Activation of Phospholipase C in Human B Cells Is Dependent on Tyrosine Phosphorylation

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Abstract

Cross-linking of the surface antigen receptor on B lymphocytes has been demonstrated to lead to activation of phospholipase C (PLC) with subsequent increases in production of inositol phosphates and diacylglycerol. In turn, these second messengers increase cytosolic free calcium ([Ca^{2+}]) and activate the serine threonine phosphotransferase protein kinase C (PKC). These processes are thought to play a major role in B cell activation and proliferation. However, the mechanism linking the B lymphocyte antigen receptor to phospholipase C remains to be identified. We demonstrate herein that activation of the antigen receptor on human lymphocytes, in addition to activation of PLC, increases tyrosine phosphorylation of specific substrates. Tyrophostins, a new class of tyrosine kinase inhibitors which compete for substrate binding site of specific tyrosine kinases have recently been synthesized. Preincubation of B lymphocytes with two different tyrophostins blocked anti-IgM-induced proliferation, oncogene expression, tyrosine phosphorylation, increases in [Ca^{2+}], and production of inositol phosphates. The same inhibitors were without effect on B cell proliferation induced by phorbol esters and cation ionophores which directly activate PKC and increase [Ca^{2+}] thus bypassing PLC. These findings strongly indicate that tyrophostins do not exhibit significant nonspecific toxicity and suggest that they act proximal to PLC. The ability of the tyrophostins to block increases in [Ca^{2+}] and inositol phosphate production, after activation of the B cell antigen receptor, indicates that a tyrosine kinase acts as an essential link between the B cell antigen receptor and PLC. (J. Clin. Invest. 1991. 87:1114–1118.) Key words: inositol-trisphosphate (IP_{3}) • protein kinase C (PKC) • phospholipase C (PLC) • protein tyrosine kinase (PTK) • 12-0-tetradecanoylphorbol acetate (TPA)

Introduction

Cross-linking the antigen receptor on B cells with anti-Ig antibody mimics the effect of antigen (1–3). Activation of the B cell antigen receptor leads to phospholipase C (PLC)^-mediated breakdown of membrane phosphatidylinositols which produces two intracellular second messengers, namely inositol trisphosphate (IP_{3}) and diacylglycerol (DAG) (4, 5). In turn, IP_{3} increases cytosolic free calcium ([Ca^{2+}]), while DAG is a physiologic activator of the serine threonine-specific phosphotransferase, protein kinase C. This bifurcating pathway is considered to initiate the cascade of biochemical events leading to B cell activation as well as clonal expansion (4, 5). Consistent with recent reports, we have shown that triggering the antigen receptor on B cells results in a rapid increase in tyrosine phosphorylation of a number of proteins (6–9). Activation of PLC and resultant increases in [Ca^{2+}], induced by the epidermal growth factor, platelet derived growth factor, and T cell receptors, is dependent on tyrosine kinase activity of the receptors (10, 11). To test whether activation of PLC was similarly dependent on tyrosine phosphorylation in B cells, we determined the effect of a series of specific tyrosine kinase inhibitors, designated tyrophostins, on anti-IgM-induced increases in PLC activity. Tyrophostins, in contrast to genesten (12), quercetin (13), and herbimycin, which compete for the ATP binding site and thus may interfere with other kinases and other ATP-dependent processes, compete for the substrate binding sites of tyrosine kinases (14–16). The marked structural differences between tyrosine and serine or threonine leads to a marked specificity for tyrosine kinases.

Using specific tyrophostins, we demonstrate that PLC activation, cell proliferation, oncogene expression, and tyrosine phosphorylation are blocked in parallel. This suggests that a tyrosine kinase acts as an intermediary between the B cell antigen receptor and phospholipase C.

Methods

Cell preparation. Tonsil tissue was obtained from patients undergoing tonsillectomy. Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation. T cell depletion was accomplished by removing, by Ficoll-Hypaque centrifugation, the cells that resettled with 2-aminoethoxyl-isothiouronium bromide-treated sheep erythrocytes. Adherent cells were removed by adherence to plastic dishes for 60 min at 37°C. The resulting B cell-enriched population was < 5% OKT11^- (CD2, T cell marker) and > 95% B1^+ (B cell marker) as measured by immunofluorescence staining. Surface Ig was present on > 95% of the cells.

Reagents. Affinity purified goat antibodies directed against human IgM were purchased from Tago Inc. (Burlingame, CA). For prolif-
ation assays, anti-IgM was cross-linked to CNBr treated sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ) as previously described (17). Ionomycin and 12-O-tetradecanoylphorbol 13-acetate (TPA) were from Calbiochem-Behring Corp. (San Diego, CA). [3H]Thymidine was obtained from Amersham Corp. (Arlington Heights, IL). 125I-labeled protein A was obtained from ICN Pharmaceuticals, Inc. (Irvine, CA). Polyclonal anti-phosphotyrosine antibodies were a kind gift from Dr. T. Pawson (University of Toronto). In addition, affinity purified polyclonal anti-phosphotyrosine antibodies were purchased from Upstate Biotechnology Inc., New York. These antibodies gave identical results to those provided by Dr. T. Pawson. Plasmid DNA for c-fos and β-actin were obtained from the American Type Culture Collection (Rockville, MD).

Tyrophostins were derived from benzylidemalononitrile (BMN) nucleus. These compounds resemble the moieties, tyrosine and erbstatin at the same time. The second cyano group in BMN was found to increase the biological activity as previously described (14–16). The guidelines for selection of compounds included compounds soluble in water as well as in mildly hydrophobic solvents. Such solubility properties allow the compounds to traverse the cell membrane of intact cells.

Cell proliferation. B lymphocytes were incubated in round bottom tissue culture plates (Costar, Cambridge, MA). As indicated, 4 h before termination of the culture 1 μCi [3H]thymidine (6.7 Ci/mmol) was added. The cells were then harvested and samples counted in a liquid scintillation counter.

Western blotting. Western blotting was performed essentially as described (18). B cells were stimulated as indicated, sedimented, and lysed by boiling SDS. Proteins were separated on SDS-PAGE, transferred to nitrocellulose, and blotted with the anti-ptyr antibodies. Antibody reactivity was detected with 125I-labeled protein A and autoradiography.

[Ca2+]i measurement. [Ca2+]i was measured with indo-1 as previously described (5).

Phosphoinositid production. B cells were incubated overnight in inositol-free RPMI (Media Preparations Services, Toronto, Canada) supplemented with glutamine, mercaptoethanol, and 10% FCS. Cells (106) were incubated in inositol-free RPMI with 100 μCi of [3H]inositol (New England Nuclear, Lachine, Quebec) for 4 h. Cells were then incubated with LiCl (20 mM) for 15 min to prevent breakdown of myo-inositol 1-phosphate (IP) and myo-inositol 1,4-bisphosphate (IP2). Cells were incubated with anti-IgM antibodies as indicated. Cells were lysed as described and inositol phosphates isolated by passage over Dowex 1 × 8 columns as previously reported (5). [3H]Inositol was determined by scintillation counting.

Phosphoinositid-bisphosphate phospholipase C assay. Cells were lysed by hypotonic stress, membranes, and cytosol were prepared by density gradient centrifugation. PLC was assayed in final volume of 40 μl containing 25 mM sodium phosphate (pH 6.7), 1 mM sodium pyrophosphate, 40 mM KCl, 0.4 mM EGTA, 0.8 mM CaCl2, 0.5% octylglucoside, and 0.2 mM [3H]phosphatidylinositol-4,5-bisphosphate (5 Ci/mol). After incubation at 37°C for 10 min the reaction was stopped by rapid cooling on ice and by adding 5% trichloroacetic acid. The precipitate was removed and radioactivity present in 0.5 ml of the supernatant was measured as previously described (11, 19).

Northern blot analysis. RNA was extracted from cells in guanidium isothiocyanate according to the method of Chomczynski and Sacchi (20). Purified RNA (10 μg) was electrophoresed, subsequently transferred to nitrocellulose, blotted, and hybridized with labeled probes as previously described (21).

Results

We used Western blotting with specific polyclonal anti-phosphotyrosine antibodies to test whether cross-linking the B cell antigen receptor with anti-IgM antibodies results in increases in tyrosine phosphorylation. These polyclonal anti-phosphotyrosine antibodies have been demonstrated to react specifically with phosphotyrosine and not with phosphoserine or phosphothreonine (18).

Incubation of B lymphocytes with anti-IgM antibodies induced a rapid increase in tyrosine phosphorylation of three major bands with molecular weights 74, 93, and 110 kD and three minor bands with molecular weights of 46, 57, and 145 kD (Fig. 1). Tyrosine phosphorylation was detected within 30 s of addition of anti-IgM antibodies with maximal increases in tyrosine phosphorylation observed 3-5 min later (not presented). Previous reports have demonstrated that binding of antibody to the antigen receptor on human B cells (6, 7) and murine B cells (8, 9) was associated with increased phosphorylation of tyrosine residues. However, a direct link between this early event and proliferation of B cells has not been established. As indicated in Table I, the tyrophostins, AG-30 and AG-126, blocked B cell proliferation induced by anti-IgM antibodies bound to beads. AG-30 and AG-126 did not alter B cell proliferation induced by TPA and ionomycin, indicating that the inhibition of anti-IgM-induced proliferation was not due to nonspecific toxicity. In contrast, AG-183, AG-294, and AG-376, which are effective inhibitors of purified epidermal growth factor receptor tyrosine kinase activity (14, 15), had little effect on anti-IgM-induced proliferation at similar concentrations (Fig. 2). Remarkably, AG-30 and AG-126 are relatively ineffective inhibitors of the EGF receptor, tyrosine kinase (19; and Levitzi et al., unpublished observations).

As indicated in Fig. 1, preincubation with 50 μM of AG-30 and AG-126 blocked anti-IgM-induced tyrosine phosphorylation. Note that preincubation with the tyrophostins did not decrease the phosphotyrosine levels to zero, but rather resulted in a pattern that was indistinguishable from the pattern of nonstimulated cells. At least 2 h of preincubation was required to

Figure 1. Effect of tyrophostins on anti-IgM antibody-induced tyrosine phosphorylation. Anti-IgM antibodies (20 μg/ml) were added to B cell cultures in the presence or absence of the tyrophostins AG-30 (50 μM) and AG-126 (50 μM). Cells were incubated with the tyrophostins 2 h before and terminated 3 min after the addition of anti-IgM antibodies. After electrophoresis and blotting, the phosphoryrosine-containing proteins were detected using phosphotyrosine antibodies and 125I-labeled protein A. The resultant autoradiogram is displayed.
consistently block tyrosine phosphorylation (not presented), which is similar to observations in other cell types (14, 16). Once again, AG-183, AG-294, and AG-376 had little effect on anti-IgM-induced tyrosine phosphorylation (not presented).

One of the earliest events following cross-linking the B cell antigen receptors is an increase in $[\text{Ca}^{2+}]_i$ (5). This is believed to result, at least in part, from PLC-mediated increases in IP₃ production (4, 5). Since increases in tyrosine phosphorylation preceded increases in $[\text{Ca}^{2+}]_i$, but mimicking PLC activation (by treatment with TPA and ionomycin), did not increase tyrosine phosphorylation (not presented), tyrosine phosphorylation may be an upstream event required for PLC activation. We tested this possibility by blocking anti-IgM-induced increases in tyrosine phosphorylation with AG-30 and AG-126 and measured the effect on ligand-induced increases in $[\text{Ca}^{2+}]_i$ and IP₃. We also measured IP levels since it is the major metabolic product of IP₃ in these cells wherein further metabolism was blocked with lithium. As indicated in Fig. 3, AG-30 and AG-126 inhibited anti-IgM-induced changes in $[\text{Ca}^{2+}]_i$, IP, and IP₃ production in a dose-dependent manner. The effects of AG-30 and AG-126 on ligand-induced increases in $[\text{Ca}^{2+}]_i$, IP, and IP₃ production were roughly parallel. Similar to the effects on anti-IgM-induced tyrosine phosphorylation, at least 2 h of preincubation was required to consistently block anti-IgM-induced changes in $[\text{Ca}^{2+}]_i$, IP, and IP₃ production (not presented).

One of the earliest correlates of mitogenesis in a variety of cell types is the increased expression of the nuclear proto-oncoprotein, c-fos (22, 23). Expression of this gene increases within 5 min and reaches maximal expression ~ 30 min after stimulation of human B cells by mitogens (24). It has been postulated that this protein regulates the subsequent expression of other genes in the cascade which ultimately culminates in proliferation. Because of its rapid onset, c-fos expression was used to study the effect of AG-30 and AG-126 on anti-IgM-induced B cell activation. Northern blot analysis of total cytoplasmic RNA extracted from B cells incubated for 30 min with anti-IgM indicated that these agents increased fos expression (Fig. 4). Consistent with our previous observations (24), TPA and ionomycin or anti-IgM induced a 7.5- and 6-fold increase in c-fos expression, respectively. Preincubation of B cells with AG-30 or AG-126 for 2 h before treatment with anti-IgM prevented the anti-IgM-induced increases in c-fos RNA expression (Fig. 4). In contrast, AG-30 and AG-126 did not inhibit the TPA and ionomycin-induced increase in c-fos RNA expression in B cells (not presented). This finding suggests that the tyr-
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In the absence or presence of AG-30 (50 μM) or AG-126 (50 μM). RNA was extracted after 30 min of incubation and analyzed by Northern Blots as described in Methods. The same blot was probed first with a c-fos probe, then stripped and reprobed with the β-actin probe. Densitometric analysis indicated that in this experiment, treatment with anti-IgM alone resulted in sixfold increase in c-fos but no change in β-actin mRNA levels.

Figure 4. Effect of tyrphostins on levels of c-fos and β-actin RNA after stimulation with anti-IgM antibodies. Tonsil-derived B cells (3 × 10^6 cells/ml) were incubated with anti-IgM (20 μg/ml) or with TPA (10^4 M) and ionomycin (1 μg/ml) in the presence or absence of AG-30 (50 μM) or AG-126 (50 μM). RNA was extracted after 30 min of incubation and analyzed by Northern Blots.

Discussion

In this study, we have demonstrated that tyrphostins AG-30 and AG-126 prevent anti-IgM-induced proliferation, oncogene expression, tyrosine phosphorylation, and increases in [Ca^{2+}], IP, and IP3. In contrast, AG-183, AG-294, and AG-376, in similar concentrations which are effective inhibitors of purified epidermal growth factor receptor tyrosine kinase activity (14–16), had little effect on anti-IgM-induced changes in any of these processes. In addition, AG-30 and AG-126 did not alter proliferation induced by TPA and ionomycin, indicating that they did not exhibit significant nonspecific toxicity at the concentrations used.

The ability of the tyrphostins to block anti-IgM-induced increases in [Ca^{2+}], IP, and IP3 indicates that a tyrosine kinase likely acts as an intermediary between the B cell antigen receptor and PLC. This is further indicated by the lack of a direct effect of AG-30 and AG-126 on the enzymatic activity of PLC. Since PLCγ has been demonstrated to be a substrate for the tyrosine kinase activity of both the EGF and PDGF receptors, it is attractive to hypothesize that PLCγ is a substrate for the tyrosine kinase activated by the B cell antigen receptor. Antigen-induced tyrosine phosphorylation of a band at 145 kD is consistent with this possibility.

The results reported herein with the tyrphostins are similar to those reported in T cells using genistein (11). However, in contrast to the tyrphostins which inhibit tyrosine phosphorylation by competing for the substrate binding site, genistein and herbimycin compete for the ATP binding site and have a greater potential for nonspecific effects. Nevertheless, the data

![Figure 3](image-url)

**Figure 3.** The effect of tyrphostins on anti-IgM-induced inositol phosphate production and changes in [Ca^{2+}]. (Top) Tonsil B cells were incubated overnight with [H]inositol. Cells were incubated with anti-IgM antibody (20 μg/ml) for 15 min, in the presence or absence of various concentrations of AG-30 (A), or AG-126 (B). Cells were lysed and inositol phosphates separated as described in Methods, and values for IP1 (○) and total IP (□) were determined. Control values (medium alone) were 714±71 cpm for IP1 and 13,490±1,450 cpm for total IP (IP1 + IP2 + IP3). Results represented the mean±SD for four replicates pooled from three individual experiments. (Bottom) Cells (2 × 10^7) were loaded with Indo-1 by a 30-min incubation with 2 μM of the acetoxymethylester derivative. 1 × 10^7 cells were preincubated with various concentrations of either AG-30 (C) or AG-126 (D) for 2 h. After extensive washing, cells were added to Ca^{2+}-containing medium. The baseline was determined and 20 μg of anti-IgM antibodies added. The values represent the mean±SEM increase in [Ca^{2+}], of six different determinations.

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suggest that in B cells as well as in T cells, a tyrosine kinase acts as an intermediary between the antigen receptor and PLC. The ability of TPA and ionomycin, which mimic the effects of the products of PLC, diacylglycerol, and IP₃, and bypass the inhibitory effect of the tyrophostins, suggests that a tyrosine kinase is located upstream but not downstream of the mediators.

In summary, two specific tyrophostin tyrosine kinase inhibitors, AG-30 and AG-126, blocked anti-IgM-induced proliferation, oncogene expression, tyrosine phosphorylation and increases in [Ca²⁺]ᵢ, IP₃, and IP₃. They were without effect on TPA and ionomycin-induced B cell proliferation. Taken together, this indicates that a tyrosine kinase acts as an intermediary between the B cell antigen receptor and PLC. The identity of this kinase remains to be demonstrated. The ability of some but not all tyrophostins to block anti-IgM-mediated activation of PLC may provide an important probe to assist in identifying the kinase. Further, the involvement of tyrosine kinase activity of many oncogene products in the expression of the transformed phenotype identifies these proteins as potential targets for selective chemotherapy and thus, for rational drug design.

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References


