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THE ROLE OF TYROSINE PHOSPHORYLATION IN SIGNAL TRANSDUCTION THROUGH SURFACE Ig IN HUMAN B CELLS

Inhibition of Tyrosine Phosphorylation Prevents Intracellular Calcium Release[1]

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Cross-linking surface Ig on human B cells, or the TCR complex on T cells leads to the rapid appearance of newly tyrosine phosphorylated proteins. This is associated with inositol phospholipid turnover and a rise in intracellular calcium. Incubation of human B or T lymphocytes with the tyrosine kinase inhibitors, herbimycin and genistein, inhibits new tyrosine phosphorylation after receptor-linked activation. This is associated with complete abrogation of the increase in intracellular calcium in these lymphocytes and inhibition of inositol phospholipid turnover. Herbimycin- and genistein-treated lymphocytes are nevertheless still capable of responding to aluminum fluoride with a rise in intracellular calcium. These data support the contention that a B cell-associated protein tyrosine kinase regulates signal transduction via phospholipase C. CD45, the membrane associated protein tyrosine phosphatase, and PMA that activates protein kinase C, both inhibit the calcium response in B lymphocytes induced by receptor cross-linking. PMA and cross-linking CD45 both induced the appearance of tyrosine phosphorylated proteins in human B cells, although the pattern is quite distinct from that seen when surface Ig is cross-linked. However, the induction of new tyrosine phosphorylation by anti-μ does not appear to be affected by these reagents. Although this may reflect an insensitivity of the tyrosine phosphorylation assay, it could indicate that regulation of the calcium response and regulation of the tyrosine kinase can be independent processes.

Triggering of both mammalian T and B lymphocytes via their Ag-specific receptors has been shown to initiate the breakdown of phosphatidylinositol bisphosphate by PLC[4] to the two second messengers, IP_3, and diacylglycerol. Diacylglycerol activates PKC, IP_3 initiates the release of intracellular calcium, and the combined action of both messengers eventually leads to downstream changes such as entry into cell cycle. Together, the activation of PKC by phorbol esters and the elevation of the $[Ca^{2+}]_i$ concentration using calcium ionophores mimic the downstream effects of receptor stimulus, suggesting that calcium release and PKC activation constitute a signal sufficient to drive lymphocyte mitogenesis (1). An attractive feature of this signal transduction system is that IP_3 entrains a set of oscillations in the intracellular calcium concentration. In other systems, the frequency of the calcium oscillations is proportional to the intensity of the stimulus, and determines the strength of the response (2). Frequency encoded signals have the advantage over their analogue counterparts in that they are less susceptible to noise, i.e., small changes in the intracellular calcium concentration are not interpreted as signals. This provides an accurate means of quantitative conversion of input at the cell membrane into the output of the cellular response. For lymphocytes, which are selected on the basis of the affinity of their Ag-specific receptors for Ag, such a system has great potential advantage, as it allows each lymphocyte to respond appropriately to the strength of the stimulus at the cell surface.

The cellular machinery that translates input at cell surface receptors into the intracellular message is poorly understood. In human and murine B cells, the intracytoplasmic portions of IgM and IgD contain only three amino acids, which suggests a role for associated proteins able to transduce the receptor input (3). It now seems likely that in B cells, such as the CD3 complex in T cells, surface IgR are associated with a protein complex. IgM is associated with a 34-kDa protein (4, 5) encoded by the mb-1 gene (6) and IgD is associated with similar but distinct proteins (7, 8). How are these proteins linked to the subsequent activation of phopholipase-C (PLC)? Accessory proteins are likely to be necessary for signal transduction as indicated by recent work showing that the expression of the sIg proteins alone is insufficient to trigger PLC (9). Recently, we showed that cross-linking either Ig or class II molecules on the surface of human B cells, in addition to activating PLC, leads to the de novo appearance of several PTP, suggesting that PTK are activated when Ag-specific receptors are cross-linked (10). The timing and relationship of events in the signal transduction pathway were not clear from these results. We have addressed that issue in the experiments described

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[4] Abbreviations used in this paper: PLC, phospholipase C; AlF_4^-: Aluminum fluoride; IP_3: inositol-trisphosphate; PKC, protein kinase C; PTP, tyrosine phosphorylated proteins; PTK, protein tyrosine kinases; sIg, surface Ig.

here. We show that treatment of both normal lymphocytes and B cell lines with herbimycin (11), an agent that reverses the transforming potential of oncogenic PTK by degrading them (12), abolishes the rise in $[Ca^{2+}]_i$ after cross-linking of sIg. In contrast, cells still respond to $AlFl_4^-$ with a rise in $[Ca^{2+}]_i$. Treatment of lymphocytes with phorbol esters and ionomycin did not lead to the appearance of the major PTP. These results are consistent with a model that places the activation of PTK before the activation of PLC in the signal transduction pathway. Furthermore, they indicate that activation of PTK is a critical step in the activation of PLC. In the light of these results, we propose a revised model for signal transduction in B lymphocytes.

MATERIALS AND METHODS

Chemicals. PMA, the calcium ionophore A23187, avidin, dibutyryl cyclic AMP, and phenyl phosphate were obtained from Sigma Chemical Co. (St. Louis, MO); N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride, and 1-(5-isoquinolinylsulfonyl-2-methylpiperazine, which are potent inhibitors of kinases including PKC (13), were obtained from Seikagaku America, Inc. (St. Petersberg, FL); herbimycin A, an inhibitor of PTK (14), was a kind gift of Dr. Uehara (Institute of Microbial Chemistry, Tokyo, Japan); genistein another inhibitor of PTK was obtained from ICN (Costa Mesa, CA); $AlFl_4^-$ was prepared from stock solutions of aluminum chloride and sodium fluoride obtained from Sigma. $AlFl_4^-$ was prepared just before experimentation by mixing 600 mM sodium fluoride with 1 mM aluminum chloride in a ratio of 5:2. Of this solution 140 μl were added to 1 ml of cells. Aluminum chloride mixed with sodium chloride in the same ratios and of the same molarity was used as a control.

Preparation of lymphoid cells. Tonsillar B cells were isolated as described elsewhere (15), and were more than 95% $CD20^+$. They were separated into different density fractions on discontinuous Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) gradients of 55, 45, and 35%. Cells that pelleted less than 55% Percoll were used for these studies. The Ramos Burkitt's lymphoma line was kindly provided by Dr. D. Pious (University of Washington, Seattle, WA). PBL were isolated in the standard manner using Lymphoprep (Robbins Scientific, Sunnyvale, CA). To determine whether calcium was released from intracellular stores, B cells were preincubated for 5 min in medium containing EGTA at a final concentration of 8 mM, made up from a 100-mM stock made up in PBS, pH 7.6.

Antibodies. A polyclonal, affinity-purified $F(ab')_2$ goat anti-human μ-chain-specific serum (Jackson Immunoresearch Labs, Avondale, PA) was used to cross-link sIgM receptors. The 9.4 mAb, which recognizes all chains of CD45 (16), was prepared as a biotinylated conjugate as described (17); δ-TA4.1 (IgG1) recognizes IgD (18); G28.5 (IgG1) that recognizes the CD40 Ag (19) was used as a control antibody; G19.4 (IgG1) is a mAb that recognizes the CD3 complex and G17.2 (IgG1) is specific for CD4 (20). G17.2 and G19.4 mAb were conjugated together as described for phycobiliproteins mAb conjugates (17).

Detection of tyrosine phosphorylated proteins. New PTP was measured by immunoblotting with a purified rabbit anti-phosphotyrosine antibody, prepared as described previously (21). The specificity of the anti-phosphotyrosine antisera was confirmed by inhibiting binding with phenyl phosphate (Fig. 1*A*). To prepare samples, 5×10^6 cells/ml/sample were stimulated as indicated, rapidly pelleted by centrifugation and lysed in 200 μl boiling SDS sample buffer containing 20 μM orthovanadate. Samples were boiled for 5 min and stored at −70°C until analysis on 10% SDS polyacrilimide gels, and transferred to immobilon (Millipore, Corp., Bedford, MA). Immunoblots were incubated for 3 h with 0.25 μg/ml anti-phosphotyrosine antisera, followed by washing and development with 1 μCi/ml high sp. act. ^{125}I-protein A (ICN). Prestained high m.w. markers (Bethesda Research Laboratory, Gaithersburg, MD) were run on each gel and migration positions are indicated by in the left hand margin of the gel.

Analysis of intracellular $[Ca^{2+}]_i$ mobilization. The method used has been described in detail (22). Tonsillar B cells and the Ramos B-cell line were loaded with Indo-1 by incubation with its acetoxymethyl ester (Molecular Probes, Junction City, OR) at 1 μM concentration for 45 min (37°C). Cells were washed once and kept at room temperature in the dark until analysis was performed with either an Ortho Cytofluorograph 50HH (Ortho Diagnostic System, Westwood, MA) or a FACStar Plus (Becton Dickinson, San José, CA). An argon laser was used for UV excitation. Violet fluorescent emission was detected at 383 to 407 nm and the blue emission was detected at 480 to 520 nm, the ratio of Indo-1 violet to blue fluorescence was digitally calculated for each individual cell and displayed as a function of elapsed time. The entire analysis was performed at 37°C. Aliquots of 5×10^5 cells were used for each experiment with a typical flow rate of 600 cells/s. The ratio of Indo-1 violet to blue was calibrated against $[Ca^{2+}]_i$ as described previously (22). An Indo-ratio of one represents an intracellular calcium concentration of 130 nM, an indo ratio of 2, 340 nM, an indo-ratio of 3, 700 nM, and an indo-ratio of 4, 1500 nM. In inhibition studies, lymphoid cells were adjusted to 5×10^6 cells/ml and treated for 30 min at 37°C with either medium, PMA (2 ng/ml), biotinylated CD45 mAb (10 μg/ml) followed by avidin (50 μg/ml), dBcAMP (1 mM), forskolin (10^{-4} M), or herbimycin A (1 to 10 μM) or genistein (100 μM) before stimulating cells with 10 μg/ml polyclonal, affinity purified $F(ab')_2$ goat anti-human μ-chain-specific antisera. The viability of cells incubated overnight with genistein or herbimycin A or as a control, DMSO only, was similar (>90%).

Measurement of inositol phosphates. The Burkitt's line, Ramos, was labeled for 24 h with medium containing 2 μCi of ^{3}H inositol/10^8 cells. A total of 10 μg/ml anti-IgM was added to cells at a density of approximately 3×10^7 per ml of medium at 37°C. Samples (300 μl) were taken at 10, 30, 60, and 300 s and quenched in 0.5 ml of cold 20% TCA with added phytate (sodium hexaphosphate, Sigma; 250 μg/ml) and phytate hydrolysate (25 μg phosphate/ml) (23). Cell lysates were spun and the pellet was washed with TCA. The combined supernatants were washed five times with 2 ml of hydrated ether. Ether was allowed to evaporate from the final aqueous phase, to which 100 μl of 50 mM EDTA was added. Samples were stored at −20°C for later analysis by HPLC.

HPLC analysis of aqueous cell extracts was performed using a Partisiphere 5 SAX column with pre-column and guard cartridge (Whatman, Clifton, NJ). Eluted ^{3}H-labeled compounds were identified by comparison with elution times of authentic tritiated standards from Amersham (Arlington Heights, IL) (Ins-1-P, Ins-1,4-P_2, Ins-1,4,5-P_3, and Ins-1,3,4,5-P_4 or New England Nuclear (Boston, MA) (Ins-1,3,4-P_3). The tritiated standards were added quantitatively to ^{3}H-inositol-labeled cell lysates after addition of TCA, and gradients were calibrated with internal nucleotide standards (cAMP, UMP, and ATP; Sigma). The gradient used elution buffers of ultrapure water and monobasic ammonium phosphate (Sigma) at pH 3.8, and separated at least 15 inositol-containing compounds. The gradient commenced as 100% water, advancing to 0.05 M ammonium phosphate over 25 min. AP concentrations were then held at 0.2 M for 10 min, increased from 0.54 to 0.56 M over 30 min, held at 0.8 M for 15 min then at 1.6 M for 10 min, and finally maintained at 2.0 M for 15 min to terminate the gradient. Fractions of 0.5 min were collected and counted by liquid scintillation chromatography with correction for quench. The fractions identified as ins-1,4,5-trisphosphate, ins-1,3,4,5-tetrakisphosphate, ins-1,3,4-trisphosphate, and ins-1,4-bisphosphate were expressed as a fraction × 10000 of the total number of counts eluted from the hplc column, most of which are in the inositol fraction. We have found that this value is large, and is unaffected by changes in the level of total inositol phosphates that might be induced by experimental treatment. This normalization controls for variation between samples in the extraction procedure.

RESULTS

Cross-linking surface IgM on human B cells through surface Ig but not CD40 induces new protein tyrosine phosphorylation. As we have previously reported (10), new PTP is detectable within 1 min after cross-linking sIg on dense human tonsillar B cells (Fig. 1*A*). Phenyl phosphate (40 mM) completely inhibited the binding of the anti-phosphotyrosine antisera (Fig. 1*A*). Other mAb including G28.5 that recognizes the CD40 Ag did not induce the appearance of these bands, indicating that the effect was specific for Ig cross-linking (Fig. 1*B*). In contrast, signaling through the CD40 molecule is associated with rapid phosphorylation on serine and threonine residues and has profound costimulatory effects with anti-Ig (24). We detected tyrosine phosphorylations on major protein bands approximately 55, 61, 74, 82, 88, 98, 115, 150, and 190 kDa in size. We have previously shown that the set of newly tyrosine phosphorylated proteins detected after cross-linking class II or sIg on B cells is similar (10). T cells also exhibited similar induc-

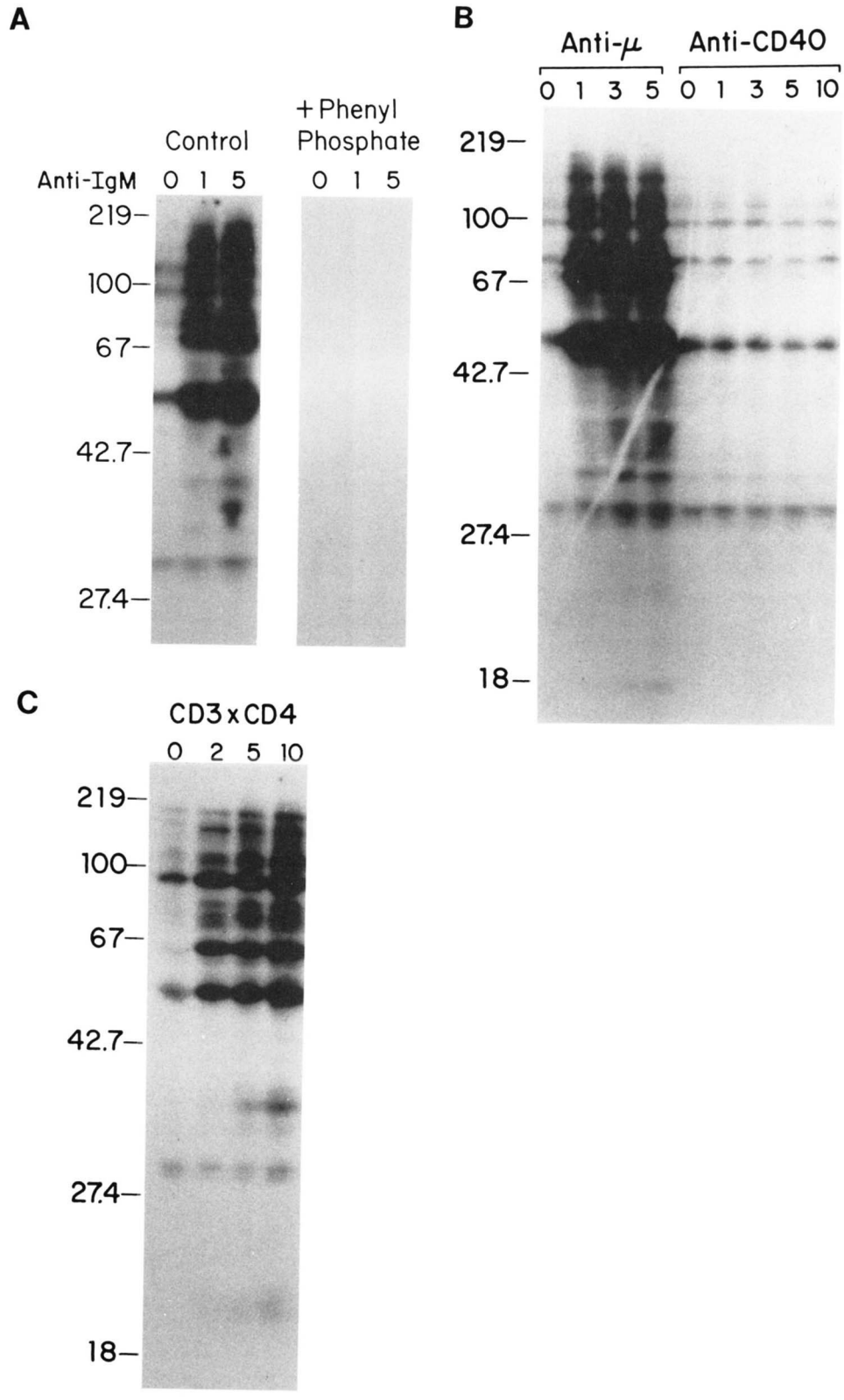

Figure 1. Induction of tyrosine phosphorylation by cross-linking of surface IgM (*A*) and CD40 (*B*) on human dense tonsillar B cells and CD3 and CD4 on T cells (*C*). Phosphorylation of proteins on tyrosine was detected by immunoblotting with an antibody specific for phosphotyrosine as described in *Materials and Methods*. The migration positions of the m.w. markers are indicated. Surface IgM was ligated by the addition of goat $F(ab')_2$ anti-μ (25 μg/2.5 $\times$ 10^7 cells/5 ml) without further cross-linking. Samples at each time point containing 5 $\times$ 10^6 cells were rapidly pelletted by centrifugation, the supernatant aspirated, and the cell pellet was lysed in hot SDS buffer followed by 5 min of boiling to denature tyrosine phosphatases. Phenyl phosphate completely inhibited the binding of the anti-phosphotyrosine antisera (*A*) indicating the specificity. As a control, CD40 was ligated using a mAb G28.5 at a final concentration of 1 μg/ml (*B*). CD3 and CD4 were co-cross-linked using a heteroconjugate of two mAb, G19.4 and G17.2, used as previously described (30) at a final concentration of 1 μg/ml.

tion of tyrosine phosphorylation when CD3 and CD4 on T cells were cross-linked by a heteroconjugate of 2 mAb 19.4, and 17.2 prepared as described (17). Although many of the substrates in T and B cells have similar m.w., there are distinct substrates (Fig. 1 *B* and *C*), suggesting that cross-linking Ag receptors on B and T cells may lead to tyrosine phosphorylation of proteins unique to these cell types.

Rise in intracellular calcium associated with cross-linking sIg is abrogated by PTK inhibitors, although $AlFl_4^-$ can bypass this inhibition. We and others have shown that cross-linking surface Ig on B cells is linked with phosphatidylinositol turnover and a rise in intracellular calcium (3, 10, 25, 26). We tested whether the PTK inhibitors, herbimycin and genistein, affected the calcium response. Herbimycin (11) has been shown to inhibit the transformation of fibroblasts by oncogenic PTK (12, 14) by directly affecting PTK activity. We measured

intracellular calcium concentrations in herbimycin- and genistein-treated peripheral B and T lymphocytes after activation through sIg and CD3 (Fig. 2), and also for the B cell line, Ramos (data not shown). The results were clear. Herbimycin inhibited the calcium response in T (Fig. 2*A*) and B cells (Fig. 2 *B* and *C*) when used at 10 μM. Genistein also inhibited intracellular calcium release in a dose dependent manner (data not shown). The inhibition did not appear to be non-specific as $AlFl_4^-$ applied to herbimycin-treated cells induces a calcium response comparable to that seen in normal cells (Fig. 3). The kinetics of the $AlFl_4^-$-induced calcium response is slow compared with anti-Ig-treated cells. This presumably reflects the time it takes for $AlFl_4^-$ to form from sodium fluoride and aluminum chloride, to enter the cell, and to bind to G proteins. The rise in $[Ca^{2+}]_i$ occurs in the presence of EGTA suggesting $AlFl_4^-$ releases calcium from intracellular stores (data not shown). $AlFl_4^-$ activates heterotrimeric G proteins by mimicking the GTP-bound activated state (27). In many cells, $AlFl_4^-$ will initiate phosphatidyl inositol turnover, presumably by acting on G proteins, and this is also true in B cells (26, 28). It is still not clear whether $AlFl_4^-$ stimulates a G protein directly coupled to phospholipase C, as has been suggested for the muscarinic receptor, or whether it acts on a G protein regulating intracellular calcium stores (29). Whatever the

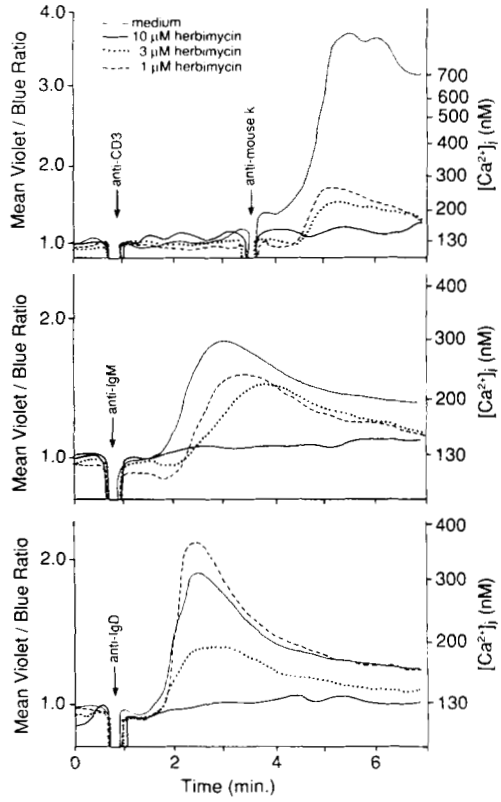

Figure 2. Dose-dependent inhibition of the rise in $[Ca^{2+}]_i$ in human B and T lymphocytes by the PTK inhibitor, herbimycin. Cells, prepared as described in the *Materials and Methods*, were incubated for 18 h at 5 × 10^6/ml with varying concentrations of herbimycin (1 to 10 μM) or 0.1% DMSO solvent for 18 h. They were washed once and then loaded with indo-1 dye as described in *Materials and Methods*. Cells were prewarmed for 5 min at 37°C before analysis. After 1 min, 10 μg CD3 mAb 19.4 was added, followed by cross-linking with an anti-mouse-κ reagent (*top*), or 10 μg goat F(ab')$_2$ anti-μ (*middle*) or 10 μg δ-TA4.1 (*bottom*) to 1 ml of cell suspension at a final concentration of 10^6 cells/ml. A FITC-anti-CD20 mAb, 1F5, was used to stain and then selectively gate on B cells (middle and bottom) as described (22). Resting lymphocytes have an Indo ratio of 1 which represents an $[Ca^{2+}]_i$ of 130 nM.

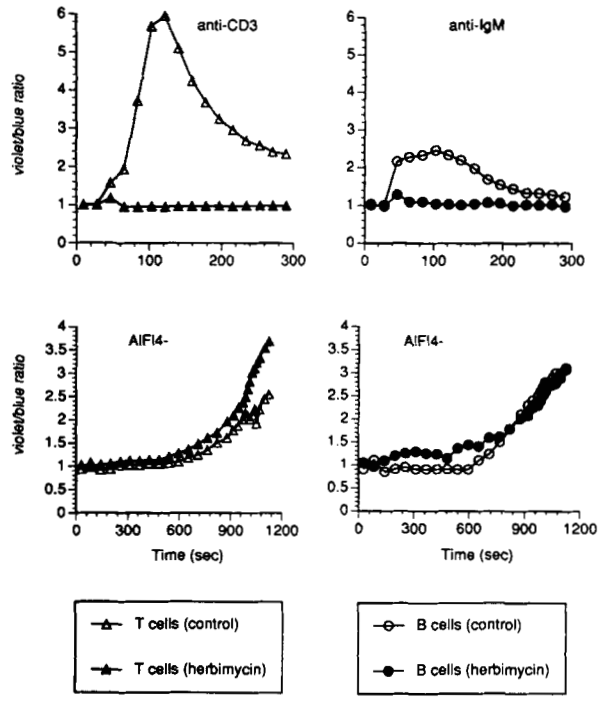

Figure 3. Comparison of calcium responses in herbimycin and control treated human B and T cells after treatment with anti-CD3 (*top left*), anti-IgM (*top right*), or $AlFl_4^-$ in T (*bottom left*), and B cells (*bottom right*). In the Indo analyses, the B and T cell responses were analyzed on gated cells using the Chronys software analysis program (Becton Dickinson, San José, CA). Dense tonsillar B and T cells at 5 × 10^6 cells/ml were incubated for 18 h with 10 μM herbimycin or with medium containing 0.1% DMSO, the solvent for herbimycin alone. Cells were washed once and resuspended to a final concentration of 5 × 10^6/ml. They were then stimulated with goat F(ab')$_2$ anti-μ (25 μg/2.5 × 10^7 cells/5 ml) or 10 μg 19.4 (anti-CD3) or 140 μl of a mixture of aluminum chloride and sodium fluoride prepared as described in *Materials and Methods*.

mechanism, our results do show that herbimycin-treated cells are capable of making a calcium response.

Induction of new tyrosine phosphorylation is inhibited by PTK inhibitors, herbimycin and genistein. The appearance of newly PTP suggested that signal transduction in B cells, as has been reported in T cells (30), might be regulated by a PTK. In addition, PTK inhibitors abrogate the rise in $[Ca^{2+}]_i$ after receptor cross-linking in lymphocytes. We tested whether the PTK inhibitors affected the appearance of PTP. The viability of dense tonsillar B cells was not affected by overnight incubation (18 h) with an optimal dose of 10 μM herbimycin. The B cell Burkitt's lymphoma line Ramos had a viability of 90% after overnight incubation with 10 μM herbimycin; dead cells were removed by centrifugation through Percoll. Cells at 10^7/ml were then stimulated with an optimal dose of polyclonal goat anti-human IgM F(ab')$_2$ serum. Control cells that had been incubated with 0.1% DMSO (solvent for the herbimycin), were similarly treated. Samples were taken at time 0 and at intervals thereafter and analyzed for PTP. Figure 4 shows that herbimycin inhibits new PTP in both normal dense tonsillar B cells and the B cell line Ramos. In separate experiments, we have found that the PTK inhibitor, genistein, profoundly inhibits the appearance of new PTP induced by anti-Ig. The minimum dose of genistein that completely inhibited the calcium response (100 μg/ml) also completely inhibited the appearance of new PTP.

Pretreatment with antibodies to CD45 inhibits rise in intracellular calcium but does not appear to affect directly PTK activity associated with cross-linking of

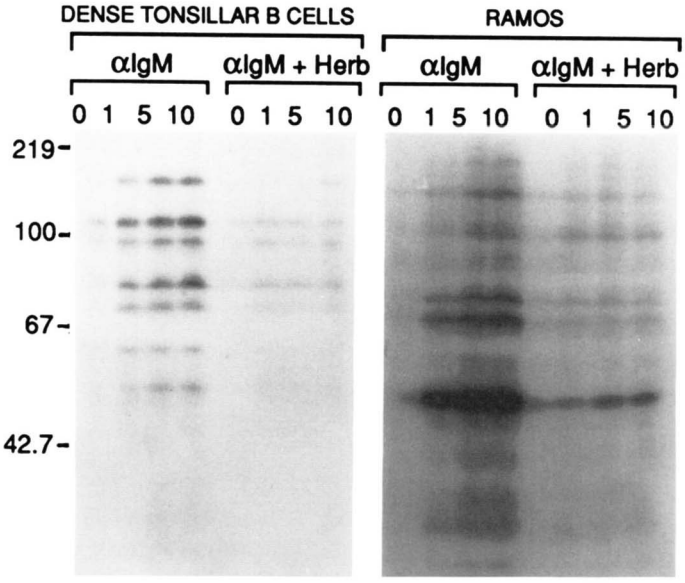

Figure 4. Inhibition of tyrosine phosphorylation by herbimycin. Dense tonsillar B cells and the Burkitt's lymphoma line, Ramos at 5×10^6 cells/ml were incubated with an optimum dose, 10 μM herbimycin for 18 h or with the solvent for herbimycin alone, 0.1% DMSO. Dense tonsillar B cells had more than 95% viability after this treatment; Ramos cells were over 90% viable by trypan blue exclusion. Dead cells were removed by centrifugation over a Percoll gradient. Cells were washed once and resuspended to a final concentration of 5×10^6/ml. They were then stimulated with goat F(ab')$_2$ anti-μ (25μg/2.5×10^7 cells/5 ml). Immunoblots were prepared as described in the legend to Figure 1.

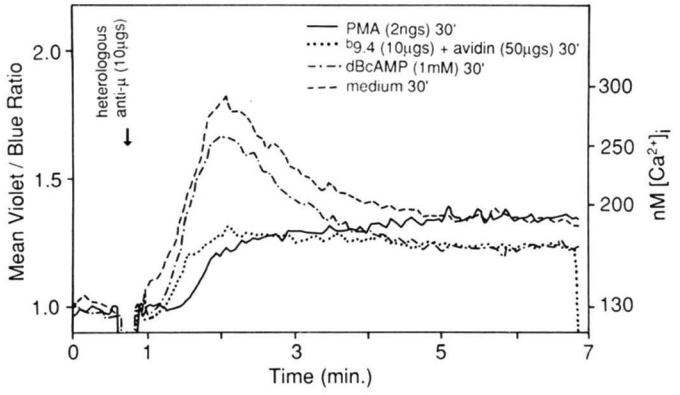

Figure 5. Effect of pretreatment of dense tonsillar B cells with dibutyryl cAMP (1 mM for 30 min), PMA (10 ng/ml for 30 min) and CD45 (biotinylated 9.4 mAb 10 μg/ml followed by cross-linking with 40 μg avidin for 30 min) on the subsequent rise in $[Ca^{2+}]_i$ after cross-linking with 10 μg goat F(ab')$_2$ anti-μ. Dense tonsillar B cells were prepared as described in *Materials and Methods*.

sIg. We have previously shown that the calcium signal in both B and T cells (10, 31) can be abrogated at least in vitro by the ubiquitous membrane protein tyrosine phosphatase, CD45 (32). The differential regulation of isoforms of this molecule in T and B cells suggest it may play a critical regulatory role in signal transduction. The most obvious way in which it might do this would be to regulate the activity of the PTK directly. To test this, we preincubated B cells with the mAb 9.4 that recognizes the common determinant of the CD45 molecule. In this situation the rise in $[Ca^{2+}]_i$ after cross-linking of surface Ig is inhibited (Fig. 5) especially in the presence of EGTA (data not shown). The appearance of newly PTP was not inhibited (Fig. 6). It is possible that the polyclonal anti-phosphotyrosine antisera used for the immunoblots may not detect all PTPs, but if relevant PTK activity was a direct target for the PTPase, CD45, one would expect a decrease in the intensity of PTP. In contrast, CD45 cross-

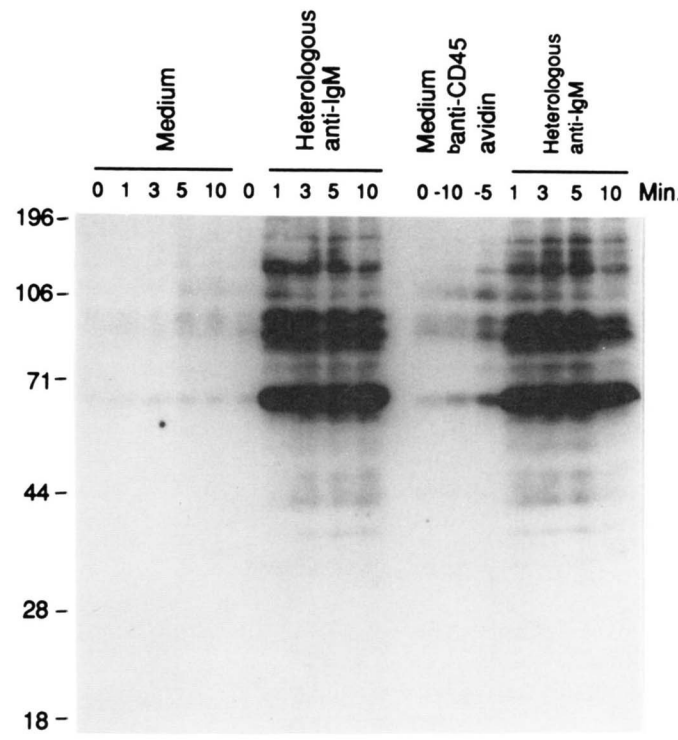

Figure 6. Effect of cross-linking CD45 on the induction of new PTP by anti-μ. Dense tonsillar B cells were pretreated with either medium or cross-linked CD45 (biotinylated 9.4 followed by 40 μg avidin for 30 min before the addition of 10 μg goat F(ab')$_2$ anti-μ. Immunoblots were made from cell extracts as described in Figure 1. Pretreatment with CD45 mAb had no discernible effect on the subsequent PTP pattern induced by anti-μ, but after further cross-linking with avidin-induced new PTP alone.

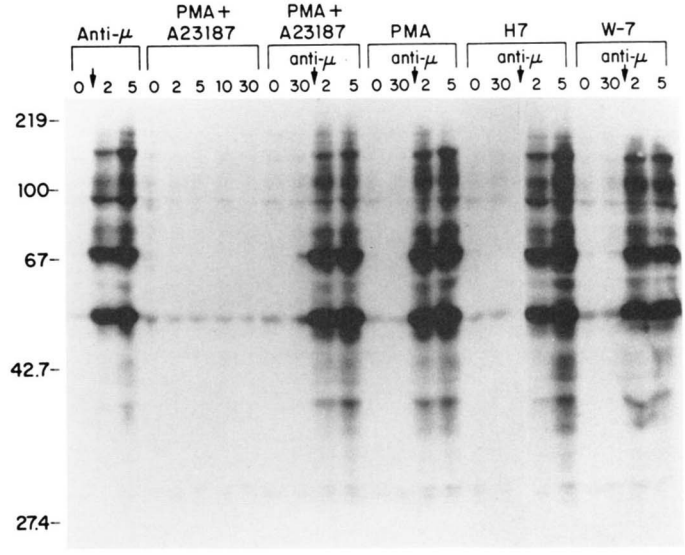

Figure 7. Pretreatment of B cells with PMA, either in combination with ionomycin or with the serine/threonine kinase inhibitors, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride and 1-(5-isoquinolinylsulfonyl-2-methylpiperazine), had no effect on the subsequent ability of anti-μ to induce PTP in dense tonsillar B cells. Dense tonsillar B cells were pretreated for 30 min with the reagents prior to stimulation with goat F(ab')$_2$ anti-μ sera at a final concentration of 10 μg/ml.

linking alone led to the appearance of PTP, and did not appear to affect the induction of PTP by the subsequent addition of anti-Ig (Fig. 7). This effect has also been seen in several experiments where potential contamination between adjacent lanes has been excluded and has also been reported in human T cells (33).

Agonists of PKC and protein kinase A inhibit rise in intracellular calcium but not new PTP. Pretreatment of

B cells with phorbol esters to activate PKC inhibits their subsequent response to anti-Ig (34), and this has been linked to inhibition of the calcium response (35). We confirmed that pretreatment with 10 nM PMA for as little as 5 min inhibits the subsequent rise in $[Ca^{2+}]_i$ induced by cross-linking surface Ig in human B cells (Fig. 5). We tested whether PMA exerted its influence by regulating PTP. PMA alone or in combination with the ionophore, ionomycin, did not induce the pattern of new PTP seen with anti-Ig (Fig. 6). In some experiments a new 40-kDa tyrosine phosphorylated band was seen (data not shown). The reason for interexperiment variability is unknown, but the data are consistent with the observations of Nel et al. (36, 37) who have identified the 40-kDa MAP-2 kinase as a substrate for PTK in lymphocytes. PMA and ionophores induce substantial proliferation in human B cells, indicating that the majority of new PTP observed after receptor activation are not essential for proliferation itself, but may rather control the signal transduction process. Although pretreatment with PMA alone inhibited the rise in $[Ca^{2+}]_i$, it had no effect on the induction of PTP by anti-Ig.

Similarly, activation of protein kinase A has been shown to inhibit signal transduction through the inositol phospholipid pathway. Analogues of cAMP had a modest inhibitory effect on the calcium response (Fig. 5) but no effect was seen on PTP (data not shown). This is similar to data reported for murine T cells (38).

Pretreatment with genistein inhibits turnover of inositol phosphates induced by anti-Ig signaling in B cell line, Ramos. Inhibiting PTK activity in B cells inhibited the rise in $[Ca^{2+}]_i$ associated with anti-Ig signaling. To test directly whether PLC was inhibited in the presence of tyrosine kinase inhibitors, we compared inositol phosphate turnover in genistein and control treated Ramos B cells which had been loaded with ^{3}H-Inositol. In this experiment genistein was used at 100 μg/ml with 5% DMSO as solvent. The viability of cells in genistein or DMSO only in a series of expeiments was similar (>90%). Although incubation with the DMSO solvent alone inhibited inositol phosphate turnover to some extent, the inhibition was much more profound in those cells that had also received genistein (Fig. 8). This data suggests the inhibition of PTK activated following anti-Ig signaling is directly correlated with the failure to activate PLC, rather than to an indirect mechanism whereby a PTP might be involved in raising $[Ca^{2+}]_i$ secondary to PLC activation.

DISCUSSION

This paper provides evidence that the activation of PTK and new PTP is a critical step in the activation of PLC in human B cells. This is comparable to the findings recently reported for murine B cells (39), and for T cells (30) using the PTK inhibitor, genistein. We found genistein and herbimycin had similar effects and completely inhibited the appearance of new PTP. The inhibition by herbimycin and genistein of new PTP is correlated directly with the abolition of the rise in $[Ca^{2+}]_i$, which is associated with inhibition of phosphoinositide turnover. This is unlikely to be due to a non-specific effect of herbimycin, as AlF_4^-, which activates G proteins directly (27), leads to a rise in $[Ca^{2+}]_i$ in herbimycin-treated cells, and the $[Ca^{2+}]_i$ increase induced by AlF4− in normal cells is associated with enhanced phosphoinositide turnover (F. M. McConnell, unpublished observations). Herbimycin-treated B cells also could not be induced to proliferate with phorbol esters and calcium ionophores (data not shown). This is not surprising as critical events during the cell cycle are regulated by cellular PTK such as *src* (40).

Pretreatment of lymphocytes with phorbol esters has previously been shown to inhibit the calcium response after cross-linking of surface IgR. It has been suggested that this effect is due to receptor desensitization (35). Our current data confirm that PMA inhibits the $[Ca^{2+}]_i$ response in human B cells, but we found PMA to have little or no effect on the induction of PTP by anti-μ. Within the limits of our assay, PTK activity did not appear to be affected directly by the activation of PKC, although we do not exclude the possibility that PKC acts to modify the response of a PTK substrate(s). PKC has been shown to affect PLC activity negatively (34, 41, 42), and may also have indirect effects on the calcium response. For example, PKC may indirectly activate protein kinase A (43) which can phosphorylate the IP_3 receptor, reducing its IP_3 sensitivity by a factor of 10 (44). Consistent with such a desensitization, we observed in our current study that analogues of cAMP partially inhibited the calcium release.

The conclusion that, once it is activated, the calcium response might be regulated independently of the PTK, may resolve a paradox created by the receptor desensitization theory (35). Receptor desensitization of the calcium pathway is a rapid event, but lymphocytes require the presence of a stimulus for several hours to initiate cell division (45, 46). Our data show that the capacity for PTP of substrates persists when the calcium signal is suppressed. The activation of receptor-linked PTK may therefore entrain parallel pathways that can thereafter be regulated independently.

Our results support the conclusion that new PTP is a critical event in the activation of PLC. CD45, the membrane associated PTPase (32) expressed on all cells of hemopoietic origin (47), has been implicated in the regulation of lymphocyte activation (31, 48). It seemed possible that it might play a role in the regulation of the PTK activity assayed indirectly by measuring the appearance of PTP after activation. Cross-linking CD45 with mAb inhibited the rise in $[Ca^{2+}]_i$ observed after addition of anti-Ig as we have found previously for class II (10), sIg, CD19, and Bgp95 on B cells, and CD3 and CD2 on T cells (31). Cross-linking CD45 alone lead to the appearance of some new PTP, and CD45 did not suppress the ability of anti-Ig to induce new PTP. This is open to more than one interpretation. Cross-linking CD45 might inhibit its normal phosphatase function, leading to the increased tyrosine phosphorylation observed. Alternatively, CD45 might also be involved in the dephosphorylation of negative regulatory tyrosine residues in the PTK (49) as has been reported for *Ick* in T cells (50). The analysis of tyrosine phosphorylation patterns in $CD45^-$ mutant lines which also fail to signal calcium may help to resolve this point (51).

The dissociation of the inhibition of the calcium signal from the induction of new PTP suggests that CD45 is having additional effects. The PTP assay system used here maybe insufficiently sensitive to detect subtle changes in phosphorylation of individual substrate(s). In the study reported here, we did not detect different patterns of PTP, but in human T cells, differences have been

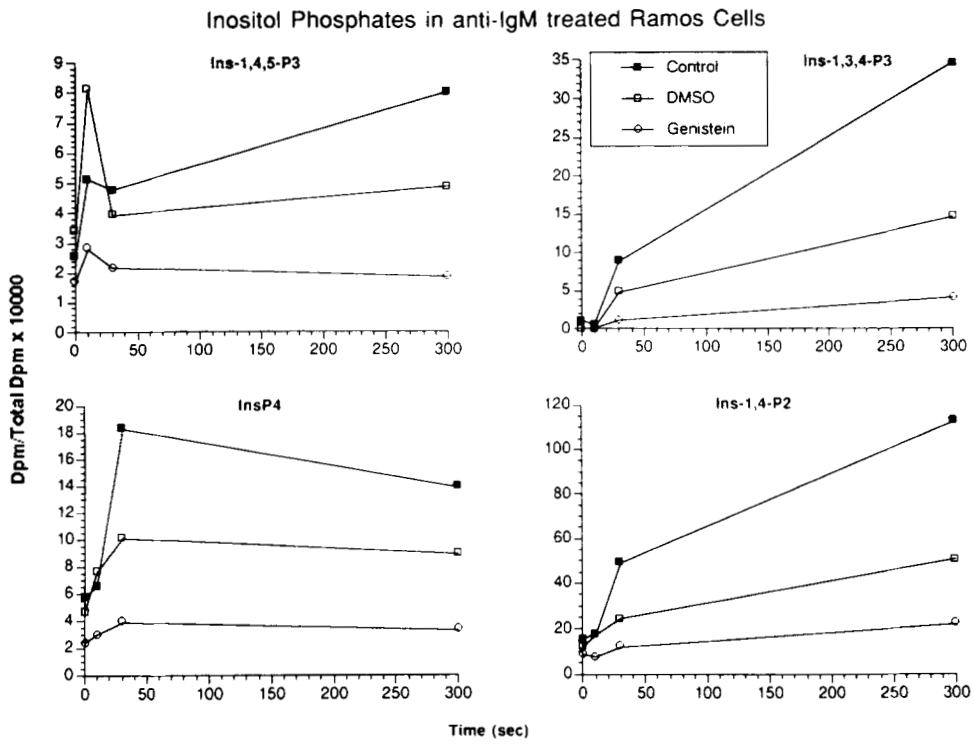

Figure 8. Inositol phosphate turnover in Ramos cells labeled with ^{3}H-Inositol. Cells were preincubated with nothing, DMSO solvent, or Genistein dissolved in DMSO. Samples were taken at different time points as indicated.

observed in the intensity of phosphorylation of a 35 and 150 kDa substrate (52). Several recent studies have shown that PLC is tyrosine phosphorylated following activation through PTK (53, 54) and dephosphorylation of these tyrosine residues inactivates the enzyme. Our data are compatible with CD45 exerting its effect by dephosphorylating PLC. The activation of PLC is likely to be a crucial point of control in lymphocyte signal transduction. Receptor-linked PTK that transmit mitogenic signals usually activate PLC, generating IP_3 and entraining calcium oscillations. These experiments raise the possibility that signal transduction through the PTK may involve independent regulation of the calcium signal and tyrosine kinase activity. One attractive idea is that the overall consequences of PTK activation are determined by the frequency of calcium oscillations entrained by PI turnover, implying that the activity of PLC is a critical site of regulation.

The recent identification of B cell-specific PTK (55) is consistent with the notion that PTK control signal transduction in B lymphocytes at least in part by activating PLC. Recently, many proteins have been shown to become both physically associated and tyrosine phosphorylated by receptor-linked PTK (49). The involvement of GTP-binding proteins like the GTP-ase activating protein may explain the observed GTP-dependence of signal transduction in mammalian lymphocytes (26, 28).

After B cell activation many substrates are tyrosine phosphorylated. In human B cells the pattern is quite similar after activation through surface Ig or class II molecules (10). There are many PTP substrates in activated B and T cells with similar m.w., but there are also distinct substrates. It seems likely that some of the distinguishing substrates include the PTK itself and the proteins that link the Ag-receptor with the PTK. The identified protein components of the CD3 complex and of the proposed Ig complex have homology, sharing conserved tyrosine residues (56) that are therefore likely candidates for tyrosine phosphorylation.

In summary our data indicate that signal transduction in B cells proceeds by the activation of a PTK, which catalyses the activation of PLC, probably via the formation of an intermediate protein complex of tyrosine phosphorylated proteins. The potent synergistic effect of calcium on B cell proliferation provides a potential control point in the mitogenic pathway. The observation that activation of PKC by PMA, Protein kinase A by cAMP, and the tyrosine phosphatase CD45 by mAb in vitro, all inhibit the calcium signal suggests that these proteins may influence the consequences of PTK activation indirectly.

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