Vol. 137, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1986 Pages 500-506

# CROSS-LINKING OF SURFACE IMMUNOGLOBULIN ON B LYMPHOCYTES INDUCES BOTH INTRACELLULAR $Ca^{2+}$ RELEASE AND $Ca^{2+}$ INFLUX: ANALYSIS WITH INDO-1

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Received April 1, 1986

SUMMARY: The new $Ca^{2+}$-probe indo-1 has a high fluorescence intensity, which allows low intracellular dye loadings. Stimulation of indo-1-loaded mouse B cells with anti-Ig antibodies provoked rapid rise of free cytoplasmic $Ca^{2+}$ from 100 nM to >1 μM, followed by a decline to a plateau at 300-400 nM. The initial rapid rise was not detected in quin2-loaded cells, presumably due to the $Ca^{2+}$-buffering effects of the dye. The sustained $Ca^{2+}$ increase was due to influx, whereas the initial rise was caused by release from intracellular stores. The magnitudes of $Ca^{2+}$ release and inositol trisphosphate release were closely correlated. Concanavalin A does not provoke inositol trisphosphate release in mouse B cells. It did not induce a rapid initial $Ca^{2+}$ rise in indo-1-loaded B cells either, but only a sustained increase to 200-300 nM. Finally, $Ca^{2+}$ influx induced by both anti-Ig and concanavalin A were not affected by membrane depolarization 

A wide variety of ligands induce in their target cells a rapid rise in cytoplasmic free $Ca^{2+}$ ($[Ca^{2+}]_i$). This is generally associated with degradation of phosphatidylinositol bisphosphate ($PIP_2$) to diacylglycerol and inositol trisphosphate ($IP_3$), and recent evidence indicates that $IP_3$ causes (part of) the increase in $[Ca^{2+}]_i$ by releasing $Ca^{2+}$ from intracellular stores [1].

The most popular current method for measuring $[Ca^{2+}]_i$ is to monitor the fluorescence of intracellularly trapped quin2 [2]. Quin2 has, however, some limitations, the most serious being a low fluorescence intensity. To overcome cell autofluorescence, it is therefore neccessary to load relatively large amounts of the dye into cells, which significantly buffers $[Ca^{2+}]_i$ transients.

Recently, $Ca^{2+}$ indicators with high fluorescence intensities have been developed, which can be used at much lower concentrations [3]. We have now

ABBREVIATIONS USED: Anti-Ig, anti-immunoglobulin antibodies; $[Ca^{2+}]i$, free cytoplasmic Ca concentration; Con A, concanavalin A; $IP_3$, inositol trisphosphate; $PIP_2$, phosphatidylinositol bisphosphate.

0006-291X/86 $1.50

used one of them, indo-1, to study $[Ca^{2+}]_i$ in murine B lymphocytes incubated with anti-immunoglobulin antibodies (anti-Ig) or concanavalin A (Con A). Anti-Ig stimulates quiescent B cells to enter cell cycle by cross-linking their antigen receptors. It induces rapid breakdown op $PIP_2$, and increases $[Ca^{2+}]_i$ in these cells [4]. Con A also causes B cell activation, but only provokes $Ca^{2+}$ mobilization, with minimal breakdown of $PIP_2$ [M.K. Bijsterbosch and G.G.B. Klaus, submitted]. These earlier studies, using quin2, suggested that the $[Ca^{2+}]_i$ increase induced by both agents was largely due to influx. We now show that quin2 does not detect a rapid, $IP_3$-mediated intracellular release of $Ca^{2+}$ induced by anti-Ig, which can be readily demonstrated with indo-1.

## EXPERIMENTAL

**Reagents:** Indo-1 (acetoxymethyl ester and potassium salt) was from Molecular Probes, Junction City, Or, U.S.A. Quin2 acetoxymethyl ester was from Amersham International, Amersham, U.K. Quin2 (free acid) and Con A (type V) were from Sigma, St. Louis, Mo, U.S.A. Molar absorption coefficients of indo-1 and quin2 were taken from refs. 3 and 5. Affinity-purified $F(ab')_2$ fragements of rabbit anti-mouse Fab antibodies, henceforth called anti-Ig, were prepared as described previously [4]. All other chemicals were analytical grade.

**Cell preparations:** Splenic B cells (ca. 90% surface Ig-positive) were prepared from 3-6 months old male (CBA x C57BL/10)$F_1$ mice by killing T cells and removing adherent cells as described earlier [4].

**Assay of inositol trisphosphate:** Levels of $[^3H]IP_3$ in B cells prelabelled with $[^3H]$inositol were measured as described in detail earlier [4].

**Determination of free $Ca^{2+}$ in vitro:** Quin2 and indo-1 were dissolved in buffers containing 120 mM KCl, 20 mM NaCl, 1 mM $MgCl_2$, 10 mM MOPS, and 1 mM EGTA plus 0.1-0.9 mM $CaCl_2$ (pH 7.05) to final concentrations of 0.5 and 6.5 µM, respectively. Their fluorescences were measured using a Perkin Elmer MPF-4 spectrofluorimeter in a cell kept at 37 °C. Excitation and emission wavelengths were 339 and 500 nm for quin2, and 340 and 390 nm for indo-1. The signals were calibrated by adding $CaCl_2$ to approx. 0.5 mM excess (for $F_{max}$), followed by setting free $Ca^{2+}$ to <1 nM by adding EGTA and Tris to 10 and 40 mM (for $F_{min}$). Free $Ca^{2+}$ concentrations in the buffers were calculated from the observed fluorescence F by:

$$Ca^{2+} = Kd \times [(F-F_{min})/(F_{max}-F)]$$

The $K_d$ values for quin2 and indo-1 under these conditions were taken to be 115 nM and 250 nM, respectively [2,3].

**Determination of $[Ca^{2+}]_i$:** Cells at 50 x $10^6$/ml in Hanks' balanced salt solution, containing 0.5% gelatin and 20 mM HEPES (pH 7.2), received the acetoxymethyl esters of quin2 or indo-1 to final concentrations of 2 or 15 µM, respectively. Both were added from stock solutions in DMSO (final DMSO concentration <0.25%). After 45 min at 37 °C, the cells were washed twice and resuspended to 5-10 x $10^6$/ml in Hanks' solution containing 0.5% bovine serum albumin and 10 mM HEPES (pH 7.3). Cell viability was >98%. The suspensions were stored on ice until use. Under these conditions, leakage of quin2 and indo-1 from the cells was <1 % per hour. Shortly before fluorescence measurements, aliquots were washed and resuspended to 5-10 x $10^6$/ml in Hanks' solution plus 10 mM HEPES (pH 7.3). After 5-10 min preincubation at 37 °C, fluorescence was measured as described above. The suspensions were stirred

periodically during measurements. After each run, $F_{max}$ was obtained by lysing the cells with 0.05% Triton X-100 in the presence of 10 uM diethylenetriaminepentaacetic acid. $F_{min}$ was determined by subsequent addition of EGTA and Tris as above (for indo-1) or $MnCl_2$ to 0.2 mM (for quin2, $F_{min}$ corrected for effect of $Mg^{2+}$) .$[Ca^{2+}]i$ was calculated with the formula given above. Intracellular dye loads were determined comparing fluorescences of cell lysates and known concentrations of the dyes and assuming a cellular volume of 110 $um^3$ [6].

## RESULTS

The free $Ca^{2+}$ concentrations measured by quin2 and indo-1 were compared in buffers in which free $Ca^{2+}$ levels were varied by altering $Ca^{2+}$/EGTA ratios. Fig. 1 shows that over the biologically important range of 40-1000 nM the free $Ca^{2+}$ concentrations measured by indo-1 and quin2 are strongly correlated. Levels measured with quin2 were, however, 16 $\pm$ 2% (means $\pm$ S.E.M., n=18) lower than those measured with indo-1. This small discrepancy is probably due to minor inaccuracies in the $K_d$ values of the dyes.

Subsequently, B cells were loaded with either quin2 or indo-1. The resting values measured with quin2 and indo-1 were very similar: 107 $\pm$ 4 nM and 114 $\pm$ 3 nM, respectively (means $\pm$ S.E.M., n=12). When cells loaded with indo-1 were stimulated with anti-Ig, $[Ca^{2+}]_i$ rose within 30 sec to a maximum of >1 $\mu$M and then rapidly declined to a plateau of 300-400 nM (Fig. 2A, left panel). In quin2-loaded cells, $[Ca^{2+}]_i$ rose to the plateau at 300-400 nM, but the rapid transient increase was not detected. This is presumably because the dye loading (1.42 $\pm$ 0.14 mM; means $\pm$ S.E.M., n=4), and thus $Ca^{2+}$-buffering, in

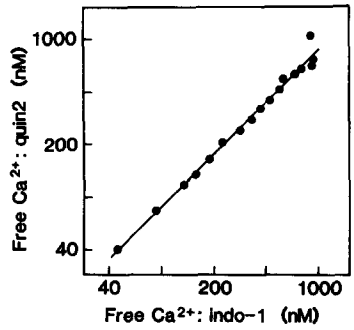


Figure 1: **Free $Ca^{2+}$ levels measured with quin2 and indo-1 in vitro.** The levels of free $Ca^{2+}$ in a series of $Ca^{2+}$/EGTA buffers was measured with quin2 and indo-1 as described in detail in the Experimental section. Each point gives free $Ca^{2+}$ levels measured with both quin2 and indo-1 in a particular buffer (results from two separate experiments).

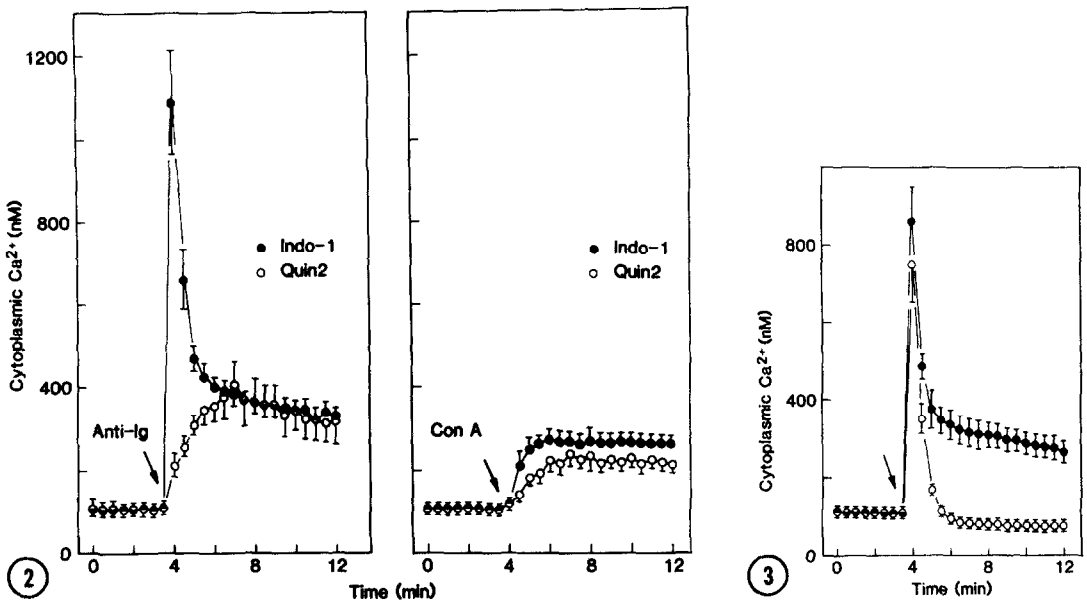


Figure 2. **Effects of anti-Ig and Con A on $[Ca^{2+}]_i$ in B cells loaded with quin2 or indo-1.** B cells containing indo-1 (●) or quin2 (○) received anti-Ig and Con A as indicated (final concentrations 50 and 2.5 μg/ml, respectively). Intracellular dye loadings were 1.42 ± 0.14 mM and 0.09 ± 0.01 mM for quin2 and indo-1, respectively. $[Ca^{2+}]_i$ was calculated from the fluorescence signals, which were corrected for dilution due to additions made, as decribed in the Experimental section. $[Ca^{2+}]_i$ in controls (medium added) did not change appreciably. Results are means ± S.E.M. of 4 replicates obtained from 4 separate experiments.

Figure 3. **Effects of anti-Ig on $[Ca^{2+}]_i$ in B cells in the presence of normal and low extracellular $Ca^{2+}$.** B cells were loaded with indo-1 and incubated in normal medium (1.3 mM $Ca^{2+}$). At the time indicated by the arrow, anti-Ig was added to 50 μg/ml. Approx. 15 sec before this addition, the cells received EGTA to 1.8 mM (○), or medium (●). Addition of EGTA immediately reduced extracellular $Ca^{2+}$ to <250 nM. $[Ca^{2+}]_i$ was calculated as in Fig. 2. Results are means ± S.E.M. of 3 replicates obtained from 3 separate experiments.

these cells was much higher than in cells containing indo-1 (0.09 ± 0.01 mM; means ± S.E.M., n=4). Addition of Con A to B cells, on the other hand, yielded very similar results in cells loaded with either dye (Fig. 2, right panel).

We next studied the contributions of extracellular and intracellular $Ca^{2+}$ to the response induced by anti-Ig in indo-1-loaded B cells (Fig. 3). After reduction of extracellular $Ca^{2+}$ to <250 nM by EGTA, the rapid transient increase in $[Ca^{2+}]_i$ was unaffected, but the sustained increase seen in the presence of extracellular $Ca^{2+}$ was completely abolished.

These results suggest that the rapid transient $[Ca^{2+}]_i$ rise is due to release of $Ca^{2+}$ from intracellular stores. Recent studies indicate that $IP_3$ causes the release of $Ca^{2+}$ from intracellular stores [1,7]. We therefore

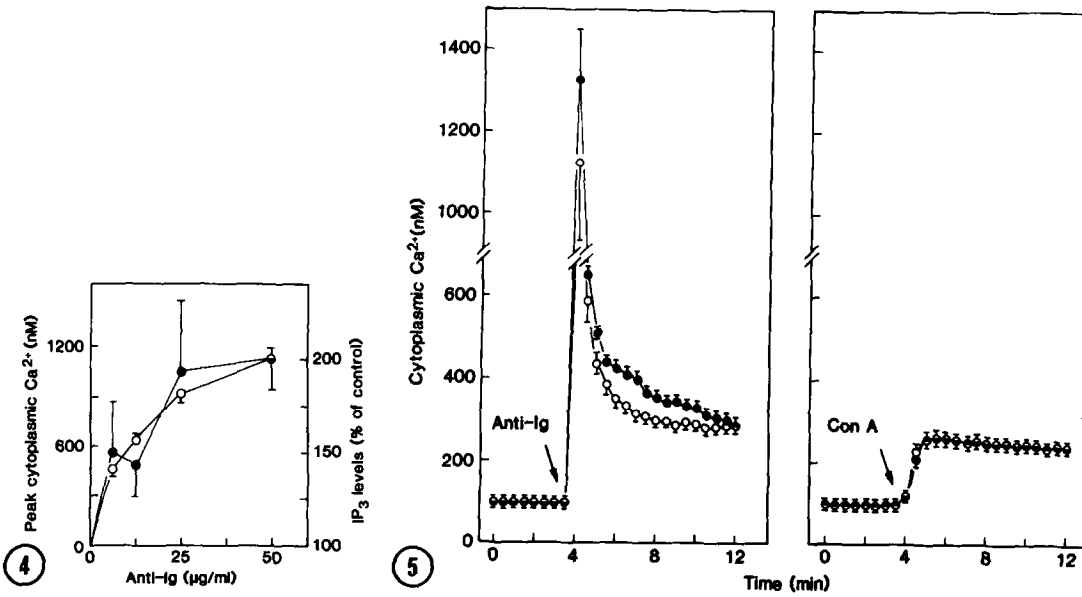


Figure 4. **Dose-dependence of induction of peak $[Ca^{2+}]_i$ and $IP_3$ release by anti-Ig.** Indo-1-loaded B cells were stimulated with 0-50 µg/ml anti-Ig. $[Ca^{2+}]_i$ reached maximum values after approx. 30 sec, and is given (O) with control levels (medium added: 104 ± 5 nM) subtracted. $[^3H]IP_3$ levels (●) were determined 30 sec after adding 0-50 µg/ml anti-Ig to $[^3H]$inositol-labelled B cells. The results are expressed as % of the levels found with medium alone: 0.19 ± 0.01% of the total cellular radioactivity (108,000 dpm). All points are means ± S.E.M. of 4 replicates from 4 separate experiments.

Figure 5. **Effects of anti-Ig and Con A on $[Ca^{2+}]_i$ in B cells in the presence of normal and high extracellular $K^+$ levels.** Indo-1-loaded B cells in normal medium (●; 6 mM $K^+$, 142 mM $Na^+$) or high $K^+$ medium (O; 70mM $K^+$, 78 mM $Na^+$) received anti-Ig or Con A as indicated (final concentrations 50 and 2.5 µg/ml, respectively). $[Ca^{2+}]_i$ was calculated as in Fig. 2. Points are means ± S.E.M. of 3 replicates from 3 separate experiments.

compared the magnitude of the initial $[Ca^{2+}]_i$ peak induced by increasing doses of anti-Ig with changes in $IP_3$ levels. Fig. 4 shows that these two responses are closely correlated.

The sustained increase in $[Ca^{2+}]_i$ is apparently due to influx of extracellular $Ca^{2+}$. Since in many other cell types influx of $Ca^{2+}$ is controlled by the cell membrane potential [8], we studied $[Ca^{2+}]_i$ in indo-1-loaded cells in media containing normal (6 mM) and depolarizing (70 mM) concentrations of $K^+$ (Fig. 5). Resting $[Ca^{2+}]_i$ values were the same in cells in normal and high $K^+$ medium (103 ± 3 nM; means ± S.E.M., n= 9). Depolarization had only a slight effect on $Ca^{2+}$ influx in cells stimulated with anti-Ig, whereas the response induced by Con A was not affected at all.

## DISCUSSION

Our data clearly demonstrate that the new $Ca^{2+}$ probe indo-1 is a much better tool than quin2 for measuring $[Ca^{2+}]_i$ in living cells. Using indo-1, we show that anti-Ig induces in B cells an extremely rapid, massive release of $Ca^{2+}$ from intracellular stores (which is presumably $IP_3$-mediated), followed by prolonged influx of extracellular $Ca^{2+}$. The rapidity of the initial response indicates that the cells respond quite synchronously to stimulation.

The main advantage of indo-1 over quin2 is its much brighter fluorescence, which allows lower dye loadings. The resulting lower $Ca^{2+}$-buffering allowed detection in indo-1-loaded cells of a rapid transient rise in $[Ca^{2+}]_i$ that was not seen with quin2 (Fig. 2). Another major feature of indo-1 is that $Ca^{2+}$ alters the wavelength of its fluorescence emission, which allows $Ca^{2+}$ determinations to be made by measuring the emission ratios at two wavelengths [3]. However, this method requires sophisticated instrumentation. The method we employ, measuring emission at one wavelength followed by cell destruction and calibration, uses a standard spectrofluorimeter. Its validity is apparent from Figures 1 and 2.

We found that the rapid transient rise in $[Ca^{2+}]_i$ induced by anti-Ig in indo-1-loaded cells is due to release of $Ca^{2+}$ from intracellular stores. In a wide variety of cell types (including B cells), addition of exogenous $IP_3$ to permeabilized cells provokes the release of $Ca^{2+}$ from intracellular stores [1,7]. A comparison of the capacity of various doses of anti-Ig to induce $IP_3$ release and the initial $[Ca^{2+}]_i$ peak revealed a close correlation (Fig. 4). Our $IP_3$ assay does not distinguish between $IP_3$ isomers, but in earlier experiments (done with M. Berridge and J. Heslop) we found that anti-Ig induces mainly release of 1,4,5-$IP_3$, the isomer capable of inducing intracellular $Ca^{2+}$ release [1]. Our data therefore support the hypothesis that mobilization of intracellular $Ca^{2+}$ by anti-Ig is mediated by 1,4,5-$IP_3$ generated from degradation of $PIP_2$. This view is further corroborated by the finding that Con A, which does not provoke substantial $IP_3$ release in B cells, fails to induce the transient $[Ca^{2+}]_i$ rise (Fig. 2).

There are various mechanisms regulating the influx of $Ca^{2+}$ into cells [8]. Some cell types possess voltage-gated $Ca^{2+}$ channels. Depolarization of their plasma membranes leads to massive $Ca^{2+}$ influx, which can be inhibited by $Ca^{2+}$ channel blockers like verapamil [9]. Others have $Ca^{2+}$ channels that open by a voltage-sensitive mechanism upon ligation of receptors on their surface [10]. Entry of $Ca^{2+}$ via these channels can also be inhibited by the blockers mentioned above. In yet other cell types, resting and stimulated $Ca^{2+}$ levels are unaffected by membrane depolarization, nor is influx of $Ca^{2+}$ susceptible to inhibition by $Ca^{2+}$ channel blockers [11]. The depolarization experiments (Fig. 5), plus the lack of effect of verapamil (not shown), indicate that mouse B cells belong to the latter group. The results of Clevers et al. [12], who found that $Ca^{2+}$ channel blockers inhibit anti-Ig-induced $[Ca^{2+}]_i$ increase in human B cells, suggest, however, that there may be species differences.

ACKNOWLEDGMENTS: M. K. Bijsterbosch and K. Rigley were supported by fellowships from the Wellcome Trust. We wish to thank Mary Holman for her technical assistance.

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