

Phospholipid methylation: A biochemical signal modulating lymphocyte mitogenesis

(arachidonic acid/prostaglandins/concanavalin A/methylation inhibition)

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ABSTRACT Phospholipid methylation in murine T lymphocytes but not B cells was stimulated by mitogenic lectins such as concanavalin A and phytohemagglutinin, and the methylation was then returned to the control level by the concomitant activation of phospholipase A₂. A parallelism between dose-response curves of concanavalin A for phospholipid methylation and thymidine incorporation was found. Inhibition of either synthesis or degradation of methylated phospholipids resulted in a decrease in the thymidine incorporation. Although prostaglandins such as the E and F series were the main products of arachidonic acid released by phospholipase A₂ activation, inhibition of synthesis of these compounds by indomethacin did not reduce the thymidine incorporation significantly. These results suggest that the mitogenesis of murine T lymphocytes is triggered by the activation of both phospholipid methyltransferase(s) and phospholipase A₂.

The binding of macromolecules such as antibodies and lectins to the lymphocyte surface triggers the stimulation of DNA synthesis and mitogenesis (1). Recent studies have shown that the mobility and distribution of receptors on the cell surface are modulated by the binding of lectins and have suggested that the signal transduction is associated with the changes in both the outside and the inside of the cell membrane complex (2). Because our previous studies had demonstrated that phospholipid methylation can alter the structure and function of biomembranes (3, 4), we examined the effect of mitogenic lectins on this biochemical process in the lymphocyte. We report that concanavalin A (Con A), a mitogen that stimulates murine T cells, also induces a transient activation of phospholipid methylation with concomitant increase of degradation of methylated phospholipids in lymphocytes.

EXPERIMENTAL PROCEDURES

Murine spleen cells were purified from BALB/c mice by Ficoll/Hypaque density centrifugation (5). The cells (50×10^6) were incubated in 10 ml of 1% fetal calf serum/99% RPMI 1640 medium containing 1 mCi (1 Ci = 3.7×10^{10} becquerels) of L-[methyl-³H]methionine (72 Ci/mmol) or 10 μ Ci [1-¹⁴C]-arachidonic acid (55.5 mCi/mmol) at 37°C in a humidified atmosphere of 5% CO₂/95% air for 1 hr. To measure the phospholipid methylation, 0.2 ml of the cell suspension was transferred to a test tube and the reaction was started by the addition of Con A (final concentration, 2 μ g/ml). To stop the reaction, 0.5 ml of 10% trichloroacetic acid containing 10 mM methionine was added. After centrifugation at $10,000 \times g$ for 10 min, the precipitates were washed with 0.5 ml of 10% trichloroacetic acid and then were extracted with 3 ml of chloroform/methanol (2:1, vol/vol) as described (3). To measure the release of [1-¹⁴C]arachidonic acid and its metabolites, the

excess radioactive arachidonic acid was removed after 1-hr incubation by washing the cells three times with fresh media containing 0.5% fatty acid-free albumin. The reaction was started by the addition of Con A and terminated by the addition of 1 ml of ice-cold 10 mM phosphate-buffered saline, pH 7.4. After centrifugation at $4000 \times g$ for 5 min an aliquot (500 μ l) of the supernatant was measured for radioactivity.

The metabolites of arachidonic acid such as prostaglandin A, B, E, and F series were analyzed by thin-layer chromatography in silica gel G plates with a solvent system of the upper phase of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (90/20/20/100, vol/vol), after the extraction with ethyl ether.

Phospholipids were analyzed by two-dimensional thin-layer chromatography with the solvent systems of chloroform/methanol/ammonia (60/35/5, vol/vol) and chloroform/methanol/acetone/acetic acid/water (50/10/20/10/5, vol/vol) or by one-dimensional chromatography with a solvent system of propionic acid/1-propanol/chloroform/water (3/2/2/1, vol/vol) as described (4). Incorporation of [³H]thymidine (1.0 μ Ci per tube, specific activity, 5 Ci/mmol) was measured after 45 hr of incubation as described (5).

RESULTS

Stimulation of Phospholipid Methylation by Mitogenic Lectins. When lymphocytes prepared from murine spleen were incubated with Con A, the incorporation of the [³H]methyl group from L-[methyl-³H]methionine into lymphocyte lipids increased considerably in the first 10 min and then gradually decreased to the control level (Fig. 1A). To compare the dose-response curves of Con A for mitogenesis and phospholipid methylation, identical culture conditions were employed. About the same concentrations of Con A were required to elicit maximal [³H]methyl incorporation into the lipid fraction at 10 min and the subsequent [³H]thymidine incorporation at 45 hr (Fig. 2). High doses of Con A were inhibitory for both events. The parallelism between the dose-response curve for phospholipid methylation and mitogenesis of lymphocytes suggests that enhanced phospholipid methylation represents an integral step in the mitogenic process.

To support our contention further, the specificities of other lectins were examined for mitogenesis and phospholipid methylation (the details will be published elsewhere). When lymphocytes from murine spleen were employed, the mitogenic lectins Con A, phytohemagglutinin from *Phaseolus vulgaris*, *Wistaria floribunda* mitogen, and *Pisum sativum* mitogen caused a transient stimulation followed by a decrease of phospholipid methylation. However, nonmitogenic lectins such as *Wistaria floribunda* agglutinin and *Bauhinia purpurea* lectin did not affect phospholipid methylation significantly.

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Abbreviations: Con A, concanavalin A; 3-deaza-SIBA, 5'-deoxy-5'-isobutylthio-3-deazaadenosine.

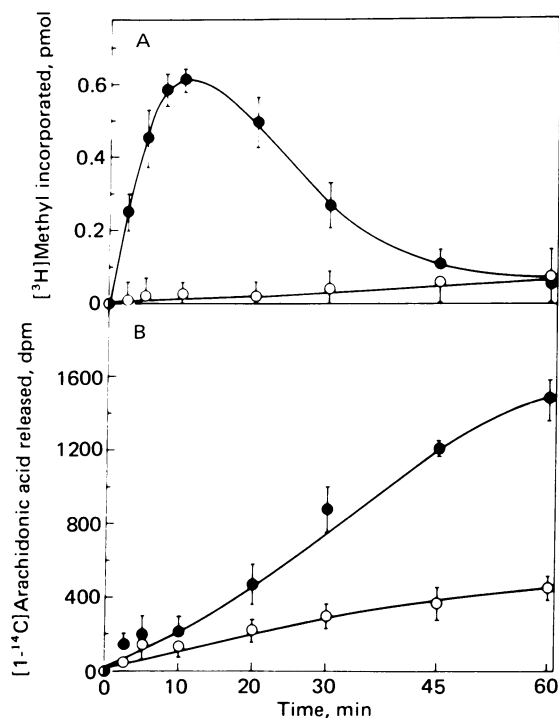


FIG. 1. Effect of Con A on phospholipid methylation (A) and arachidonate release (B) in murine spleen cells. Phospholipid methylation was measured as described in the text, using 10^6 cells. Each point represents the mean \pm SD ($n = 4$). ●, With Con A; ○, control without Con A. During the preincubation, approximately 0.6 pmol of [^3H]methyl group was incorporated into the lymphocyte lipids. This value was subtracted from the total amounts of phospholipid methylation, because no significant increase was observed thereafter. The splenocytes were pre-labeled with [^{14}C]arachidonic acid as described in the text. Under the conditions of preincubation used, 15%, 5%, 5%, 10%, and 60% of the [^{14}C]arachidonic acid were incorporated into phosphatidylethanolamine, phosphatidyl-*N*-monomethylethanolamine, phosphatidyl-*N,N*-dimethylethanolamine, phosphatidylserine (plus phosphatidylinositol), and phosphatidylcholine, respectively, when these lipids were separated and quantified by thin-layer chromatography. About 10% of the arachidonate incorporated into phospholipids was released after Con A treatment. The released radioactivity, expressed as [^{14}C]arachidonic acid released, included free arachidonic acid and its metabolites as described in the text. Counting efficiency was 41% for ^3H and 81% for ^{14}C .

Selective Stimulation of Phospholipid Methylation in T Cells. Lymphocytes prepared from spleens of *nude* mice are deficient in classical T cells and do not respond to Con A by increased mitosis. Con A did not significantly change phospholipid methylation in these lymphocytes. When B cells in normal spleen cultures were selectively destroyed by treatment with anti-IgG and complement, phospholipid methylation was not affected. However, when T cells of normal splenocytes were destroyed by treatment with anti- θ and complement, the stimulation of phospholipid methylation by Con A disappeared. Furthermore, Con A caused a larger stimulation of phospholipid methylation in murine thymocytes than in normal splenic lymphocytes. All of these observations suggest that Con A stimulates phospholipid methylation in T cells but not B cells. The same specificity was shown for increased DNA synthesis.

Concomitant Activation of Phospholipid A_2 by Mitogenic Lectins. The stimulation of lymphocytes with Con A also caused a release of [^{14}C]arachidonic acid and its metabolites from phospholipids pre-labeled with this fatty acid (Fig. 1B). Arachidonic acid is known to be incorporated into the 2 position of glycerophospholipids (6). As previously shown (7), the main

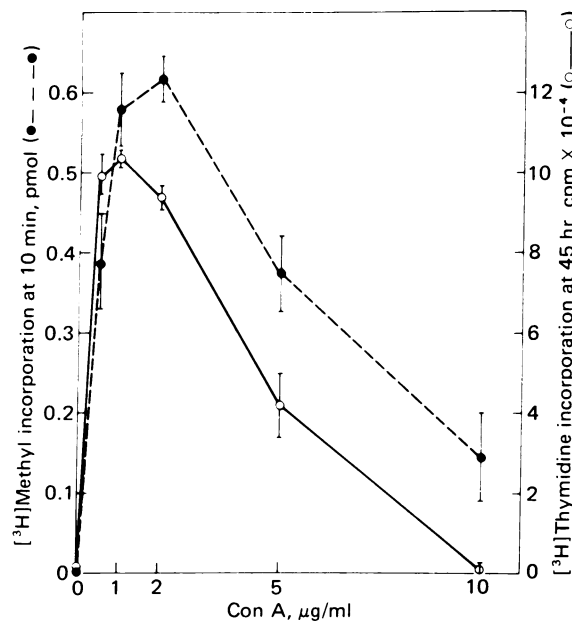


FIG. 2. Effect of various concentrations of Con A on phospholipid methylation and thymidine incorporation. Phospholipid methylation of lymphocytes 10 min after stimulation with Con A and thymidine incorporation 45 hr after the Con A stimulation were measured as described in the text. Each point represents the mean \pm SD ($n = 4$).

metabolites of arachidonate are prostaglandins. The amounts of free arachidonic acid and the prostaglandin E, F, and B (plus A) series were 30%, 14.2%, 4%, and 13.5%, respectively, at 10 min, and 17.3%, 14.3%, 6.8%, and 13.7%, respectively, at 60 min. An unidentified metabolite(s) remained at the origin with the solvent system employed. This unidentified metabolite represented 10–30% of the total radioactivity in the media. The stimulation of arachidonic acid release by the mitogenic lectin suggests that phospholipase A_2 , an enzyme that removes unsaturated fatty acids from phospholipids, is activated by Con A. This was supported by the observation of a 2- to 3-fold increase in the accumulation of [^3H]lysophosphatidylcholine, a product of phospholipase A_2 , 10 min after the stimulation with Con A as measured by thin-layer chromatography. After 30 min, the level of lysophosphatidylcholine returned to that of the control, indicating that this compound was further metabolized. The release of arachidonate and its metabolites as well as the decrease of methylated phospholipids could not be detected when Ca^{2+} was removed from the medium, indicating the requirement of Ca^{2+} for this lipase as previously described (5).

Inhibition of Phospholipid Methylation Reduces Lymphocyte Mitogenesis. To further examine the relationship between the activation of phospholipid methylation and mitogenesis, 5'-deoxy-5'-isobutylthio-3-deazaadenosine (3-deaza-SIBA), an analogue of the methyltransferase inhibitor S-adenosylhomocysteine (8), was added to the culture medium. The degree of inhibition of phospholipid methylation was dependent upon the incubation period with this compound prior to the exposure to Con A (Fig. 3). There was a clear association between the inhibition of phospholipid methylation and the inhibition of thymidine incorporation. The maximal inhibition was attained by incubating the lymphocytes with 10 μM 3-deaza-SIBA for 60 min before adding Con A. No inhibition was observed when these reagents were added simultaneously. Furthermore, other transmethylation reactions such as nucleotide methylation and binding of [^3H]Con A to lymphocytes

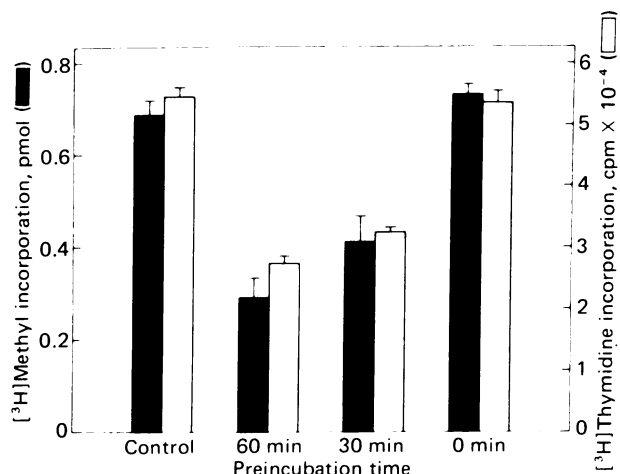


FIG. 3. Effect of 3-deaza-SIBA on phospholipid methylation and thymidine incorporation. Spleen lymphocytes were preincubated with 10 μ M 3-deaza-SIBA for various intervals as indicated. Phospholipid methylation at 10 min and thymidine incorporation at 45 hr after the addition of Con A (2 μ g/ml) were measured as described in the text. 3-Deaza-SIBA was generously provided by P. K. Chiang, National Institute of Mental Health. Each bar represents the mean \pm SD ($n = 4$). Under these conditions, no significant inhibition of thymidine incorporation by 3-deaza-SIBA was observed in the control cells (1860 \pm 225 cpm).

were not significantly inhibited under these conditions (unpublished data). The inhibition of both phospholipid methylation and thymidine incorporation was dose dependent. Preliminary experiments have shown that the inhibition of phospholipid methylation by 3-deaza-SIBA resulted in a marked decrease in the release of arachidonate and its metabolites into the media. These data suggest that arachidonate originates from the methylated phospholipids. Comitogens such as the Ca^{2+} ionophore A-23187 and phorbol esters, which, when added alone, increased [³H]thymidine incorporation to much lesser degree than Con A (1), caused only an increase in the degradation of phospholipids as measured by the release of [¹⁴C]-arachidonic acid and its metabolites. These observations indicate that, after increased methylation, the degradation of the phospholipids is also involved in the stimulation of mitogenesis. Because phospholipase A_2 is concomitantly activated by lectins, attempts were made to inhibit both phospholipase activity and mitogenesis of lymphocytes by the use of the phospholipase A_2 inhibitors mepacrine (quinacrine) and tetracaine (6). These compounds decreased both the release of arachidonic acid and the incorporation of [³H]thymidine (Table 1). However, these drugs might be toxic to the cells after 45 hr because they also inhibited the thymidine incorporation in the control cells. In-

Table 1. Effect on thymidine incorporation in lymphocytes of drugs that affect the arachidonate metabolism

Drug	Thymidine incorporation, cpm	
	Without Con A	With Con A
None	2091 \pm 232	176,511 \pm 8,357
Tetracaine, 1 μ M*	1497 \pm 237 [†]	93,129 \pm 12,323 [†]
Mepacrine, 1 μ M*	728 \pm 30 [†]	81,237 \pm 17,207 [†]
Indomethacin, 1 μ M* [‡]	1758 \pm 262	149,162 \pm 6,911

Thymidine incorporation was measured with and without 2 μ g of Con A as described in the text. Data are mean \pm SEM ($n = 6$).

* Tetracaine, mepacrine, or indomethacin at 1 μ M inhibited the arachidonate release by 38%, 45%, or 5%, respectively.

[†] Significant at $P < 0.05$ in comparison with "None."

[‡] Indomethacin at 1 μ M almost completely blocked prostaglandin synthesis.

domethacin, an inhibitor of prostaglandin synthesis, showed no inhibition of [³H]thymidine incorporation, suggesting that the prostaglandins synthesized mainly after the release of arachidonate do not affect the DNA synthesis of the lymphocytes. However, it is possible that other metabolites of arachidonic acid generated by lipo-oxygenase might have some effect on the mitogenesis.

DISCUSSION

Our present results demonstrate that activation of murine T lymphocytes by mitogenic lectins causes a transient increase and decrease of methylated phospholipids. This transient stimulation of phospholipid methylation is not observed with nonmitogenic lectins. Mitogenic lectins also cause activation of phospholipase A_2 to generate arachidonic acid and lysophosphatidylcholine. Arachidonate is further metabolized to prostaglandins. Inhibition of phospholipid methylation by 3-deaza-SIBA brought about the reduction of thymidine incorporation. However, inhibition of prostaglandin synthesis by indomethacin had little effect on mitogenesis, whereas phospholipase A_2 inhibitors such as tetracaine and mepacrine appeared to be toxic to the cells. All of these results indicate that the stimulation of phospholipid methylation with the concomitant activation of phospholipase A_2 is an important signal for the subsequent mitogenesis of the lymphocytes. The exact roles of arachidonate, lysophosphatidylcholine, and their metabolites in lymphocyte mitogenesis remain to be established. Lysophosphatidylcholine has been reported to increase guanylate cyclase (9). Transient accumulation of lysophosphatidylcholine might play a role in the generation of cyclic GMP, a nucleotide involved in mitogenesis (1).

Adenosine deaminase deficiency causes immunological insufficiency, including ablastosis (10, 11). Recent studies with inhibitors of the deaminase have demonstrated that one of the most prominent metabolic changes is an intracellular accumulation of *S*-adenosyl-L-homocysteine, a competitive inhibitor of transmethylation reactions (3, 4, 12). *S*-Adenosylhomocysteine analogues, including 5'-deoxy-5'-isobutylthioadenosine, have been reported to inhibit blastogenesis (13). These observations are consistent with our present results. The cellular level of *S*-adenosyl[³H]methionine formed by the incubation with [*methyl*-³H]methionine was markedly reduced by the stimulation with Con A at 10 min and then gradually recovered (unpublished data). These observations suggest that transmethylation reactions including phospholipid methylation are stimulated by Con A. When 3-deaza-SIBA was used, it inhibited phospholipid methylation but not incorporation of [³H]methyl group into the proteins and nucleotides. Because the turnover of phosphatidylcholine has been reported to increase immediately after the stimulation with lectins (1), it is likely that phospholipid methylation is disturbed in patients with adenosine deaminase deficiency.

Our work with rat erythrocytes has demonstrated that two methyltransferases of phospholipids are asymmetrically distributed in the membrane (3). The successive methylation of phosphatidylethanolamine present on the cytoplasmic side of the membrane causes a rapid translocation of the lipids to the outer surface. The accumulation of the intermediate phosphatidyl-*N*-monomethylethanolamine within the membrane results in a marked increase in membrane fluidity (14). Transient changes of membrane fluidity found after stimulation of lymphocytes with lectins (5) might be the result of the activation of phospholipid methylating enzymes. An increase of Ca^{2+} influx has also been reported as one of the early events (1). Preliminary results suggest that the inhibition of phospholipid methylation blocks Ca^{2+} influx stimulated by lectins. In rat

reticulocyte ghosts and intact HeLa cells, phospholipid methylation is stimulated by binding of ligands to β -adrenergic receptors which, in turn, enhances the lateral mobility and coupling of the receptors to adenylate cyclase (4). It may be possible to correlate phospholipid methylation with redistribution of the receptors by employing monovalent, divalent, and tetravalent Con A.

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