B CELL ACTIVATION

III. B Cell Plasma Membrane Depolarization and Hyper-Ia Antigen Expression Induced by Receptor Immunoglobulin Cross-linking Are Coupled*

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We have previously reported B lymphocyte plasma membrane depolarization resultant from receptor immunoglobulin cross-linking by anti-immunoglobulin (1) and specific antigen (2). These findings extend observations of Taki (3) and Kiefer et al. (4), who have reported an early decrease in the plasma membrane potential of T and B lymphocytes in response to mitogens. The mutually exclusive induction of depolarization in T and B cells by concanavalin A (Con A)[1] and lipopolysaccharide (LPS) respectively, and the failure of LPS-unresponsive C3H/HeJ B cells exposed to LPS (5) to undergo membrane depolarization suggests that changes in ion flux manifest by decreased membrane potential may be important in mitogenesis. In other somatic cell systems (6–9), plasma membrane depolarization has been demonstrated to be important for activation and entry into cell cycle, and has further been postulated to be an initial and critical event in these processes. Our studies (1, 2) indicate that while there is a strong correlation between depolarization and entry into cycle by B lymphocytes in response to mitogens, anti-immunoglobulin, and thymus-independent antigens (TI), plasma membrane depolarization is itself an insufficient signal to support transition from G_0 to G_1. This conclusion is based upon results that demonstrate that while thymus-dependent (TD) antigens (2), 50 mM K^+ (10), and phorbol diesters (11) are able to facilitate decreased plasma membrane potential, none stimulate a significant proportion of cells to undergo G_0 to G_1 transition. In view of the distinction observed between TD and TI antigens, it is possible that membrane depolariation is a manifestation of but one of multiple signals required for B cell activation.

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[1] *Abbreviations used in this paper:* Ba, *Brucella abortus* tube antigen; BSS, balanced salts solution; Con A, concanavalin A; $DxSO_4$, dextran sulfate; FCS, fetal calf serum; LPS, lipopolysaccharide; MHC, major histocompatibility complex; mIa, membrane-associated, I-region–associated antigen; OVA, ovalbumin; SRBC, sheep erythrocytes; TD, thymus-dependent; TI, thymus-independent; TNP, trinitrophenol.

A hallmark of the two signal hypothesis for B lymphocyte activation as originally proposed by Bretscher and Cone (12) is that antigen-receptor immunoglobulin interaction results in transduction of an initial signal across the plasma membrane. It was proposed that this signal alone, while preparing the cell to receive a second signal, would be insufficient to drive B lymphocyte proliferation and differentiation into antibody-secreting cells. These later events would require a second signal(s), delivered by T cells in the case of TD antigens or by mitogenic moieties in the case of TI antigens. In the case of TD antigen-driven activation, the necessary second signal may be delivered by antigen-specific, I-region–restricted helper T cells or their secreted factors (13, 14). The Ia restriction of T-B collaboration (15), along with recent observations that antigen (2) and anti-immunoglobulin (16) binding to (by) B cells induces increased I-A expression, and observations that high I-A B cells are more receptive to I-region–restricted T cell help (17) suggest that I-A "induction" may play an important role in thymus-dependent antibody responses. Specifically, an essential effect of antigen binding to cell surface immunoglobulin during TD responses may be generation and transduction of a signal resultant in heightened mIa expression.

The mechanism of signal transduction involved in coupling of receptor immunoglobulin cross-linking to heightened I-A antigen expression is of obvious importance. Evidence discussed earlier suggests that changes in plasma membrane potential could be integral in this process. In view of this possibility, we undertook this study to assess the role of plasma membrane depolarization in receptor cross-linking–mediated induction of heightened I-A expression. Our results demonstrate a correlation between stimuli and conditions that induce decreased membrane potential, and those that stimulate hyper-I-A antigen expression. Further, inhibition of ligand-induced plasma membrane depolarization using valinomycin results in inhibition of hyper-I-A antigen expression. Finally, passive (non-ligand–mediated) plasma membrane depolarization by 50 mM K^+ results in hyper-I-A antigen expression. Taken together these data suggest that plasma membrane depolarization is a central and essential intermediary in the cell surface Ig-mediated signal transduction mechanism.

Materials and Methods

Mice. BALB/k mice (6–8 wk old), which are H-2^k in the I-A subregion of the MHC, were used in all experiments. They were bred and maintained at the Duke University Division of Laboratory Animal Resources.

Culture Conditions. Spleens were removed aseptically from animals killed by cervical dislocation. They were teased apart and forced through a fine mesh steel screen. Erythrocytes were lysed by treatment with Gey's solution. Resulting cells were washed three times with balanced salts solution (BSS). T cells were removed by treatment with anti–Thy-1 (hybridoma T24, 18) plus complement. The resulting cells, henceforth referred to as B cells, were washed three times in BSS and cultured at concentrations indicated in the text in 1 ml of media in Costar 24-well cluster plates (Costar, Data Packaging, Cambridge, MA). Medium was RPMI 1640 containing 10% fetal calf serum (FCS; Lot 100273; Sterile Systems, Logan, UT), glutamine, streptomycin, penicillin, 5×10^{-5} M 2-mercaptoethanol, nonessential amino acids, sodium pyruvate, insulin, and oxaloacetic acid.

Preparation of Anti-Fab. $F(ab')_2$ fragments of rabbit anti-mouse Fab were prepared as described previously (1).

Mitogens. Lipopolysaccharide (Lot 3123-25; Difco Laboratories Inc., Detroit, MI) was used at a final concentration of 50 μg/ml. Dextran sulfate ($DxSO_4$, molecular weight

500,000; Gibco Laboratories, Grand Island, NY) was used at a final concentration of 20 μg/ml.

Antigens. Trinitrophenol–*Brucella abortus* tube antigen (TNP-Ba), TNP_{5}-ovalbumin (OVA), and TNP_{40}-Ficoll were prepared as described previously (2). Concentrations of antigens used were those demonstrated to be optimal for induction of primary antibody responses in vitro in the presence of appropriate primed T cell populations (19–21, data not shown).

TNP-binding Cell Isolation. TNP-binding cells were isolated by a modification (22) of the phosphorylcholine-binding cell isolation procedure of Cambier and Neale (23). Cells isolated in this manner are typically >97% Ig^+, <2% Thy-1^+, and >70% rebound antigen in a rosette assay utilizing TNP-Ba or TNP-sheep erythrocytes (SRBC) (22).

Antisera. The hybridoma 10-2.16 secreting monoclonal anti-I-A^k (24) was obtained from the Salk Institute, San Diego, CA. Its antibody product, which recognizes products of the I-A subregion of the H-2 complex in mice with the H-2^k haplotype (24), was biotinylated as previously described (25–27).

Immunofluorescent Staining and Cytofluorimetric Analysis. Cells were stained and analyzed using a Cytofluorograf System 50-H (Ortho Diagnostic Systems Inc., Westwood, MA) as previously described (27). The relative frequencies of high I-A antigen expressing cells among mixed populations were determined as follows: Histograms of anti-I-A–stained B cells from cultured, unstimulated and stimulated, ie., mitogen, anti-Fab, antigen, or K^+ populations were constructed and compared (see Fig. 4). The inflection point was then determined and the area to the right of this point (high I-A antigen expression) and the total area were integrated using the Cytofluorograf distribution analyzer. In all cases, regardless of stimulation with anti-Ig or $50mk^+$ with or without Valinomycin, cells exposed to fluoresceinated avidin without prior exposure to biotinylated anti-I-A were <5% positive. In all experiments, forward narrow angle light scatter was used as a second parameter to facilitate exclusion of dead cells from the analyses.

Determination of Relative Membrane Potential. Relative membrane potential was measured by cytofluorimetric analysis of cells stained with the membrane potential sensitive dye 3,3′-dipentyloxacarbocyanine iodide [$DiOC_5(3)$] as described in detail elsewhere (1). The relative frequencies of depolarized cells within mixed populations was determined as follows: Histograms were generated using unstimulated and stimulated (i.e. mitogen, anti-Fab, antigen, or K^+) cells and superimposed. The inflection point was then determined and the area to the left of this point (low membrane potential; depolarization) and the total area were integrated using the Cytofluorograf distribution analyzer. In all experiments, forward narrow angle light scatter was used as a second parameter to facilitate exclusion of dead cells from the analyses.

Results

Equal Frequencies of B Cells Are Stimulated to Depolarize and Express Elevated Levels of mI-A Antigen by anti-Fab Antibodies and Mitogens. We and others have reported decreased plasma membrane potential upon B cell stimulation by anti-Fab (1) and mitogens (3–5). This event is detectable within 5 min and maximal by 1 h following exposure to anti-Fab (1). Somewhat later (first detectable by 3–12 h, 16) after exposure to anti-Fab (16) or mitogens (27) B cells increase in expression of mI-A antigen. The ultimate goal of these studies was to determine the relationship between these events. Specifically, is membrane depolarization causally related to induction of increased mIa expression, or is it an epiphenomenon? Our initial approach to this question was to establish whether a correlation exists between induction of depolarization and induction of hyper-I-A antigen expression. Presented in Fig. 1 are data comparing the frequency of B cells depolarized by LPS and $DxSO_4$ or anti-Fab at 2 h and the frequency of B cells in companion cultures that express hyper-mI-A antigen 24 h after stimula-

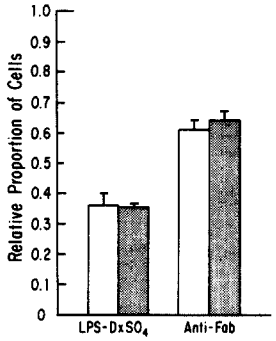

FIGURE 1. Relative frequency of B cells exhibiting decreased plasma membrane potential and hyper-I-A antigen expression in response to mitogens or $F(ab')_2$ anti-Fab. B cells at 2×10^5 cells/ml in 1-ml cultures were incubated with no stimulus, LPS-$DxSO_4$ (50 $\mu g/\mu l$ and 20 $\mu g/\mu l$, respectively) or anti-Fab (25 μg/ml) for 2 h before analysis for depolarization or 24 h before I-A antigen expression analysis. Analyses were performed as described in the text. Values represent the relative proportion of cells, in each population, that exhibited decreased plasma membrane potential (light bars) on elevated I-A antigen expression (shaded bars) relative to unstimulated cultures. In all cases, backgrounds from unstimulated companion cultures have been subtracted. Each value is the mean of three separate experiments ± SEM. Each experimental value was derived from analysis of 100,000 cells.

tion. As can be seen, for both stimuli there exists no significant ($P<0.001$) difference between the frequency of cells depolarized and the frequency for cells that subsequently express elevated levels of I-A antigen, thereby suggesting a correlation between the frequencies. These results are consistent with the possibility that all B cells that are depolarized by mitogen or anti-Fab subsequently go on to express elevated levels of I-A antigen. A more direct approach to this question might be to sort depolarized cells, reculture them, and then later analyze mI-A expression. Unfortunately, $DiOC_5[3]$, used to assess membrane potential, has extremely deleterious effects on cell physiology. Therefore, this approach is not feasible.

Correlation Between Antigen-induced Depolarization and Increased mI-A Antigen Expression by Isolated TNP-binding Cells. We have previously demonstrated (2) plasma membrane depolarization of TNP-binding B cells induced specifically by both TD and TI antigens. If, as the previous results for mitogens and anti-Fab suggest, B cell membrane depolarization signals B cell expression of increased mI-A antigen, one would predict that both TD and TI antigens should also induce an increase in the mI-A antigen expression. Further, the antigen should induce an equal proportion of cells to depolarize and to increase in mI-A antigen expression. These predictions were tested by determination of the relative frequency of isolated TNP-binding B cells induced to depolarize by TNP-carrier conjugates or by carrier alone and the frequency of those cells that express increased mI-A antigen in response to the same stimuli. The results of these experiments are presented in Fig. 2. The relative frequency of cells depolarized and stimulated to hyper-I-A antigen expression are presented normalized to responses to anti-Fab. Normalization to the response to anti-Fab was necessary to facilitate comparison of results obtained from multiple experiments. As can be seen, carriers alone were unable to induce a significant response, as measured

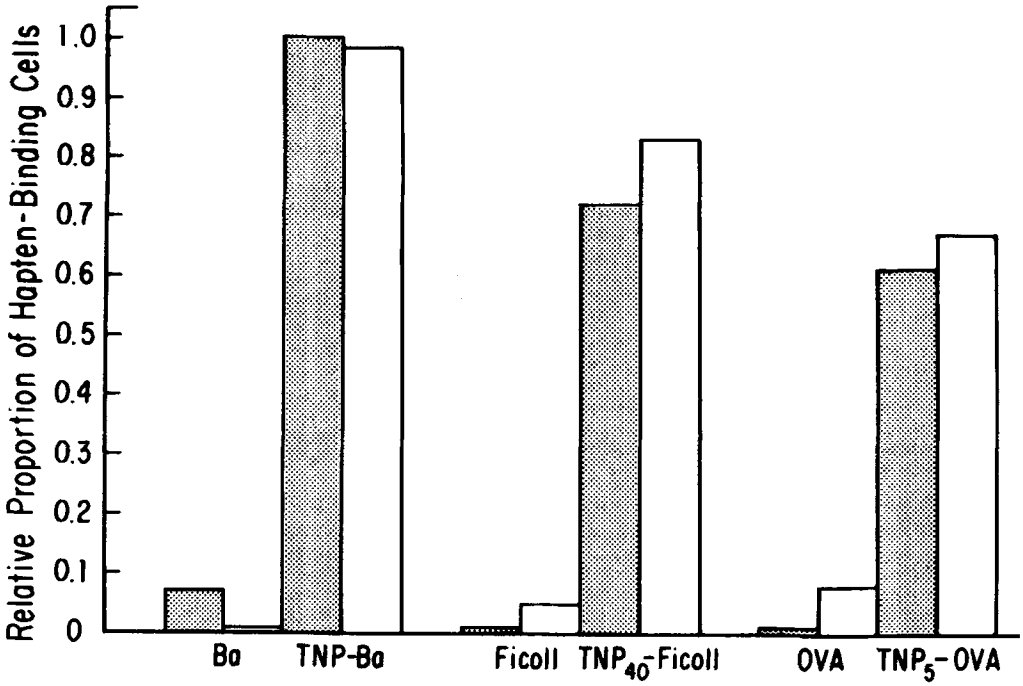

FIGURE 2. Relative frequency of TNP-binding B cells exhibiting decreased plasma membrane potential and hyper-I-A antigen expression in response to specific antigens. Isolated TNP-binding B cells at 2×10^5 cells/ml in 1-ml cultures were incubated for 2 h (depolarization) or 24 h (I-A antigen expression) with no stimulus, anti-Fab (25 $\mu g/\mu l$), Ba (0.01% vol/vol), TNP-Ba (0.01% vol/vol), Ficoll (10 ng/ml), TNP_{40}-Ficoll (10 ng/ml), OVA (100 ng/ml), or TNP_5-OVA (100 ng/ml). Analyses of relative membrane potential and I-A antigen expression were as described in the text. Values represent the proportion of cells within each population that exhibit decreased plasma membrane potential (shaded bars) or elevated I-A antigen expression (light bars) relative to the response to anti-Fab. In all cases, backgrounds from unstimulated companion cultures have been subtracted. Most values are the mean of three separate experiments. Each experimental value was derived from analysis of 10,000 cells.

by either assay. In contrast, all of the haptenated carriers were able to stimulate a significant proportion of the cells to depolarize and to exhibit hyper-I-A antigen expression. If the background responses of cells exposed to carriers alone are subtracted, the proportion of cells with antigen-induced decreased membrane potential is, in all cases, not significantly different from the proportion that expresses heightened-I-A antigen. Therefore, consistent with results derived using mitogen- and anti-Fab–stimulated B cells, a strong correlation exists between antigen-induced membrane depolarization and increased I-A antigen expression.

Inhibition of Anti-Fab–induced Depolarization Results in Inhibition of Increased Expression of mI-A Antigen. Data presented suggest that plasma membrane depolarization and heightened-mI-A antigen expression may be coupled. We investigated this question more directly by assessing the effect of inhibition of anti-Fab–induced depolarization on the subsequent change in mI-A antigen expression. As an inhibitor we used the potassium ionophore valinomycin. Valinomycin affects plasma membrane potential by allowing K^+ ions to freely cross the

membrane (28). At appropriate concentrations, valinomycin inhibits the decrease in plasma membrane potential induced by anti-Fab (Monroe and Cambier, unpublished observations). In view of this, we examined the effect of various concentrations of valinomycin on induction of membrane depolarization and hyper-mI-A antigen expression by anti-Fab. It should be noted that at doses $\leq 10^{-7}$ M, valinomycin had no adverse effect on cell viability as indicated by the fact that viable cell recovery at all doses tested was within 10% that of cultures containing anti-Fab alone. As can be seen in Fig. 3, at concentrations greater than 10^{-10} M valinomycin markedly inhibits anti-Fab–induced depolarization. When valinomycin is present at 10^{-7} M the response induced by anti-Fab is <10% of the control response (anti-Fab alone). Fig. 3 also depicts the effect of valinomycin on anti-Fab–induced hyper-mI-A antigen expression. The dose vs. response curves seen for the two responses are indistinguishable. These results suggest that membrane depolarization may be an essential event in anti-Ig–induced hyper-mI-A antigen expression.

B Cell Plasma Membrane Depolarization Induced by Elevated Extracellular Potassium Causes Hyper-I-A Antigen Expression. Data presented above suggest that membrane depolarization may be necessary for anti-Ig–induced hyper-mI-A antigen expression. We then determined whether membrane depolarization itself is sufficient to induce subsequent hyper-mI-A antigen expression. To test this possibility, we examined the effect of "passive" depolarization of murine B lymphocytes by supplementation of the cultures with 50 mM K^+. In previous studies and control experiments conducted here, this treatment induced plasma membrane depolarization by virtually 100% of murine B cells. B cells were cultured for 24 h with or without 50 mM K^+ or anti-Fab, then stained and

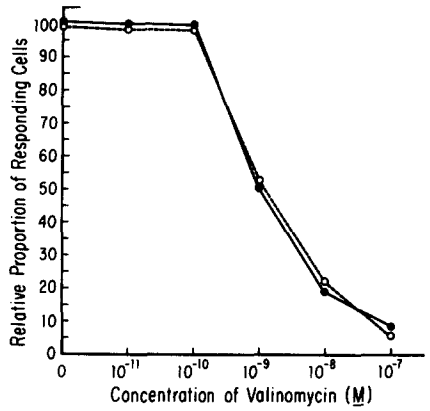

FIGURE 3. Effect of valinomycin-mediated inhibition of anti-Fab–induced depolarization on subsequent I-A antigen expression. B cells at 2×10^5 cells/ml in 1-ml cultures were incubated for 2 h (depolarization) or 24 h (I-A antigen expression) in the presence of 25 $F(ab')_2$ anti-Fab/ml and valinomycin. The valinomycin was diluted into media from a stock solution of 9×10^{-4} M in absolute ethanol. After incubation, the frequency of cells, in each population, that exhibited decreased plasma membrane potential (●) and increased I-A antigen expression (○) was determined as described in the text. For all values, backgrounds from unstimulated companion cultures have been subtracted. All values are normalized to the response observed in cultures containing anti-Fab 25 μg/ml in the absence of valinomycin. Each determination was made using 10,000 cells.

analyzed to quantitate surface I-A antigen expression. In Fig. 4 are presented histograms depicting the relative I-A antigen expression on B cells cultured with no stimulus, anti-Fab, or 50 mM K^+. Consistent with results of Mond et al. (16), we observe a dramatic increase in the level of mI-A antigen expression by B cells stimulated with anti-immunoglobulin (Fig. 4*a*). Most importantly, we observed an increase in mI-A antigen expression by those B cells cultured with 50 mM K^+ (Fig. 4*b*). The increased staining observed was not nonspecific, since in control experiments $B6D2F_1$ B cells stimulated with potassium increased in I-A expression, as detected with an $I\text{-}A^{b,d}$ monoclonal, while no staining (of $B6D2F_1$ cells) was detectable using the $I\text{-}A^{b,d}$ monoclonal (data not shown). When BALB/k mice were used, appropriately reciprocal results were observed. This increase was not due to changes in tonicity resulting from supplementation with K^+, since supplementation of medium with an equivalent amount of Na^+ (which does not induce depolarization by these cells) did not result in induction of altered mI-A antigen expression by these cells (data not shown). Cells exposed to K^+ for 24 h remained viable as determined by narrow angle light scattering and trypan blue exclusion. Further, these cells were as responsive to subsequent exposure to LPS as are normal B cells, with 40–50% entering cell cycle within 48 h of stimulation (data not shown). As is evident from comparing the histograms of the stimulated cells, the mean fluorescence intensity of K^+-depolarized cells was not as high as that of the anti-Fab–stimulated cells. Although the increased expression induced by K^+ is not as great as that induced by anti-Fab, the kinetics of the increase appears to be the same, being detectable by 10–12 h and maximal 24 h after

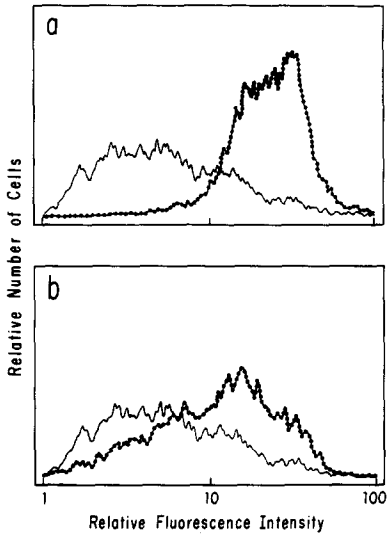

FIGURE 4. Induction of increased antigen expression by anti-Fab or elevated extracellular potassium. (*a*) B cells at 2×10^5 cells/ml were cultured for 24 h in 1-ml cultures with (●—●—●—●) or without (——) 25 μ/m of anti-Fab/ml. Following incubation, cells were stained for mI-A antigen expression and analyzed by flow cytometry using the parameters forward, narrow angle light scatter, to facilitate exclusion of dead cells, and log of integrated fluorescence to determine relative marker expression. Each histogram represents analysis of 10,000 cells. (*b*) Same as above, except cells were cultured in media with (●—●—●) or without (——) supplementary 50 mM K^+.

stimulation. Thus, for unknown reasons, induction by 50 mM K^+ appears somewhat less efficient than induction by anti-Fab. Taken together, these results and those of the previous experiments suggest that a cause-and-effect relationship exists between plasma membrane depolarization and subsequent heightened-mI-A antigen expression by cells responding to antigen or anti-immunoglobulin antibodies.

Discussion

In previous studies (1, 2) we have demonstrated that B cell plasma membrane depolarization results from receptor immunoglobulin cross-linking by antigen or anti-immunoglobulin. In the studies described here, we investigated the relationship between membrane depolarization and increased mI-A antigen expression induced by ligand binding to mIg. Our results demonstrate that TI as well as TD antigens stimulate depolarization and subsequent mI-A antigen hyper-expression and that the frequency of B cells depolarized by mitogen or anti-Ig equals the frequency of cells that exhibit hyper-I-A expression in response to these stimuli. Results of experiments in which valinomycin was utilized to block membrane depolarization suggest that depolarization is required for the increased mI-A antigen expression and, thus, that a cause-and-effect relationship may exist between these two physiologic changes. A cause-and-effect relationship between membrane depolarization and increased mI-A antigen expression is further supported by results demonstrating that "passive" (non-ligand–mediated) depolarization of murine B cells by K^+ also induces hyper-I-A antigen expression.

These results bear significant implications regarding the mechanisms of receptor immunoglobulin–mediated signal transduction. In view of the data reported here, we can operationally divide this transduction mechanism into two phases. First is the coupling of receptor immunoglobulin cross-linking to membrane depolarization. Studies in progress in our laboratory indicate that changes in phospholipid metabolism and protein kinase C activation are important intermediary events in this coupling mechanism (Cambier, J. C., J. G. Monroe, and Coggeshall, manuscript in preparation).

The second phase of the receptor immunoglobulin–mediated transduction mechanism involves coupling of membrane depolarization to increased mI-A expression. While the exact mechanism of coupling of these events is unknown, evidence from other systems provides some insight. In studies of neuroblastoma (29) and neurosecretory cells (7), membrane depolarization has been implicated in facilitating opening of voltage-dependent Ca^{+2} channels allowing Ca^{+2} influx and subsequent increases in cGMP levels. Both of these events have been demonstrated to be of critical importance in lymphocyte activation (30–32). Increased intracellular calcium, through calmodulin, may mediate changes in cellular metabolism and gene expression at multiple levels resulting in altered expression of cell surface markers including I region–encoded antigens. In the case of I-A, induction of increased antigen expression by anti-Ig or mitogens is blocked by cyclohexamide (16) and actinomycin D (33), suggesting that new RNA and protein synthesis are necessary. Similarly, induction of hyper-I-A expression by passive depolarization using 50 mM K^+ is also blocked by cyclohexamide (data not shown). Thus data are consistent with anti-Ig (and K^+)–

mediated activation occurring via membrane depolarization, which is sufficient to drive increased expression of genes encoding I-A antigens.

Studies reported here, and elsewhere (1, 2, 10, 27) indicate that the receptor Ig cross-linking initiated cascade may cease after expression of increased mI-A. We hypothesize that progression of cells in this poised, high I-A state, to G_1 requires additional signals provided by TI carrier or Ia-restricted helper T cells. Increased I-A expression facilitates the interaction between poised B cell and Ia-restricted, antigen-specific T cell. This hypothesis is supported by evidence that cross-linking of receptor Ig prepares the B cell for an optimal interaction with, Ia-restricted, antigen-specific T cells (34), that this interaction involves T cell recognition and binding to I region–encoded antigens (35), and that this interaction is necessary for B cell blastogenesis (13, 14), indicative of G_0 to G_1 transition (33). Recent evidence (36) demonstrating enhancement of antibody responses by anti-I-A antibodies suggests that I region antigens may play an important T cell signal transducing role in B cell immune responses. Studies to better define the multiplicity and mechanism of signaling required for induction G_0 to G_1 transition by TD antigens are in progress.

Summary

We report investigation of the relationship between ligand-induced B cell plasma membrane depolarization and increased expression of membrane-associated, I-A subregion encoded (mI-A) antigens. Results demonstrate that equal frequencies of B cells are stimulated to undergo membrane depolarization and to increase mI-A expression in response to mitogen, anti-Ig, and thymus-independent (TI) or thymus-dependent (TD) antigens. Further, a cause-and-effect relationship between these two events is suggested by results that demonstrate that inhibition of anti-Fab–induced depolarization by valinomycin also inhibits the subsequent increase in mI-A antigen expression and "passive" (non-ligand–mediated) depolarization of murine B cells by K^+ results in hyper-mI-A antigen expression. Based upon these results we hypothesize that antigen-mediated receptor cross-linking results in signal transduction via membrane depolarization, which is resultant in increased mI-A antigen synthesis and cell surface expression. This increase in mI-A antigen density may render the B cell more receptive to subsequent interaction with I-region–restricted helper T cells.

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