Activation of Ca^{2+}-dependent K^+ Channels in Human B Lymphocytes by Anti-Immunoglobulin

Stephen L. MacDougall, Sergio Grinstein, and Erwin W. Gelfand
Division of Immunology and Rheumatology, Research Institute, Hospital for Sick Children, Toronto, Ontario, Canada, M5G 1X8; and Department of Biochemistry, University of Toronto, Ontario, Canada M5G 1X8

Abstract

Many mammalian cell types exhibit Ca^{2+}-dependent K^+ channels, and activation of these channels by increasing intracellular calcium generally leads to a hyperpolarization of the plasma membrane. Their presence in B lymphocytes is as yet uncertain. Crosslinking Ig on the surface of B lymphocytes is known to increase the level of free cytoplasmic calcium ($[Ca^{2+}]_i$). However, rather than hyperpolarization, a depolarization has been reported to occur after treatment of B lymphocytes with anti-Ig. To determine if Ca^{2+}-dependent K^+ channels are present in B lymphocytes, and to examine the relationship between intracellular free calcium and membrane potential, we monitored $[Ca^{2+}]_i$ by means of indo-1 and transmembrane potential using *bis*(1,3-diethylthiobarbituric)trimethine oxonol in human tonsillar B cells activated by anti-IgM. Treatment with anti-IgM induced a biphasic increase in $[Ca^{2+}]_i$ and a simultaneous hyperpolarization. A similar hyperpolarization was induced by ionomycin, a Ca^{2+} ionophore. Delaying the development of the $[Ca^{2+}]_i$ response by increasing the cytoplasmic Ca^{2+}-buffering power delayed the hyperpolarization. Conversely, eliminating the sustained phase of the $[Ca^{2+}]_i$ response by omission of external Ca^{2+} abolished the prolonged hyperpolarization. In fact, a sizable Na^+-dependent depolarization was unmasked. This study demonstrates that in human B lymphocytes, Ca^{2+}-dependent K^+ channels can be activated by crosslinking of surface IgM. Moreover, it is likely that, by analogy with voltage-sensitive Ca^{2+} channels, Na^+ can permeate through these ligand-gated Ca^{2+} "channels" in the absence of extracellular Ca^{2+}.

Introduction

Binding of foreign antigens to resting B lymphocytes activates and induces these cells to proliferate, producing an expanded and functionally responsive population. It is well established that the initial steps of this activation process can be mimicked in vitro by crosslinking surface Ig (sIg),[1] which function as antigen receptors on the membrane of B lymphocytes (1). This can be achieved using antibodies directed against sIg.

The biochemical events underlying activation of B lymphocytes by antigens or by anti-Ig are not completely understood. One of the earliest responses associated with crosslinking of sIg is an increase in the level of cytoplasmic free calcium ($[Ca^{2+}]_i$). This was initially inferred from measurements of $^{45}Ca^{2+}$ distribution (2) and later confirmed by direct determinations of $[Ca^{2+}]_i$ using fluorescent probes such as quin2 (3). At least part of the Ca^{2+} that appears in the cytoplasm originates from intracellular stores, inasmuch as anti-sIg can elevate $[Ca^{2+}]_i$ in cells suspended in Ca^{2+}-free media containing EGTA (3, 4). The source of intracellular Ca^{2+} is believed to be the endoplasmic reticulum, which has been shown to release Ca^{2+} when challenged with inositol 1,4,5-trisphosphate, one of the products of the hydrolysis of phosphatidylinositol 4,5-biphosphate by phospholipase C (5, 6). The latter enzyme is known to be activated in B lymphocytes when sIg is crosslinked (6–8).

Most cell types analyzed thus far, including some populations of lymphocytes, display K^+ channel activity in their plasma membranes (9–11). One particular type of K^+ channel is of potential importance to the activation process, since its activity is modulated by the cytoplasmic concentration of Ca^{2+} (see references 12 and 13 for reviews). The open state of the channel becomes predominant as $[Ca^{2+}]_i$ increases above the resting level, bringing the membrane potential (E_m) closer to the K^+ equilibrium potential (E_K). Because the resting potential of lymphocytes approximates 50–60 mV (negative inside) and their intracellular K^+ concentration has been reported to be 130–140 mM, opening of these K^+ channels in cells suspended in normal media (containing 3–5 mM K^+) is therefore expected to bring about a hyperpolarization.

Contrary to this expectation, a sizable depolarization has been reported to follow activation of B lymphocytes with anti-sIg (14, 15). The ionic mechanism underlying this change in potential has not been defined. This depolarization was detected by means of flow cytometry using cyanine dyes (15, 16). Due to limitations inherent to this technique, the measurements were performed at least 10 min after addition of the anti-sIg, and depolarization progressed over a 60–120-min period (15). No information is available regarding the magnitude of the E_m changes at earlier times. In contrast to the reported E_m changes, the increased $[Ca^{2+}]_i$ observed in response to crosslinking of sIg is evident within seconds (2–4, 17). Thus, it is conceivable that the rapid elevation in $[Ca^{2+}]_i$ is accompanied by opening of K^+ channels and hyperpolarization, which may then be superseded by a slower depolarization.

The aim of the present experiments was to perform continuous measurements of the E_m of B lymphocytes immediately after stimulation with anti-sIgM, to determine whether Ca^{2+}-gated K^+ channels are activated in human B lymphocytes in response to sIgM crosslinking. For this purpose we performed spectroscopic measurements using the potential-sensitive dye

Dr. Gelfand's present address is National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson St., Denver, CO 80206. Address correspondence to Dr. Gelfand at this address.

Received for publication 11 March 1987 and in revised form 7 July 1987.

1. *Abbreviations used in this paper:* AM acetoxymethylester; BAPTA, 1,2-*bis*-(2-aminophenoxy)ethane-*N,N,N',N'* tetraacetic acid; *bis*-oxonol, *bis*(1,3-diethylthiobarbituric)trimethine oxonol; $[Ca^{2+}]_i$, free cytoplasmic calcium; E_K, K^+ equilibrium potential; E_m, membrane potential; NMG, *N*-methyl-D-glucamine; NMG^+, *N*-methyl-D-glucammonium$^+$ ion; sIg, surface Ig.

J. Clin. Invest.

0021-9738/88/02/0449/06 $2.00
Volume 81, February 1988, 449–454

bis(1,3-diethylthiobarbituric)trimethine oxonol (*bis*-oxonol), a negatively charged lipid-soluble fluorophore. Unlike the cyanine dyes, which are positively charged, *bis*-oxonol does not accumulate in the mitochondrial matrix, and reports primarily the potential across the plasma membrane (16, 18). Our results indicate that a Ca^{2+}-induced hyperpolarization is indeed observed shortly after crosslinking of sIg. Moreover, we demonstrate that a comparatively large depolarization is unmasked when anti-sIgM is added to B lymphocytes suspended in Ca^{2+}-free medium. This depolarization is shown to be mediated by influx of Na^+, conceivably through the ligand-activated Ca^{2+} channels.

Methods

Reagents. *Bis*-oxonol, and the acetoxymethylester (AM) forms of indo-1 and 1,2-*bis*-(2-aminophenoxy)ethane-*N*,*N*,*N'*,*N'* tetraacetic acid (BAPTA) were obtained from Molecular Probes, Junction City, OR. Goat anti-human IgM, $F(ab')_2$ fragments of goat anti-human IgM and anti-β_2-microglobulin were from Atlantic Antibodies (Scarborough, ME). EGTA and gramicidin were purchased from the Sigma Chemical Co. (St. Louis, MO). Ionomycin was from Calbiochem-Behring Corp. (San Diego, CA).

Solutions. RPMI 1640 was obtained from Gibco (Grand Island, NY). Normal phosphate-buffered saline was prepared as described (19). Stock solutions of *bis*-oxonol, indo-1 AM, BAPTA AM, gramicidin, and ionomycin were made up in dimethylsulfoxide. EGTA and $CaCl_2$ stocks were made up in aqueous solutions. For E_m and intracellular calcium measurements, the buffers contained (millimoles): 140 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 3 KCl, 10 glucose, and 10 Hepes (pH 7.3). Where indicated, NaCl was isoosmotically replaced with either KCl or *N*-methyl-D-glucamine (NMG) chloride. Calcium-free buffers were prepared without the addition of 1 mM $CaCl_2$, and with 0.5 mM EGTA. All buffers were adjusted to pH 7.3±0.05 and 290±5 mosmol/liter.

Cell isolation. Fresh human tonsil tissue was obtained postoperatively. The mononuclear cell fraction obtained after Ficoll-Hypaque gradient centrifugation was enriched for B lymphocytes by rosette-depleting contaminating T lymphocytes with neuraminidase-treated sheep erythrocytes (19). Greater than 90% of the cells obtained in this fashion were B lymphocytes.

Cell loading with indo-1 AM and BAPTA AM. Cells (40×10^6 in 1 ml Hepes-buffered RPMI) were incubated with 1 μM indo-1 AM, or with 1 μM indo-1 AM plus 10 μM BAPTA AM. After 30 min at 37°C, the cells were washed once in phosphate-buffered saline, once in NaCl buffer, and resuspended at 20×10^6/ml in NaCl buffer.

E_m and cytoplasmic calcium determination. E_m was measured using *bis*-oxonol and a Perkin-Elmer 650-40 spectrofluorimeter set with excitation and emission wavelengths of 540 nm (3-nm slit) and 580 nm (10-nm slit), respectively. Dye, to a final concentration of 0.3 μM, was added to cuvettes containing 2 ml of the appropriate buffer and 2×10^6 cells and the suspension was stirred continuously at 37°C until a steady fluorescence was obtained (~ 10 min). Additions were then made, and recording continued. Calibration was made by adding gramicidin (25 nM, final concentration) to cells suspended in isotonic medium containing various ratios of Na^+ and *N*-methyl-D-glucammonium$^+$ ion (NMG^+) (which does not permeate through gramicidin). E_m was calculated assuming that Na^+ and K^+ permeation through gramicidin is comparable (20).

Cytoplasmic calcium was measured with indo-1 using excitation and emission wavelengths of 331 nm (3-nm slit) and 410 nm (10-nm slit), respectively. Calibration of indo-1 fluorescence versus $[Ca^{2+}]_i$ was performed using ionomycin and Mn^{2+}, a divalent cation that permeates through ionomycin and quenches the fluorescence of the dye. $[Ca^{2+}]_i$ for a given fluorescence F was calculated as follows: $[Ca^{2+}]_i = 250 \text{ nM } (F - F_{min})/(F_{max} - F)$, where 250 nM is the reported dissociation constant for the indo-1/calcium complex (21), and F_{max} is the fluorescence obtained in Ca^{2+}-containing media in the presence of 1 μM ionomycin, a concentration producing maximal effects on the fluorescence of indo-1. F_{min} is calculated as: $F_{min} = 1/12(F_{max} - F_{auto}) + F_{auto}$, where F_{auto} is the autofluorescence, determined after addition of 2 mM $MnCl_2$ to cells pretreated with ionomycin, and 12 is the ratio of the fluorescence intensity of the indo-1/calcium complex over the fluorescence of the free acid of indo-1, at the wavelengths used.

Routinely, the cells were stimulated with 37 μg of goat anti-human IgM. In some experiments $F(ab')_2$ fragments of goat anti-human IgM were used, yielding essentially identical results.

All the experiments were performed at 37°C. Representative traces of at least three similar experiments are illustrated. Unless otherwise indicated, results are expressed as the mean±SE of the number of determinations indicated.

Results

Fig. 1 shows parallel measurements of $[Ca^{2+}]_i$ and E_m in human B lymphocytes treated with antibodies against sIgM. When measured with indo-1, the resting $[Ca^{2+}]_i$ of human tonsillar B lymphocytes averaged 176.2±5.8 nM (n = 4). As reported for rodent lymphocytes (3, 4), addition of anti-Ig to human B cells induced a rapid increase in $[Ca^{2+}]_i$. As shown in Fig. 1 *A*, the response measured with indo-1 is biphasic. This change in $[Ca^{2+}]_i$ was accompanied by a distinct hyperpolarization (Fig. 1 *D*). The resting potential, which averaged −43.6±2.7 mV (n = 5), increased transiently by 6–10 mV and then partially returned towards the resting potential (Fig. 1 *D*). This potential change was reproducibly recorded in over 20 experiments. The shape of the hyperpolarizing wave closely resembles the time course of the increase in $[Ca^{2+}]_i$, suggesting a causal relationship.

The specificity of the effect of anti-IgM was tested using a second antibody, known to bind to surface β_2-microglobulin. As illustrated in Fig. 1 *B*, no changes in $[Ca^{2+}]_i$ were detected when the cells were treated with comparable or even higher concentrations of antibody against β_2-microglobulin. Similarly, no hyperpolarization was detected when this antibody was added to cells equilibrated with *bis*-oxonol (Fig. 1 *E*). The marginal increase in fluorescence (apparent depolarization) observed is due to binding of the probe to hydrophobic domains on the antibody and was observed also in the absence of cells (not illustrated).

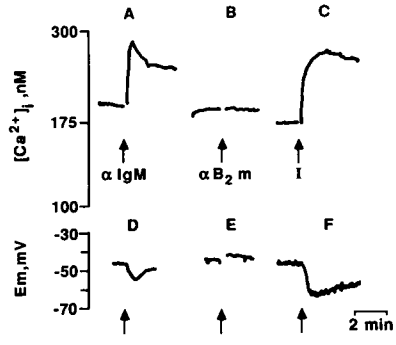
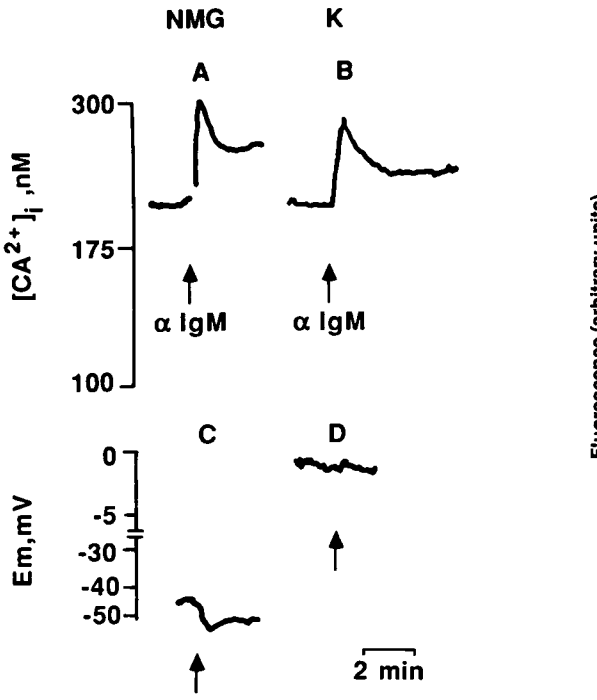


Figure 1. Effects of anti-IgM, anti-β_2-microglobulin, and ionomycin on $[Ca^{2+}]_i$ and E_m. B lymphocytes were loaded with indo-1 as described in Methods. Cells (2×10^6) were suspended in a cuvette containing 2 ml Hepes-buffered Na^+ solution. *A–C:* $[Ca^{2+}]_i$ was determined fluorimetrically using a Perkin-Elmer 650-40 spectrometer. *D–F:* E_m was assessed fluorimetrically using *bis*-oxonol (0.3 μM) in parallel cell suspensions. 37 μg of goat anti-human IgM (*A* and *D*) or goat anti-human β_2-microglobulin (*B* and *E*) were added at the times indicated by the arrows. In *C* and *F*, 25 nM ionomycin was added where indicated.



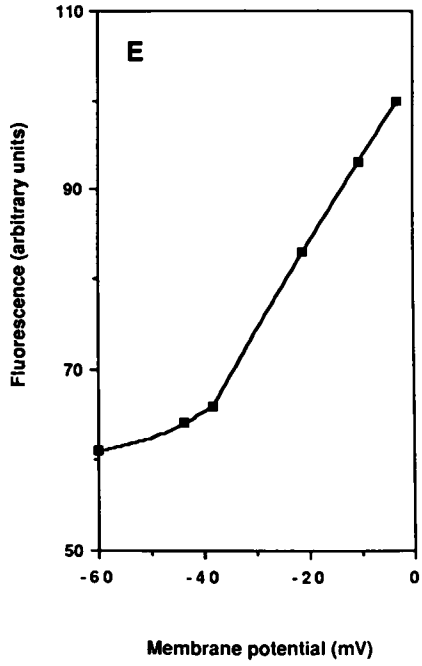


Figure 2. Effects of extracellular ion substitution on anti-IgM–induced changes in $[Ca^{2+}]_i$ and E_m. B lymphocytes were loaded with indo-1, washed and resuspended in either Hepes-buffered NMG^+ solution (*A* and *C*) or Hepes-buffered K^+ solution (*B* and *D*). *A* and *B:* $[Ca^{2+}]_i$ determinations using indo-1. *C* and *D:* determinations using *bis*-oxonol. Anti-IgM (37 μg) was added to the cell suspension at the times indicated by the arrows. E: E_m dependence of *bis*-oxonol fluorescence. A typical calibration curve, obtained by addition of 25 nM gramicidin to cells suspended in isotonic media containing varying ratios of Na^+ and NMG^+, is illustrated. E_ms were calculated from the Nernst equation as described in Methods.

That the change in potential recorded in cells treated with anti-IgM is secondary to the elevation of $[Ca^{2+}]_i$ is further indicated by the experiments in Fig. 1, *C* and *F*. Increasing $[Ca^{2+}]_i$ by addition of nanomolar concentrations of the electroneutral nonfluorescent ionophore ionomycin was accompanied by a marked hyperpolarization.[2]

If the ligand-activated Ca^{2+} pathway were conductive, a depolarization should be associated with Ca^{2+} uptake upon crosslinking of sIgM. Therefore, it must be postulated that the observed hyperpolarization is secondary to the entry of Ca^{2+}, and likely associated with Ca^{2+}-induced changes in the permeability to other ions. A hyperpolarization could be attained by increasing the permeability to K^+, whose equilibrium potential is more negative than E_m. Conversely, a reduction in the permeability to those ions with equilibrium potentials above E_m (e.g., Na^+) could also induce a hyperpolarization. This hypothesis was tested by analyzing the response of B lymphocytes to anti-IgM in media of varying cation composition (Fig. 2). Replacement of extracellular Na^+ by the impermeant organic cation NMG^+ had no significant effect on the resting $[Ca^{2+}]_i$ (184.7±6.3 nM; n = 3) of human B lymphocytes (Fig. 2 *A*), suggesting that, if present, Na^+/Ca^{2+} exchange does not play a significant role in $[Ca^{2+}]_i$ homeostasis in these cells. Similarly, the E_m was affected very little by removal of extracellular Na^+ (−47.5±3.3 mV; n = 4) (Fig. 2 *C*), indicating that the transference number of this ion is small. Stimulation of the cells with anti-IgM in Na^+-free NMG^+ medium produced an increase in $[Ca^{2+}]_i$ which was at least as large, and frequently even larger than that recorded in Na^+ medium (Fig. 2 *A*). Concomitantly, the E_m underwent a clear hyperpolarization (Fig. 2 *C*), as described for Na^+-containing media. Though comparatively small, this potential change was reproducibly recorded in five experiments. These results indicate that extracellular Na^+ is not involved in the ligand-induced Ca^{2+} uptake mechanism, and that a decrease in Na^+ conductance cannot account for the hyperpolarization. Under these conditions, a depolarization would be expected from a reduction in Na^+ conductance, since the Na^+ gradient is very large and outward; i.e., the equilibrium potential for Na^+, E_{Na} is more negative than E_m.

When extracellular Na^+ was replaced by K^+, little change in resting $[Ca^{2+}]_i$ was detected (Fig. 2 *B*). Because under these conditions the cells are depolarized (Fig. 2 *D*, see below), this implies that voltage-gated Ca^{2+} channels, if present, do not contribute significantly to $[Ca^{2+}]_i$ homeostasis. A biphasic response to anti-IgM was recorded under these conditions (Fig. 2 *B*), even though the sustained phase frequently stabilized at a level below that reported in Na^+-containing media. As expected for a membrane that is predominantly permeable to K^+, elevating extracellular K^+ to levels approximating $[K^+]_i$ (and thereby making $E_K \approx 0$) resulted in a marked depolarization (Fig. 2 *D*). Under these conditions the resting E_m averaged −3.7±1.8 mV and addition of anti-IgM produced little change in potential (Fig. 2 *D*), or occasionally a slight further depolarization. Failure to detect an anti-IgM–induced change in potential in K^+-rich medium was not due to reduced sensitivity of the probe in depolarized cells. This is indicated by the calibration curve illustrated in Fig. 2 *E*, obtained using gramicidin in cells suspended in media of varying monovalent cation composition (see Methods). The data show a linear relationship between fluorescence and E_m in the range of −35 to 0 mV, with a slope that is, in fact, steeper than that at more negative potentials. These results are consistent with the notion that binding of anti-IgM to surface IgM affects E_m due to a Ca^{2+}-induced increase in K^+ conductance.

2. The hyperpolarization induced by ionomycin was not always sustained. Frequently, a rapid transient hyperpolarization was observed, which partially subsided over the course of 5–10 min. This variability may reflect differences in the ability of individual cell batches to regulate $[Ca^{2+}]_i$ after challenge with the ionophore.

If a relationship indeed exists between the hyperpolarization and the increased $[Ca^{2+}]_i$, we reasoned that modifications in the shape of the Ca^{2+} response should be reflected in alterations of the E_m change. The biphasic nature of the $[Ca^{2+}]_i$ change induced by anti-IgM suggests that it may be a composite of Ca^{2+} released from internal stores and entry of Ca^{2+} from the external medium, as has been shown for other systems, including B lymphocytes (3, 4). We sought to dissociate these components by either omitting extracellular Ca^{2+}, or minimizing the effect of the intracellular stores on $[Ca^{2+}]_i$ by increasing the Ca^{2+}-buffering power of the cytoplasm. The results of the first approach are illustrated in Fig. 3. Omission of extracellular Ca^{2+} (plus inclusion of 0.5 mM EGTA to remove contaminating Ca^{2+}) drastically affected the shape of the $[Ca^{2+}]_i$ response to anti-IgM. Only a sharp, transient elevation of $[Ca^{2+}]_i$ was recorded, which returned to baseline within 2 min (Fig. 3 *A*). A similar transient $[Ca^{2+}]_i$ elevation was recorded when extracellular Na^+ was replaced by NMG^+ (Fig. 3 *B*). The resting E_m was not significantly affected by removal of extracellular Ca^{2+} and the attendant decrease in resting $[Ca^{2+}]_i$ (Fig. 3 *C*). In contrast, the profile of the anti-IgM response on E_m was clearly modified. Consistent with the opening of Ca^{2+}-sensitive K^+ channels, the transient elevation in $[Ca^{2+}]_i$ was accompanied by a transient hyperpolarization. However, this was followed by a marked depolarization of nearly 20 mV (Fig. 3 *C*). This depolarization was apparently due to influx of Na^+, since it was reproducibly eliminated when external Na^+ was replaced by the impermeant NMG^+ (Fig. 3 *D*). Under these conditions only a transient hyperpolarization was observed, which returned to baseline potential with a time course resembling that of the $[Ca^{2+}]_i$ transient (refer to Fig. 2, *B* and *D*). These data are consistent with the presence of Ca^{2+}-activated K^+ channels, but in addition demonstrate the existence of a ligand-induced Na^+ current unmasked by omission of extracellular Ca^{2+}. By analogy with voltage-gated Ca^{2+} channels in excitable cells (22), this current could represent Na^+ permeation through the ligand-activated Ca^{2+} channels (see Discussion).

Because the amount of Ca^{2+} released from the intracellular reservoirs by anti-IgM is finite, the magnitude of the early phase of $[Ca^{2+}]_i$ change should be inversely proportional to the cytoplasmic Ca^{2+}-buffering power. Thus, introducing into the cytoplasm increasing amounts of Ca^{2+} chelator is expected to decrease and eventually obliterate the $[Ca^{2+}]_i$ transient. To test the effect of eliminating the early phase of $[Ca^{2+}]_i$ increase on the E_m, we artificially increased the Ca^{2+}-buffering power of

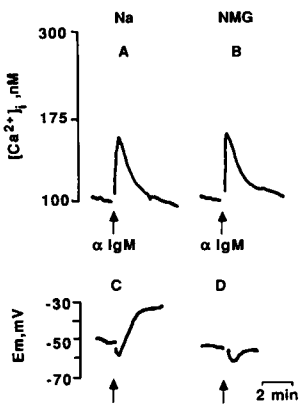


Figure 3. Anti-IgM–induced changes in $[Ca^{2+}]_i$ and E_m in calcium-free medium. B lymphocytes were loaded with indo-1 and resuspended in either calcium-free Hepes-buffered Na^+ solution containing 0.5 mM EGTA (*A* and *C*) or calcium-free Hepes-buffered NMG^+ solution containing 0.5 mM EGTA (*B* and *D*). $[Ca^{2+}]_i$ (*A* and *B*) and E_m (*C* and *D*) were monitored as described for Fig. 1. Anti-IgM (37 μg) was added to the cell suspension at the times indicated by the arrows.

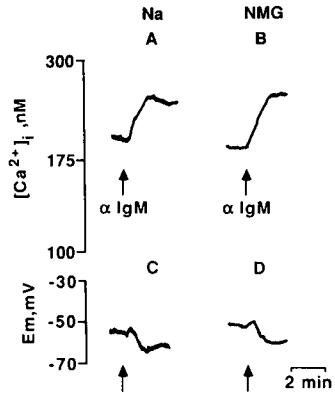


Figure 4. Anti-IgM–induced changes in $[Ca^{2+}]_i$ and E_m in cells loaded with indo-1 and BAPTA. B cells were loaded with indo-1 and BAPTA as described in Methods. Cells were washed and resuspended in either Hepes-buffered Na^+ solution (*A* and *C*) or Hepes-buffered NMG^+ solution (*B* and *D*). $[Ca^{2+}]_i$ (*A* and *B*) and E_m (*C* and *D*) were monitored as described above. Anti-IgM (37 μg) was added at the times indicated by the arrows.

the cytoplasm of B lymphocytes. This was accomplished by preloading the cells with BAPTA, a nonfluorescent chelator that can be generated in situ in the cytoplasm by incubation of intact cells with its precursor tetraacetoxymethylester. As shown in Fig. 4 *A*, the resting $[Ca^{2+}]_i$ was not significantly affected by this pretreatment, but the changes induced by anti-IgM were markedly slower and the biphasic nature of the change was virtually eliminated. A relatively monophasic increase was observed, reaching a level that was not significantly different from that recorded in cells without BAPTA (248.2±8.2 nM vs. 249.8±15.1 nM, respectively). A similar monophasic increase was recorded in Na^+-free NMG^+ medium (Fig. 4 *B*). In parallel with the elimination of the rapid $[Ca^{2+}]_i$ transient, the early hyperpolarizing phase of the E_m change was also eliminated. Instead, upon addition of anti-IgM a small but reproducible depolarization was recorded (Fig. 4 *C*). This depolarization was not likely due to Na^+ influx, as it was also noted in NMG^+ medium (Fig. 4 *D*). The delayed increase in $[Ca^{2+}]_i$ was, however, accompanied by the expected hyperpolarization. Taken together, these results indicate that the hyperpolarization associated with stimulation of the antigen receptor is due to Ca^{2+} activation of K^+ channels.

A corollary of this hypothesis is that prevention of both phases of the $[Ca^{2+}]_i$ change should entirely preclude hyperpolarization. This can be achieved by combining the two approaches used above, i.e., by suspending BAPTA-loaded cells in Ca^{2+}-free media. The results of such experiments are illustrated in Fig. 5. Under these conditions, activation of the cells with anti-IgM induced only a marginal increase in $[Ca^{2+}]_i$: from an initial level of ~ 90 nM to ≤ 100 nM. Similar results were obtained in the presence and absence of extracellular Na^+ (Fig. 5, *A* and *B*). In Ca^{2+}-free, Na^+-containing medium the activation of BAPTA-loaded cells by anti-IgM was not accom-

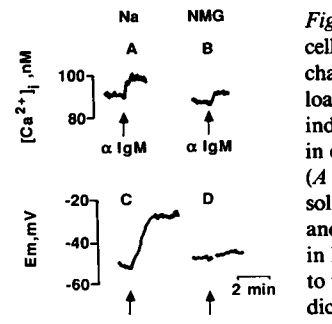


Figure 5. Effects of removal of extracellular calcium on anti-IgM–induced changes in $[Ca^{2+}]_i$ and E_m in BAPTA-loaded cells. B cells were loaded with indo-1 and BAPTA, and resuspended in either Hepes-buffered Na^+ solution (*A* and *C*) or Hepes-buffered NMG^+ solution (*B* and *D*). $[Ca^{2+}]_i$ (*A* and *B*) and E_m (*C* and *D*) were measured as in Fig. 1. Anti-IgM (37 μg) was added to the cell suspension at the times indicated by the arrows.

panied by a hyperpolarization. Instead only a large depolarization (Fig. 5 *C*), similar to the second phase reported in Fig. 3 *C*, was recorded. This depolarization was largely eliminated in NMG^+ medium, supporting the interpretation that it is mediated by Na^+ influx through ligand-activated Ca^{2+} channels.

Discussion

In this manuscript we provide evidence for the presence of a Ca^{2+}-activated K^+ conductance in the plasma membrane of human B lymphocytes. A comparable system had been described for human (23, 24) and rodent (25) T lymphocytes, but no evidence existed in the case of B lymphocytes. In fact, a recent report comparing the properties of murine B and T lymphocytes concluded that Ca^{2+}-stimulated K^+ channels exist in the latter but not the former (23). Failure to detect a Ca^{2+}-induced hyperpolarization in B cells, however, does not necessarily imply that the Ca^{2+}-sensitive K^+ channels are absent. A similar result would be observed if E_m were close to E_K, or if the Ca^{2+}-induced conductance were a small fraction of the total conductance. In addition to possible species specific properties, the discrepancy between our results and those of Wilson and Chused (23) could reflect methodological differences. Whereas continuous recording with a spectrofluorimeter was used in the present study, Wilson and Chused (23) utilized flow cytometry for their studies. This method provides discontinuous recordings of individual cells, which are by necessity averaged over the period of time required for accumulation of the desired number of cells (50,000 in the case referred to in reference 23). Moreover, due to the operational properties of the flow cytometer, a delay of variable magnitude is incurred between addition of reagents to the cells and the start of the recording. For these reasons, a transient change in E_m would be difficult to detect.

Interaction of anti-IgM with the surface membrane of human B lymphocytes increased $[Ca^{2+}]_i$ and concomitantly made the membrane potential more negative (Fig. 1). Similar findings were recently reported in the WEHI-231 B lymphoma cell line, where stimulation of the antigen receptor was found to change both $[Ca^{2+}]_i$ and the E_m (26). The hyperpolarization detected in the present study is likely due to an increase in K^+ conductance since it was eliminated and even reversed by increasing the concentration of K^+ in the suspending media (Fig. 2 *D*), but was unaffected by alterations in the equilibrium potential for Na^+ (Fig. 2 *C*) or Cl^- (not illustrated), the other major ions. Though the contribution of changes in the conductance to minor ions such as H^+ or HCO_3^- cannot be ruled out, the data can be readily explained by an elevation in K^+ conductance. Several lines of evidence indicate that K^+ conductance increases in response to a change in $[Ca^{2+}]_i$: (*a*) the shape of the E_m change resembles the time course of the $[Ca^{2+}]_i$ change (Fig. 1); (*b*) the sustained phase of the hyperpolarization is abolished by removal of extracellular Ca^{2+}, which leaves the transient $[Ca^{2+}]_i$ unaffected but precludes the sustained $[Ca^{2+}]_i$ increase (Figs. 3 and 5); (*c*) the rapid phase of the hyperpolarization is absent when the rate of the $[Ca^{2+}]_i$ increase is slowed by incorporation of BAPTA, a Ca^{2+}-buffering agent, into the cells (Fig. 4); and (*d*) the hyperpolarization is completely eliminated when both components of the $[Ca^{2+}]_i$ change are blocked (Fig. 5).

In apparent disagreement with our results, Cambier and his collaborators have reported that murine B cells depolarize in response to antibodies that crosslink sIg (14). However, it must be borne in mind that these studies utilized cyanine dyes and flow cytometry for the detection of E_m, and that measurements were started at least 10 min after addition of the anti-Ig to the cells. It is conceivable that a rapid hyperpolarization occurred that was followed by a slower depolarizing process. Alternatively, it is possible that the apparent change in plasma E_m was instead the manifestation of metabolic changes affecting the mitochondrial E_m. By virtue of their positive charge, cyanines accumulate in the mitochondrial matrix in response to the potential across the inner mitochondrial membrane, a problem that is not encountered in the case of *bis*-oxonol which is negatively charged and therefore excluded from mitochondria. Validation of the late depolarization must therefore await the use of alternative E_m probes.

Unlike the case of voltage-sensitive Ca^{2+} channels in excitable tissues, very little is known concerning the properties of the ligand-gated channels of lymphocytes. A possible similarity between these two systems was revealed by the experiments in Fig. 3, where extracellular Ca^{2+} was omitted. Under these conditions, $[Ca^{2+}]_i$ increased only transiently as a result of release of Ca^{2+} from intracellular stores. Concomitantly, a transient hyperpolarization was observed. However, this was followed by a marked depolarization that was dependent on the presence of extracellular Na^+. These observations are consistent with permeation of Na^+ through the anti-IgM activated "Ca^{2+} channel." Indeed, a sizable monovalent cation conductance through voltage-gated Ca^{2+} channels is known to appear when extracellular Ca^{2+} is removed (22, 27). This has been attributed to a change in the ion selectivity of the channel due to removal of Ca^{2+} from a binding site that is distinct from the transport moiety, since the concentrations for half-maximal saturation of transport and for control of selectivity differ markedly (28, 29). Using macroscopic potential recording techniques it is presently impossible to define whether in B lymphocytes Na^+ uptake is indeed occurring through a Ca^{2+} channel of altered selectivity or through a parallel and independent pathway. However, the finding that the Na^+-dependent depolarization in Ca^{2+}-free medium is sensitive to the Ca^{2+} channel blocker verapamil (100–200 μM, unpublished observations) suggests that a single type of channel is involved. Moreover, electrophysiological recordings in a hybridoma cell line showed that a constitutive Ca^{2+} channel could also become permeant to monovalent cations when Ca^{2+} was absent (29). Thus, the phenomenon may be more widespread than originally recognized, and applicable to nonexcitable cells as well.

The large depolarization observed in cells suspended in Ca^{2+}-free medium clearly differs from that recorded in BAPTA-loaded cells in Ca^{2+}-containing media. Not only is the latter substantially smaller, but it is also Na^+ independent. Moreover, this depolarization is also independent of the Cl^- gradient (not shown), but is instead abolished by removal of external Ca^{2+}. This can only be visualized in cells suspended in NMG^+, to prevent the large Na^+-mediated depolarization (Fig. 5 *D*). Thus, the depolarization in BAPTA-loaded cells could reflect entry of Ca^{2+} through the ligand-activated pathway. This depolarization may not be visible in cells without BAPTA for two reasons: first, the rapid increase in $[Ca^{2+}]_i$ would open the K^+ channels, inducing an offsetting hyperpolarization; second, the total amount of Ca^{2+} entering the cells is lower in untreated than in BAPTA-loaded cells (unpublished observa-

tions). This implies that a larger, and more readily detectable Ca^{2+} current is induced by anti-IgM in cells with elevated Ca^{2+} buffering.

In summary, we have demonstrated for the first time the presence of Ca^{2+}-sensitive K^+ channels in human B lymphocytes, and their activation by crosslinking of sIg with antibodies. The K^+ channels can be activated by Ca^{2+} released from intracellular reservoirs, as well as by Ca^{2+} entering the cell from the medium. Moreover, we have provided evidence for an anti-IgM–induced Na^+ current detectable only in the absence of external Ca^{2+}. This current could be mediated by Ca^{2+} channels of reduced selectivity. This may provide a convenient method for the study of ligand-induced channels in lymphocytes. The significance of these E_m changes in the processes of B cell activation and proliferation remains to be elucidated.

Acknowledgments

The authors wish to thank Roy Cheung, Gordon Mills, Chaim Roifman, Jeff Dixon, and Bill Breuer for their advice and encouragement.

This work was supported by the Medical Research Council of Canada and the National Cancer Institute (Canada). S. L. MacDougall is a Cancer Research Society Fellow. S. Grinstein is the recipient of a Medical Research Council Scientist Award.

References

1. Cooper, M. D., J. F. Kearney, W. E. Gathings, and A. R. Lawton. 1980. Effects of anti-Ig antibodies on the development and differentiation of B cells. *Immunol. Rev.* 52:29–53.

2. Braun, J., R. I. Sha'afi, and E. R. Unanue. 1979. Crosslinking by ligands to surface immunoglobulin triggers mobilization of $^{45}Ca^{2+}$ in B lymphocytes. *J. Cell Biol.* 82:755–766.

3. Pozzan, T., P. Arslan, R. Y. Tsien, and T. J. Rink. 1982. Anti-immunoglobulin, cytoplasmic free calcium, and capping in B lymphocytes. *J. Cell Biol.* 94:335–340.

4. Bijsterbosch, M. K., K. P. Rigley, and G. G. B. Klaus. 1986. Crosslinking of surface immunoglobulin on B lymphocytes induces both intracellular Ca^{2+} release and Ca^{2+} influx: analysis with indo-1. *Biochem. Biophys. Res. Commun.* 137:500–506.

5. Streb, H., R. F. Irvine, M. J. Berridge, and I. Schulz. 1983. Release of Ca^{2+} from a non-mitochondrial intracellular store in pancreatic acinar cells by inositol 1,4,5 triphosphate. *Nature (Lond).* 306:67–69.

6. Ransom, J. T., L. K. Harris, and J. C. Cambier. 1986. Anti-Ig induces release of inositol 1,4,5-trisphosphate, which mediates mobilization of intracellular Ca^{++} stores in B lymphocytes. *J. Immunol.* 137:708–714.

7. Bijsterbosch, M. K., J. C. Meade, G. A. Turner, and G. G. B. Klaus. 1985. B lymphocyte receptors and polyphosphoinositide degradation. *Cell.* 41:999–1006.

8. Coggeshall, K. M., and J. C. Cambier. 1984. B cell activation. VIII. Membrane immunoglobulin transduces signals via activation of phosphatidylinositol hydrolysis. *J. Immunol.* 133:3382–3386.

9. Latorre, R., R. Coronado, and C. Vergara. 1984. K^+ channels gated by voltage and other ions. *Ann. Rev. Physiol.* 46:485–495.

10. De Coursey, T. E., K. G. Chandy, S. Gupta, and M. D. Cahalan. 1984. Voltage-gated K^+ channels in human T lymphocytes: a role in mitogenesis? *Nature (Lond).* 307:465–468.

11. Matteson, D. R., and C. Deutsch. 1984. K channels in T lymphocytes: a patch clamp study using monoclonal antibody adhesion. *Nature (Lond.).* 307:468–471.

12. Schwartz, W., and H. Passow. 1983. Ca^{2+}-activated K^+ channels in erythrocytes and excitable cells. *Ann. Rev. Physiol.* 45:359–374.

13. Rink, T. J., and C. Deutsch. 1983. Calcium activated potassium channels in lymphocytes. *Cell Calcium.* 4:463–473.

14. Monroe, J. G., and J. C. Cambier. 1983. B cell activation. I. Anti-immunoglobulin-induced receptor crosslinking results in a decrease in the plasma membrane potential of murine B lymphocytes. *J. Exp. Med.* 157:2073–2086.

15. Klaus, G. G. B., C. M. Hawrylowicz, M. Holman, and K. D. Keeler. 1984. Activation and proliferation signals in mouse B cells. III. Intact (IgG) anti-immunoglobulin antibodies activate B-cells but inhibit induction of DNA synthesis. *Immunology.* 53:693–701.

16. Rink, T. J., C. Montecucco, T. R. Hesketh, and R. Y. Tsien. 1980. Lymphocyte membrane potential assessed with fluorescent probes. *Biochim. Biophys. Acta.* 595:15–30.

17. Ransom, J. T., D. L. Digiusto, and J. C. Cambier. 1986. Single cell analysis of calcium mobilization in anti-immunoglobulin-stimulated B lymphocytes. *J. Immunol.* 136:54–57.

18. Gulyaeva, N. V., G. I. Konoshenko, and E. N. Mokhova. 1985. Mitochondrial membrane potential in lymphocytes as monitored by fluorescent cation diS-C_3-(5). *Membr. Biochem.* 6:19–32.

19. Cheung, R. K., S. Grinstein, and E. W. Gelfand. 1982. Volume regulation by human lymphocytes. Identification of differences between the two major lymphocyte subpopulations. *J. Clin. Invest.* 70:632–638.

20. Gomez-Puyou, A., and C. Gomez-Lojero. 1977. The use of ionophores and channel formers in the study of the function of biological membranes. *Curr. Top. Bioenerg.* 6:221–257.

21. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with improved fluorescence properties. *J. Biol. Chem.* 260:3440–3450.

22. Hess, P., J. B. Lansman, and R. W. Tsien. 1986. Calcium channel selectivity for divalent and monovalent cations. *J. Gen. Physiol.* 88:293–319.

23. Wilson, H. A., and T. M. Chused. Lymphocyte membrane potential and Ca^{2+}-sensitive potassium channels described by oxonal dye fluorescence measurements. *J. Cell. Physiol.* 125:72–81.

24. Tatham, P. E. R., K. O'Flynn, and D. C. Linch. 1986. The relationship between mitogen-induced membrane potential changes and intracellular free calcium in human T lymphocytes. *Biochim. Biophys. Acta.* 856:202–211.

25. Tsien, R. Y., Y. Pozzan, and T. J. Rink. 1982. T-cell mitogens cause early changes in cytoplasmic free Ca^{2+} and membrane potential in lymphocytes. *Nature (Lond.).* 295:68–71.

26. LaBaer, J., R. Y. Tsien, K. A. Fahey, and A. L. DeFranco. 1986. Stimulation of the antigen receptor on WEHI-213 B lymphoma cells results in a voltage-independent increase in cytoplasmic calcium. *J. Immunol.* 137:1836–1844.

27. Levi, R., and L. J. DeFelice. 1986. Sodium-conducting channels in cardiac membranes in low calcium. *Biophys. J.* 50:5–9.

28. Hess, P., and R. W. Tsien. 1984. Mechanism of ion permeation through calcium channels. *Nature (Lond.).* 309:453–456.

29. Fukushima, Y., and S. Hagiwara. 1985. Currents carried by monovalent cations through calcium channels in mouse neoplastic B lymphocytes. *J. Physiol. (Lond.).* 358:255–284.