Anti-Immunoglobulin, Cytoplasmic Free Calcium, and Capping in B Lymphocytes

T. POZZAN, P. ARSLAN, R. Y. TSIEN, and T. J. RINK
Consiglio Nazionale delle Richerche Unit for the Study of Mitochondria and Institute of General Pathology, University of Padova, Padova, Italy; and the Physiological Laboratory, University of Cambridge, Cambridge CB2 3EG, England

ABSTRACT This paper examines, in mouse spleen lymphocytes, the effect of anti-immunoglobulin (anti-Ig) on the cytoplasmic free calcium concentration, $[Ca^{2+}]_i$, measured with the fluorescent indicator quin2, and the relationship of $[Ca^{2+}]_i$ to the capping of surface Ig. Anti-Ig causes a rapid rise of $[Ca^{2+}]_i$ which precedes capping. Assuming that only those 40–50% of the cells which can bind anti-Ig (the B cells) undergo a $[Ca^{2+}]_i$ response, $[Ca^{2+}]_i$ in those cells approaches 500 nM. It declines to resting levels over many minutes, roughly paralleling the formation of caps. Part of the $[Ca^{2+}]_i$ signal is due to stimulated influx across the plasma membrane, since in Ca^{2+}-free medium, anti-Ig gives a smaller and shorter $[Ca^{2+}]_i$ rise. The amplitude of this reduced transient now varies inversely with quin2 content, as if some 0.25 mmol Ca per liter of cells was released into the cytoplasm from internal stores. These stores are probably sequestered in organelles since A23187 in Ca^{2+}-free medium also causes a transient $[Ca^{2+}]_i$ rise after which anti-Ig has no effect. These organelles seem not to be mitochondria because uncouplers have hardly any effect on $[Ca^{2+}]_i$.

Though anti-Ig normally raises $[Ca^{2+}]_i$ before causing capping, there seems to be no causal link between the two events. Cells in Ca^{2+}-free medium whose stores have been emptied by A23187, still cap with anti-Ig even though there is no $[Ca^{2+}]_i$ rise. Cells loaded with quin2 in the absence of external Ca^{2+} still cap anti-Ig normally even though their $[Ca^{2+}]_i$ remains steady at below 30 nM, four times lower than normal resting $[Ca^{2+}]_i$.

Capping of surface Ig represents a dramatic reorganization of membrane components apparently involving cytoskeletal elements and requiring metabolic energy (2, 12). Formation of caps is followed by contractile activity and cell motility. Rather little is known of how ligand binding stimulates these processes although changes in ion flux and potential difference across the plasma membrane play no essential part (7). Ca^{2+} ions are well known to trigger muscle contraction and are implicated in the regulation of various forms of nonmuscle motility, so it has been attractive to suppose that a rise in cytoplasmic free Ca^{2+} mediates the B cell response to anti-Ig (2). The evidence hitherto available is entirely indirect. It is generally agreed that external Ca^{2+} is not needed. The ionophore A23187, which in other cell types raises cytoplasmic free $[Ca^{2+}]$ and stimulates Ca^{2+}-activated processes, actually inhibits capping in B lymphocytes. However this inhibitory effect, like those of local anesthetics (5) and *cis*-unsaturated fatty acids (3), is at least in part explainable (10) by depletion of cellular ATP. Recently anti-Ig has been found to cause a rapid loss of ^{45}Ca from mouse spleen cells (1). This efflux was interpreted as mobilization of Ca^{2+} from internal stores causing a rise in the level of free cytoplasmic Ca^{2+} and subsequent extrusion of the mobilized ions. There was no evidence that the Ca^{2+} movements caused capping but the correlations between the two were felt to suggest "that Ca^{2+} may have a physiological role in contractile dependent capping" (2).

In the present paper the intracellular Ca^{2+} indicator, quin2 (13, 14), has been used to assess more directly the importance of Ca^{2+} by monitoring and manipulating the cytoplasmic free Ca^{2+} concentration, $[Ca^{2+}]_i$, in mouse spleen lymphocytes. The changes in $[Ca^{2+}]_i$ brought about by anti-Ig on cap formation are analyzed and the effects of preventing such changes are examined.

MATERIALS AND METHODS

BALB/c mouse spleen lymphocytes and thymocytes were prepared as previously described (6–10) in HEPES-buffered culture medium, either H-MEM or RPMI-1640. Viability, assessed by eosin exclusion, exceeded 95%. B-cell enriched

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suspensions were obtained by either anti-Thy 1,2 antibodies and complement (1), or Percoll density gradients (4).

Cells were loaded with quin2, and the fluorescence signal used to monitor $[Ca^{2+}]_i$ as described in the preceding paper (14). For these experiments the cells were resuspended in a simplified medium containing 140 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 5.5 mM glucose, 0.5 mM $MgSO_4$, 20 mM Na HEPES, pH 7.4 at 37°C and either 1 mM $CaCl_2$ or 0.2 mM Tris EGTA.

The anti-Ig used to cap spleen B cells was rabbit anti-mouse immunoglobulin G, RaMIg, sometimes labeled with fluorescein isothiocyanate, FITC-RaMIg, and sometimes unlabeled. Capping of FITC-RaMIg was run for 7 min at 37°C unless stated otherwise, and assessed after formaldehyde fixation, as previously described (3, 5, 10). When it was necessary to measure the effect of RaMIg on the quin2 signal, unlabeled RaMIg was used, since the fluorescein fluorescence of FITC-RaMIg would overwhelm that of the quin2. To assess cap formation such cells were washed in ice-cold medium and exposed in the cold to FITC goat anti-rabbit Ig antibody to locate the rabbit anti-mouse Ig on the cell surface. The cells were then washed and resuspended in ice-cold medium and fixed with formaldehyde.

The rabbit anti-mouse antibodies were obtained from Miles-Yeda, Rehovot, Israel, and dialyzed against phosphate-buffered saline at pH 7.4 to remove preservatives. Fab'_2-RaMIg was prepared from the RaMIg by pepsin digestion, followed by Sephadex G100 chromatography. Fab'-RaMIg was prepared by reduction of the disulphide bond with 10 mM mercaptoethanol and treatment with iodoacetamide as described by Braun et al. (1). There was not sufficient final product to rechromatograph so there may have been some contamination with intact antibody. The FITC goat antibody was a kind gift from Dr. S. Sartore, University of Padua. The culture media came from Flow Laboratories (Irvine, Scotland), the A 23187 from Calbiochem, and FCCP (carbonylcyanide, *p*-trifluoromethoxyphenyl hydrazone) and oligomycin from Sigma Chemical Co. (Poole, England). Analytical grade chemicals were used throughout, where available.

RESULTS

Effects of RaMIg on $[Ca^{2+}]_i$

In resting splenocytes suspended in 1 mM Ca medium, the fluorescence of intracellular quin2 showed that it was roughly half Ca-saturated. The indicated basal $[Ca^{2+}]_i$ ranged from 100 to 150 nM, very similar to levels found in mouse thymocytes (14). Fig. 1 shows the typical effect of 40 μg/ml of RaMIg, which increased the Ca-saturation of the quin2 from 50% to some 65% indicating a rise in $[Ca^{2+}]_i$. The response showed no measurable delay and reached a maximum within 1 min, and then slowly declined over 20 min almost to the resting level. The inset in Fig. 1 shows the time course of cap formation with the same concentration of RaMIg. Clearly the $[Ca^{2+}]_i$ rise preceded capping. Fig. 2 shows that the peak of the $[Ca^{2+}]_i$ response increased with RaMIg concentration up to 40 μg/ml and then plateaued. The speed of the $[Ca^{2+}]_i$ rise also increased with RaMIg concentration.

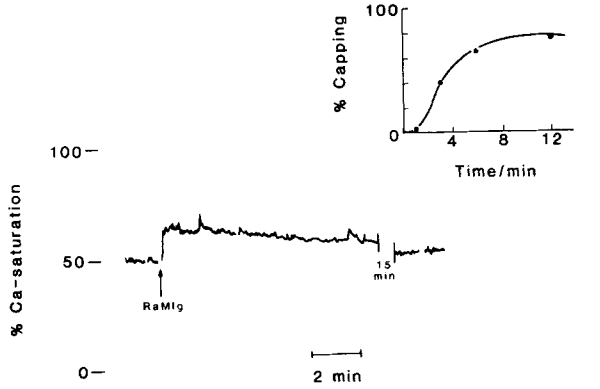

FIGURE 1 Effect of RaMIg on the fluorescence of quin2 trapped in mouse spleen lymphocytes. 10^7 splenocytes per ml, quin2 content ~1 mM, were suspended in 1 mM Ca medium. 53% of the cells were Ig^+, i.e. could be stained with FITC RaMIg. 40 μg/ml unlabeled RaMIg was added when indicated. The occasional brief random spikes on the fluorescence record are due to fluctuations of lamp intensity or small bubbles intersecting the beam, and should be ignored. The calibration marks for 0 and 100% Ca-saturation correspond to F_{min} and F_{max} as defined in the previous paper (14), and denote the fluorescence levels attained when the intracellular quin2 was released by Triton X-100, exposed to <1 nM and 1 mM $[Ca^{2+}]_i$ respectively, and the resulting limiting fluorescences corrected slightly for cell autofluorescence. The inset shows cap formation as a function of time in the same batch of cells exposed to the same concentration of RaMIg.

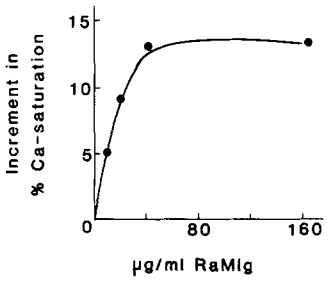

FIGURE 2 Effect of RaMIg concentration on the quin2 response. 10^7 cells/ml suspended in 1 mM Ca medium. Quin2 content 1.5 mM, 43% Ig^+. The vertical axis shows the peak percentage change in quin2 Ca-saturation after addition of the RaMIg.

TABLE I

Specificity of the Quin2 Response

Preparation	Ligand	Peak increase in quin2 saturation
		%
Unfractionated spleen cells (48% Ig^+)	RaMIg	16
	Rabbit nonimmune Ig	0
	Fab'_2 RaMIg	17
	Fab' RaMIg	1.5
B-enriched spleen cells (80% Ig^+)	RaMIg	27
Thymocytes (<1% Ig^+)	RaMIg	0

The experimental conditions were as for Fig. 1. The quin2 of these preparations was ~1.2 mM. The ligands were added to a final concentration of 40 μg/ml. These data are shown for one batch of quin2-loaded, unfractionated cells. The result with RaMIg is typical of that seen in many experiments. Nonimmune serum gave the same nil-effect with another preparation. The Fab' and Fab'_2 effects were very similar in two other cell preparations. The result shown for B-enriched cells is that obtained with the preparation giving the highest percentage of Ig^+ cells.

Table I summarizes experiments that examined the specificity of the quin2 response. Preimmune Ig was ineffective. The Fab'_2 fragment gave virtually the same response as the original RaMIg, indicating that direct interaction with the F_c receptor is not necessary. Fab'-RaMIg was almost without effect, suggesting that receptor cross-linking is required. The residual response may have been due to traces of intact antibody. The effect of RaMIg seems specific for B-cells. The quin2 responses from unfractionated spleen cell preparation, and one enriched in B-cells directly related to the percentage of Ig + ve cells. A thymocyte preparation, lacking B lymphocytes showed no quin2 response to RaMIg.

We have pointed out previously (13) that if subpopulations of cells respond in different ways, then the interpretation of a quin2 signal becomes more complicated. If, however, one assumes that a given proportion of the cells are unaffected while the rest have a uniform $[Ca^{2+}]_i$ change, one can calculate that change from the overall quin2 signal. With the mixed spleen cells it seems reasonable to suppose that only B-cells (those binding RaMIg) respond to RaMIg. We shall further assume that all B-cells respond, that the basal $[Ca^{2+}]_i$ is similar in all the cells, and that responsive and nonresponsive cell types have the same average dye content. Errors in these

assumptions will affect the quantitative estimates of B-cell $[Ca^{2+}]_i$ but not the qualitative biological conclusions. Let x be the fraction of B cells, i.e. those staining Ig^+. Define $[Ca^{2+}]_{stim}$ and F_{stim} as the $[Ca^{2+}]_i$ and fractional Ca-saturation of the quin2 in those cells. Then,

$$F_{stim} = [Ca^{2+}]_{stim}/([Ca^{2+}]_{stim} + 115)$$

because quin2 binds Ca^{2+} with a 1:1 stoichiometry and an effective dissociation constant $K_d \approx 115$ nM in an ionic environment similar to that of cytoplasm (14). The fraction of nonresponsive cells is $(1 - x)$. Let F_{rest} be the fractional saturation of quin2 in those cells, assumed to be the same as all the cells had before stimulation. Then the composite observed Ca-saturation, F_{obs}, in the population exposed to RaMIg would be $xF_{stim} + (1 - x)F_{rest}$. Solving for $[Ca^{2+}]_{stim}$, we find

$$[Ca^{2+}]_{stim} = 115 \cdot \frac{F_{obs} - (1 - x)F_{rest}}{x - F_{obs} + (1 - x)F_{rest}}$$

With this procedure one estimates that in Fig. 1 the B-cell $[Ca^{2+}]_i$ rose from 115 nM to a peak of 470 nM.

Effects of Removing External Ca^{2+} and Varying the Dye Content

Fig. 3 shows that in Ca-free medium, RaMIg can still produce some transient $[Ca^{2+}]_i$ response, which is therefore attributed to triggered Ca^{2+} release from intracellular stores. Since this response is smaller and briefer than that seen with normal external Ca^{2+}, e.g. in Fig. 1, RaMIg evidently also produces an increase in the Ca^{2+} permeability of the plasma membrane.

A release of Ca^{2+} from internal stores is one condition in which the presence of added intracellular Ca^{2+} chelator is expected (14) to significantly modify the $[Ca^{2+}]_i$ response. Fig. 3 illustrates this, showing the effect of RaMIg in Ca-free medium on cells with two different quin2 loadings. In Fig. 3*A* the cells had 1 mM quin2 and the $[Ca^{2+}]_i$ in the B cells is calculated to have risen from 90 to 210 nM. With 3 mM quin2 in the cells in Fig. 3*B*, the change is only from 90 to 130 nM, showing the greater Ca buffering damping the $[Ca^{2+}]_i$ transient. From the quin2 content and the calculated $[Ca^{2+}]_i$ change in the B cells one can obtain the amount of Ca^{2+} buffered by the quin2. This gives a minimum value for the size of the releasable pool. Some will presumably be additionally taken up on native Ca binding sites. For both sets of cells shown in Fig. 3, the amount of Ca^{2+} initially buffered on quin2, after addition of RaMIg, is around 250 μmol per liter of cell water. It seems likely that in unloaded cells the internal release of this much Ca^{2+} would transiently raise the $[Ca^{2+}]_i$ to considerably higher levels. Indeed with quin2 loadings down to ~0.3 mM, the $[Ca^{2+}]_i$ appear to peak at over 1 μM though the signals are noisy and difficult to interpret quantitatively.

Even in the presence of 1 mM external Ca^{2+}, a $[Ca^{2+}]_i$ spike which looks like release from internal stores can sometimes be distinguished from a subsequent plateau which presumably reflects a new balance point between increased plasma membrane leak and pumping. Fig. 4*A* shows cells with a low quin2 loading, 0.7 mM. The initial transient after RaMIg corresponds to >800 nM $[Ca^{2+}]_i$ in the 58% B cells, but within 30 s this sags back to a nearly steady level around 300 nM. If the quin2 loading is increased to 3 mM (Fig. 4*B*), the two components of the signal are affected just as expected for one-shot release and sustained influx/efflux balance respectively: the initial jump is still rapid but much smaller, whereas the subsequent rise is slowed but not reduced in final amplitude.

Effects of A23187

With 1 mM external Ca^{2+}, 10–20 nM A23187 elevated splenocyte $[Ca^{2+}]_i$ to the micromolar range (experiments not shown), just as seen with thymocytes (13, 14). In Ca-free medium A23187 caused a transient rise in splenocyte $[Ca^{2+}]_i$ as shown for instance in Fig. 5*A*, contrasting with minimal effects

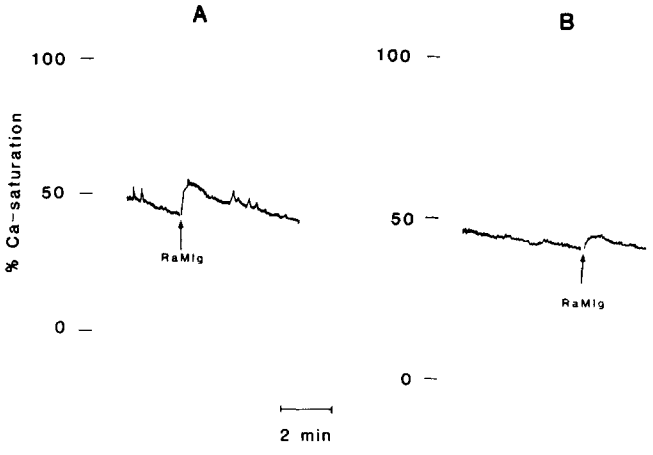

FIGURE 3 Responses of quin2 fluorescence to anti-Ig in Ca-free solution (zero added Ca, 200 μM EGTA) at different quin2 loadings. 40 μg/ml RaMIg added as indicated. Cells 50% Ig^+. (*A*) Quin2 content, 1 mM. (*B*) Quin2 content, 3 mM.

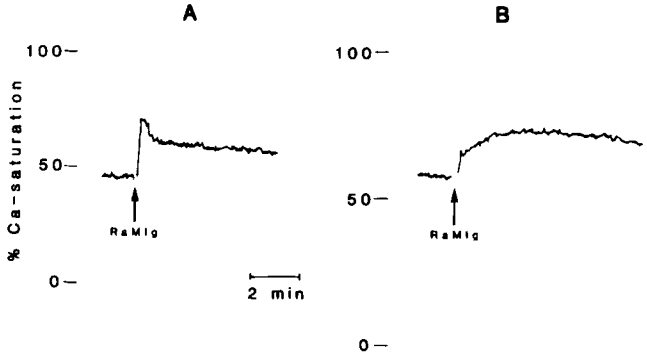

FIGURE 4 Responses of quin2 fluorescence to anti-Ig in 1 mM Ca solution at different quin2 loadings. 40 μg/ml RaMIg added as indicated. Cells 58% Ig^+. (*A*) Quin2 content, 0.7 mM. (*B*) Quin2 content, 3 mM.

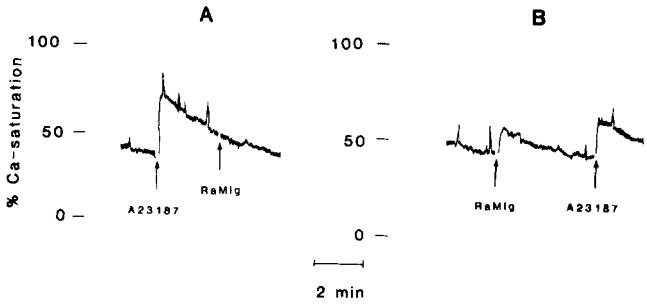

FIGURE 5 Effects of A23187 on $[Ca^{2+}]_i$ in Ca-free medium. Quin2 content, 1.4 mM, 44% Ig^+. Addition of 40 nM A23187 and 40 μg/ml RaMIg, as indicated. For unknown reasons the noise "glitches" were unusually severe in this experiment.

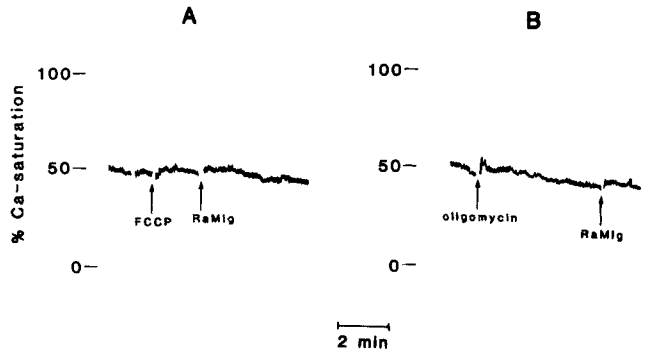

FIGURE 6 Effects of mitochondrial poisons. Quin2 content, 1 mM, 50% Ig$^+$. (*A*) 1 mM Ca solution. (*B*) Ca-free solution. 1 μM FCCP, 1 μg/ml oligomycin, and 40 μg/ml RaMIg added as indicated.

seen in thymocytes under these conditions. Since thymocytes and splenic T cells need not have the same behavior, we cannot tell if the internal stores revealed in Fig. 5*A* were only or even mainly in the B cells. If they were, the peak level of B-cell $[Ca^{2+}]_i$ would have been near 1 μM. It is generally taken that A23187 releases Ca^{2+} sequestered within organelles. In Fig. 5*A*, 40 μg/ml RaMIg, added after the $[Ca^{2+}]_i$ had returned to basal levels, was without effect. Presumably A23187 had emptied the RaMIg accessible Ca^{2+} stores and the Ca pump had extruded the internally released $[Ca^{2+}]_i$. Reversing the order of addition, as in Fig. 5*B*, shows that after recovery from the RaMIg induced $[Ca^{2+}]_i$ transient, in Ca-free medium, A23187 had a smaller effect. It is impossible to say from the present data whether this residual response to A23187 comes from additional stores in B cells or from a different cell type.

Effects of Mitochondrial Poisons

Mitochondrial poisons had little effect on basal $[Ca^{2+}]_i$ but blocked the responses to RaMIg. Fig. 6*A* shows that in 1 mM Ca^{2+} medium an "uncoupling" level of FCCP had little short-term effect on $[Ca^{2+}]_i$. This suggests that the mitochondria contain little Ca^{2+} and that the intracellular stores must be in other organelles. However, subsequent addition of RaMIg was without effect, indicating that the normal $[Ca^{2+}]_i$ responses require metabolic energy. Fig. 6*B* shows a recording in Ca-free medium where oligomycin blocked the RaMIg response. Interestingly however, the slow decline in signal, normally seen in Ca-free medium and attributed to Ca extrusion, was still present. Though FCCP releases mitochondrial Ca^{2+} while oligomycin does not, the two agents had similar effects to each other both with and without external Ca^{2+}, suggesting that their common mode of action was ATP depletion.

The Relationship between $[Ca^{2+}]_i$ and Capping

From data such as that in Fig. 1 it is clear that cap formation is not primarily responsible for the $[Ca^{2+}]_i$ rise. Table II summarizes experiments which show that the rise of $[Ca^{2+}]_i$ is not necessary for capping to occur since RaMIg induced normal capping in three conditions which greatly attenuated or abolished the $[Ca^{2+}]_i$ transient. First, cells loaded with relatively high quin2 and resuspended in Ca-free solution as in Fig. 3*B* were able to cap normally even though the $[Ca^{2+}]_i$ rise was greatly attenuated. Next, spleen cells were loaded with quin2 in Ca-free culture medium and resuspended in Ca-free saline. This treatment greatly lowered basal $[Ca^{2+}]_i$ as shown previously for thymocytes (14). Fig. 7 shows that one batch of such cells had an initial $[Ca^{2+}]_i$ below 30 nM and showed almost no $[Ca^{2+}]_i$ response to RaMIg; presumably the RaMIg-releasable Ca stores were largely depleted by the quin2. Nonetheless these cells, too, capped normally. Fig. 7 shows, incidentally, that subsequent addition of external Ca^{2+} allowed the $[Ca^{2+}]_i$ to gradually rise to the usual stimulated level. A third approach was to apply a low dose (40 nM) of A23187 in Ca-free medium. This treatment does not significantly depress ATP (10) but discharges the internal Ca^{2+}-stores (Fig. 5*A*). Subsequent RaMIg produces no $[Ca^{2+}]_i$ transient but causes normal capping.

The observed distribution of fluorescent ligand in the experiments had the appearance usually attributed to receptor capping. However, at 37°C, it is conceivable that receptor endocytosis occurring with a similar time course might be confused with capping and give rise to ambiguities in interpretation. Key experiments were therefore repeated at 20°C, when capping is slower, but endocytosis is largely abolished. Fig. 8 shows that the rate and extent of RaMIg capping in quin2-loaded cells was indistinguishable from that of controls. Additionally, cells loaded and resuspended in Ca-free medium had a $[Ca^{2+}]_i$ below 30 nM, showed only a tiny rise on addition of RaMIg similar to that in Fig. 7, yet again capped normally.

TABLE II

FITC-RaMIg Causes Capping Regardless of $[Ca^{2+}]_i$ Level

Conditions	Estimated peak $[Ca^{2+}]_i$	Capping	n
	nM	%	
Control cells in 1 mM external $[Ca^{2+}]$.	470	85	(6)
Cells with high quin2 content stimulated in Ca-free medium.	130	75	(2)
Cells loaded and stimulated in Ca-free solution.	30	82	(2)
Cells pretreated with A23187 in Ca-free solution before stimulation.	100	81	(2)

Capping was induced by the addition of 100 μg/ml FITC-RaMIg, and is expressed as a percentage of the Ig$^+$ cells. The number of experiments is denoted by *n*. Estimated values for peak $[Ca^{2+}]_i$ after anti-Ig stimulation were taken from parallel quin2 experiments with unlabeled RaMIg, as in Figs. 1, 3 *B*, 7, and 5 *A*, respectively.

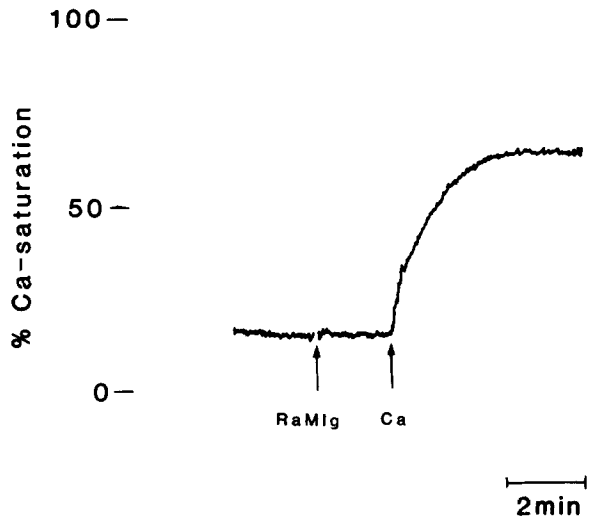

FIGURE 7 Effect of RaMIg in cells with lowered $[Ca^{2+}]_i$. Basal $[Ca^{2+}]_i$ was reduced by loading with quin2 in Ca-free saline containing 100 μM EGTA and resuspending the cells in Ca-free solution. Quin2 content, 1 mM, 53% Ig$^+$. 40 μg/ml RaMIg added as indicated.

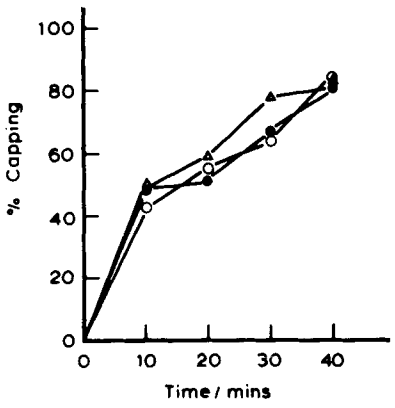

FIGURE 8 Capping at 20°C. Cells were equilibrated at this temperature for 30 min before addition of 100 μg/ml RaMIg. ▲; control, unloaded, cells. ○; cells loaded with quin2 to 1.2 mM, in normal Ca^{2+} medium. ●; cells loaded with quin2 to 1.1 mM, in Ca-free medium with 100 μM K_2EGTA. These cells were then resuspended in Ca-free solution and $[Ca^{2+}]_i$ was 23 nM.

DISCUSSION

A newly developed method for introducing a fluorescent Ca^{2+}-indicator into intact cells has allowed the first measurements of the effect of RaMIg on B-cell cytoplasmic $[Ca^{2+}]_i$. The use of quin2 to measure and to manipulate $[Ca^{2+}]_i$ was discussed in the previous paper (14), and this work with spleen cells highlights the strengths and also certain drawbacks of the technique. The results clearly show that RaMIg produces an elevation in $[Ca^{2+}]_i$ in B cells by both release from internal stores and influx from the external medium. Two factors, however, prevent accurate quantitation of the response. First the presence of sufficient quin2 to give an adequate signal can perturb the $[Ca^{2+}]_i$ transient in particular by partially buffering the internally released Ca^{2+} and blunting the initial transient. Second, the precise numerical estimate of the $[Ca^{2+}]_i$ in stimulated B cells relies on several unproven assumptions, e.g. that all the cell types in the preparation have the same basal $[Ca^{2+}]_i$, and that all the cells observed to bind RaMIg give the same $[Ca^{2+}]_i$ response. Nonetheless it seems likely that the B-cell $[Ca^{2+}]_i$ rises from ~0.1 μM to over 1 μM within seconds of exposure to RaMIg, at the concentration typically used, and remains around 0.5 μM for many minutes. Our data says little about how the RaMIg brings about the Ca^{2+} movements except that metabolic energy seems to be needed. It is possible that cross-linking of receptor-ligand complexes starts the process, and that the recovery of basal $[Ca^{2+}]_i$, in normal medium, is consequent upon receptor capping which has a roughly similar time course.

Braun et al. (1) found that RaMIg applied to mouse spleen lymphocytes reduced the cell-associated ^{45}Ca by 20 to 30% within the first 2 min. This loss could have been due to a primary acceleration of Ca efflux which would lower $[Ca^{2+}]_i$, or to internal release of Ca^{2+} raising $[Ca^{2+}]_i$ with subsequent Ca efflux. The authors offered the latter explanation, which the quin2 data supports. Our estimate for the RaMIg-releasable stores of 250 μmoles per liter cell water is ~25% of total cell Ca^{2+}. The similarity of this figure to the RaMIg induced ^{45}Ca loss suggests that the recovery to basal $[Ca^{2+}]_i$, seen for instance in Fig. 3, is achieved by Ca^{2+} extrusion rather than internal sequestration.

Our data argue against a causal role for Ca^{2+} in anti-Ig-induced capping since apparently normal capping occurred when the $[Ca^{2+}]_i$ rise was much reduced and even when $[Ca^{2+}]_i$ remained well below normal throughout. A simple scheme which seems to encompass the present evidence is shown in Fig. 9. Here, binding of anti-Ig activates independently, and in parallel, an influx of Ca^{2+}, Ca^{2+} release from

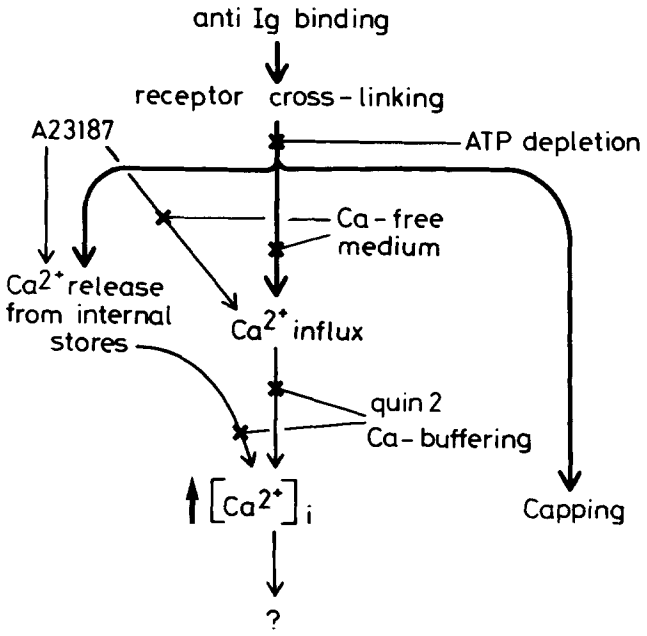

FIGURE 9 Parallel scheme for pathways of anti Ig-evoked $[Ca^{2+}]_i$ rise and capping. Crosses denote inhibition.

internal stores and capping. Under normal conditions we therefore expect the observed positive correlations between the $[Ca^{2+}]_i$ rise or increased Ca tracer loss (1) and capping. This correlation is also predicted by a sequential model in which anti-Ig binding causes first the $[Ca^{2+}]_i$ rise, which then causes the capping. But finding conditions that suppress $[Ca^{2+}]_i$ elevation without affecting capping appears to preclude the sequential model, while being quite compatible with the parallel scheme.

Effects of A23187 and certain local anesthetics and *cis*-unsaturated fatty acids have been interpreted in terms of supposed alterations of cell Ca^{2+}, and perhaps indicating a role for Ca^{2+} in capping. There are, however, other interpretations of these data. A23187, at much higher doses (>200 nM) than those used in the present study and with normal extracellular Ca^{2+}, inhibits capping, and disrupts formed caps (2). The inhibition may be partly, or even mainly, attributable to the ATP depletion associated with this treatment. However, although such data may indicate that high Ca^{2+} disorganizes the cytoskeletal rearrangements involved in capping (2) it hardly supports the role for Ca^{2+} in the capping itself. At concentrations that inhibit capping, both local anesthetics and *cis*-unsaturated fatty acids deplete cellular ATP sufficiently to explain most, or even all, of the inhibition (3, 5), while evidence of effects on lymphocyte Ca^{2+} is lacking.

The present findings raise a number of intriguing questions including the following: (*a*) What is the intracellular signal for capping, if not Ca^{2+}? Perhaps the cross-linking of surface Ig might constitute a sufficient stimulus to trigger the cytoskeletal rearrangements (7). (*b*) What is the message for internal Ca^{2+} release? Could this release be another direct consequence of receptor cross-linking, or is a soluble second messenger involved? (*c*) How does RaMIg promote Ca^{2+} influx? (*d*) Why is the $[Ca^{2+}]_i$ rise blocked by mitochondrial poisons? (*e*) Where is the releasable Ca^{2+}? All that can be said so far is that it seems not to be in mitochondria. (*f*) Perhaps most important of all, what function might the $[Ca^{2+}]_i$ rise have? The cell locomotion that follows RaMIg capping may well be Ca^{2+}-triggered (2).

Also perhaps $[Ca^{2+}]_i$ is an important regulator of B-cell differentiation and proliferation. Admittedly, unmodified RaMIg is not an effective mitogen on mouse spleen cells, possibly because capping occurs too rapidly (9). Anti-Ig is, however, mitogenic in rabbit spleen cells, and various treatments such as enzymic removal of the F_c fragment can make RaMIg mitogenic to mouse cells (8). Future investigation will need to be directed to such systems for assessment of the involvement of Ca^{2+} in effective mitogenic stimulation of B lymphocytes.

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