generate oscillatory transients.

### Materials and Methods

*Mice.* BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were used between 9 and 12 wk of age.

*Preparation of Small B Lymphocytes and TNP-binding Cells.* Small B lymphocytes were purified from spleen by modification (6) of the method of Leibson et al. (7), suspended in enriched Iscove's medium (8), supplemented with 0.1% BSA or FCS and maintained at 37°C and 5% CO<sub>2</sub> until used in experiments calling for polyclonal cells. TNP-specific cells were isolated exactly as described elsewhere (9). These cells were cultured overnight to allow for reexpression of new Ig receptors free of TNP-horse RBC ghosts.

*Antibodies and Antigens.* Affinity-purified F(ab')<sub>2</sub> fragments of rabbit antibody specific for mouse IgD (anti- $\delta$ ) or IgM (anti- $\mu$ ) and the monoclonal anti-Fc receptor antibody 2.4G2 were prepared as described (10, 11). F(ab')<sub>2</sub> rabbit anti-mouse IgG (anti-Fab) was obtained from Cappel Laboratories, (Cochranville, PA). TNP-ficoll was from Biosearch, San Rafael, CA; TNP-OVA and TNP-LPS were gifts from P. Stein and P. Dubois, Laboratory of Microbial Immunity (9).

*Calcium Measurement.* The  $[Ca^{2+}]_i$  of individual B lymphocytes or TNP-binding B cells was measured with the fluorescent  $Ca^{2+}$  indicator dye fura-2. The spectral characteristics and  $Ca^{2+}$  selectivity of fura-2 are described elsewhere (12), as are the components of the imaging system used in these experiments (5, 13). Fura-2 reports  $[Ca^{2+}]_i$  by a shift in its excitation spectrum to shorter wavelengths when it binds  $Ca^{2+}$ . The ratio of 350–385 nm excitation efficiency, measured by fluorescence at 510 nm, increases with  $[Ca^{2+}]_i$  and is independent of dye concentration. The dissociation constant of fura-2 for  $Ca^{2+}$  ( $K_d$  224 nM at 37°C), about twice that of quin2, permits sensitive measurements of  $Ca^{2+}$  transients up to the micromolar range.

Small B lymphocytes ( $10^7$ /ml) or TNP-binding cells ( $2 \times 10^6$ /ml) were loaded with 1–3  $\mu$ M or 0.5–1  $\mu$ M, respectively, of the acetoxymethylester of fura-2 (Molecular Probes, Junction City, OR) as described (4). In preliminary experiments, the 350/385 nm ratio of cells exposed to the  $Ca^{2+}$  ionophore ionomycin (1  $\mu$ M) was the same as the ratio for free dye in saturating calcium concentration, indicating that all of the intracellular dye had been hydrolyzed. 3 h after fura-2 loading, some compartmentalization of dye becomes apparent in both control and stimulated cells, although, perhaps surprisingly,  $[Ca^{2+}]_i$  distribution remained uniform throughout these cells. Measurements were therefore done within 3 h of loading.

Cells were transferred to a recording chamber composed of a stainless steel block thermostatted at 36°C, to which a coverslip was attached with vaseline. Grooves in the block permitted rapid exchange of solutions by perfusion and suction with pipettes. In preliminary experiments, glass cover slips were prepared in several ways. The simplest method was coating with 0.1% polylysine for 1 min followed by washing with 2% FCS serum. Two attempts were made to produce a surface suitable for binding antibody (2.4G2) but free of prohibitive interference with the fluorescence measurements; coverslips were either presilanized with chlorotrimethylsilane (Aldrich Laboratories, Inc., Columbus, OH) and then coated with a thin film of polystyrene solution, or else coated directly with chlorodimethylphenylsilane. All methods yielded the same B cell responses. Because more cells were lost due to medium exchanges with antibody than with polylysine adherence, the latter method was used routinely. The protocol for experiments was as follows. Cell suspensions were allowed to settle on the cover slip (about 3 min) until adherence could be established by jiggling the chamber. The medium was then changed to recording medium prepared by deleting phenol red, reducing NaHCO<sub>3</sub> to 4 mM, and increasing Hepes (pH 7.3) to 20 mM (4). A field was chosen (for maximum cell number)

and observed for several minutes, after which the stimulating reagent was added. Where indicated, ionized external calcium was reduced to <100 nM with EGTA (4, 12).  $[Ca^{2+}]_i$  levels and even intracellular gradients could be viewed and recorded in up to 100 lymphocytes simultaneously. Data were stored on digital tape for subsequent analysis. Average  $[Ca^{2+}]_i$  values for individual cells were obtained by an interactive computer routine for placing circles approximately equal to the cell diameter over each cell on the video display. Pixel values within the circle were then used to determine average  $[Ca^{2+}]_i$ .

### Results and Discussion

The  $[Ca^{2+}]_i$  of unstimulated small B lymphocytes was  $96 \pm 41$  nM (130 cells, pooled from four experiments, mean  $\pm$  SD), and corresponding values for hapten-specific cells were  $91 \pm 53$  nM (137 cells, pooled from 18 experiments). Over a 10-min observation period, slightly <10% of the cells of either preparation, and hapten-specific cells exposed only to the carriers (LPS or ficoll), exhibited either transient or sustained  $Ca^{2+}$  elevation >150 nM. We suspect that this is an artifact of the adherence method, since occasional cells (<1%) were seen to lyse. B lymphocytes or hapten-specific cells stimulated with high doses (10–60  $\mu$ g/ml) of anti-Ig (Fab,  $\delta$  or  $\mu$ ) or antigens, respectively, exhibited marked elevation of  $[Ca^{2+}]_i$  within a few seconds. The fraction of responders ( $[Ca^{2+}]_i$  >200 nM) was 95% for small B lymphocytes and 57% for hapten-specific cells stimulated with TNP-LPS or TNP-ficoll. TNP-OVA, a thymus-dependent antigen, appeared to yield similar results (two experiments).  $[Ca^{2+}]_i$  elevation occurred in ~70% of hapten-specific cells stimulated with anti-Fab (45  $\mu$ g/ml), suggesting that some cells failed to regenerate mIg. For all stimulated preparations, the averaged peak responses of individual cells were in the micromolar range.  $[Ca^{2+}]_i$  values in each population were, however, markedly heterogeneous with respect to maximum  $[Ca^{2+}]_i$ . Sequential histograms showed broad distributions of  $[Ca^{2+}]_i$  similar to those obtained in flow cytometry experiments (4).

A more interesting form of asynchrony is apparent in individual cells followed over time (Fig. 1). Antigen receptor cross-linkage results in a repetitive pattern of  $[Ca^{2+}]_i$  transients rising to the micromolar range followed by a fall to the level of unstimulated cells. This oscillatory activity predominated in acutely stimulated cells (73%, the remainder exhibiting a peak followed by a slow decline in  $[Ca^{2+}]_i$  over the observation period), and was the only pattern of  $[Ca^{2+}]_i$  elevation observed in cells examined after incubation with anti-Ig for several hours (Fig. 2B). Oscillations were irregular in amplitude, wave form, and duration, but typically lasted 15–60 s. This asynchrony accounts for the underestimation of  $[Ca^{2+}]_i$  peaks after the initial spike, as measured in curvette or flow cytometry experiments. In most fields, individual cells within small clumps could be resolved. The  $[Ca^{2+}]_i$  transients of contiguous cells were not temporally related.

The concentrations of ligands used to elicit maximum initial responses greatly exceeded those needed for cell activation. The same pattern of  $Ca^{2+}$  transients was observed however, with stimulating concentrations much lower than required for the induction of maximum size increase (Fig. 2B) measured at 24 h (4). At these low ligand concentrations spatial cytoplasmic  $[Ca^{2+}]_i$  gradients were detected within occasional cells.  $Ca^{2+}$  transients were observed in cells for up to 6 h after stimulation, and preliminary data suggest that oscillations continue at least through 18 h (not shown).

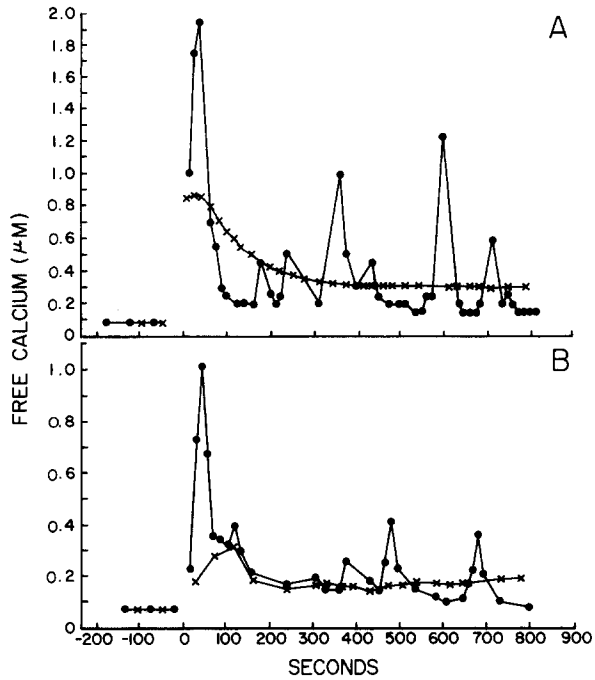


FIGURE 1. Antigen receptor cross-linkage induces repetitive calcium transients. Free intracellular calcium is shown for a single fura-2 loaded B lymphocyte (A) stimulated with 30  $\mu\text{g}/\text{ml}$  of anti- $\delta$  (closed circles) at time 0. The mean population response (x) is presented for comparison. (B) Similar data for a TNP-binding B cell stimulated with 10  $\mu\text{g}/\text{ml}$  of TNP-ficoll. The mean response (x) was calculated for responding cells only.

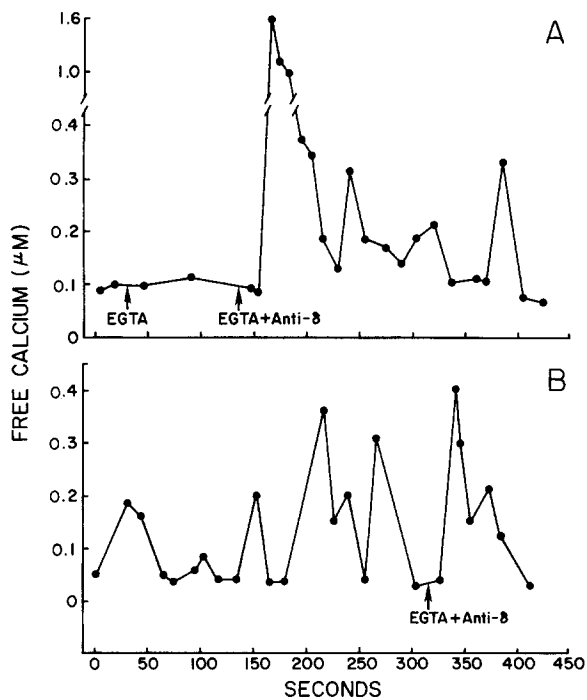


FIGURE 2. Extra cellular calcium is not required for the generation of B lymphocyte calcium transients. (A) Medium calcium was reduced to 100 nM by EDTA addition, followed by anti- $\delta$  (30  $\mu\text{g}/\text{ml}$ ) stimulation in the low calcium medium. The cell shown in B was cocultured in regular medium for 2 h with 10  $\mu\text{g}/\text{ml}$  of anti- $\mu$  before transfer to the recording chamber. Anti- $\delta$  (30  $\mu\text{g}/\text{ml}$ ) in 100 nM calcium medium was added as indicated.

It has been assumed (reviewed in reference 4) that the elevation of  $[Ca^{2+}]_i$  after an initial spike is mediated directly by inward calcium transport. Fig. 2 shows that both in cells acutely stimulated (Fig. 2A) or incubated for hours (Fig. 2B) with anti-Ig,  $Ca^{2+}$  transients of decreasing amplitude persist for several minutes after ionized medium  $Ca^{2+}$  is complexed with EGTA. Because cellular  $Ca^{2+}$  is rapidly depleted by these conditions, the effect of  $Ca^{2+}$  influx on  $Ca^{2+}$  transient amplitude cannot be quantitatively assessed, but is likely to be relatively small (initial transients in the presence or absence of EGTA are similar). The occurrence of repetitive transients in the absence of  $[Ca^{2+}]_0$  strongly suggests, however, that the induction of transients depends only on intracellular  $Ca^{2+}$  mobilization. An attractive extrapolation from these data, requiring neither phasic  $IP_3$  generation or stochastic  $Ca^{2+}$  channel activity, is that  $Ca^{2+}$  oscillation in B cells is generated by repetitive uptake and release of  $Ca^{2+}$  by endoplasmic reticulum. Consistent with this postulate,  $Ca^{2+}$  transients were diminished (not shown) by manipulations that reduce  $IP_3$  levels: preincubation of cells with protein kinase C-activating phorbol esters (14), or substitution of molar equivalent intact anti-Ig, with resulting Fc $\gamma$  receptor-mIg crosslinkage, for the standard F(ab') $_2$  reagents (4, 15). Phorbol esters alone reduce  $[Ca^{2+}]_i$  and hence may additionally regulate calcium pump activity. The modulation of  $Ca^{2+}$  transients is likely to be complex and provides a number of potential regulatory sites for mediators of lymphocyte activation.

### Summary

Our results indicate that B lymphocytes stimulated with anti-Ig or antigen exhibit repetitive  $[Ca^{2+}]_i$  transients which persist for hours. The magnitude of these transients favors an important and ongoing role for  $[Ca^{2+}]_i$  elevation in antigen driven B cell activation. Repetitive  $Ca^{2+}$  transients may prove to be a prevalent mechanism of  $Ca^{2+}$  signaling. In preliminary experiments (with L. E. Samelson and R. D. Klausner), we have observed  $Ca^{2+}$  transients in cloned T cells stimulated with antigen. Woods et al. (16) have described repetitive free  $Ca^{2+}$  transients in hepatocytes stimulated with extracellular ligands promoting glycogenolysis, and suggest that the intervals of base-line  $[Ca^{2+}]_i$  levels explain the absence of mitochondrial overload in chronically stimulated cells. These considerations apply equally to B lymphocytes and recommend caution in delineating the range of  $Ca^{2+}$ -mediated functions by prolonged coculture of cells with  $Ca^{2+}$  ionophores. Our experiments were done in a simple recording chamber with one cell type. No cell interactions were observed. Given the variety of indicator dyes now available, the technical approach we present, augmented by a more sophisticated recording chamber, is a potentially powerful tool for examining the intrinsic, and T- or accessory cell-dependent, physiology of B cell differentiation.

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