The Rapid Induction by Phytohemagglutinin of Increased α-Aminoisobutyric Acid Uptake by Lymphocytes

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ABSTRACT The effect of phytohemagglutinin (PHA) on the ability of human lymphocytes to transport the nonutilizable amino acid, α-aminoisobutyric acid (AIB) has been studied. PHA binds rapidly to plasma membrane receptor sites with half maximal binding requiring approximately 7.5 min. During the first 30 min after PHA addition to lymphocytes no change was detected in AIB transport, but then a linear increase in the initial rate of AIB transport occurred over the next 9 hr. Subsequently, the rate of AIB transport stabilized at a level 6–7 times greater than that found in control lymphocytes. The change in membrane function developed even when *de novo* protein synthesis was inhibited by 85–90% with puromycin or cycloheximide. However, the PHA effect did not occur when the lymphocytes were maintained at 4°C.

Studies of the kinetics of AIB uptake by control and PHA-treated lymphocytes demonstrated that PHA increases the V_{max} of AIB uptake by 6–7-fold (0.7 mμmole AIB per 10^6 lymphocytes/15 min versus 0.1 mμmole per 10^6 lymphocytes/15 min) without affecting the Km (Michaelis constant) of the transport system (2mM in both cases).

When fetuin was added to lymphocyte cultures to remove bound PHA, the PHA-induced increase in the rate of AIB uptake was arrested at the rate achieved during the time of prior incubation with PHA. This level of AIB transport persisted for at least 3 hr after 80% of the PHA was removed from the cell membrane.

These data demonstrate that PHA rapidly induces a change in a lymphocyte cell membrane transport function, and that the continued presence of PHA on the cell membrane is required for the full stimulatory effect to be reached. The data do not distinguish between a direct action of PHA upon the lymphocyte membrane or the possibility that PHA slowly enters into the cell where it then exerts its effect.

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INTRODUCTION

When phytohemagglutinin, a glycoprotein derived from the red kidney bean, is incubated with small lymphocytes, they undergo a series of metabolic changes resulting in morphologic transformation and mitosis. The mechanism whereby PHA [1] stimulates lymphocytes to undergo mitosis is unknown. While numerous investigators have studied a variety of metabolic alterations which occur after lymphocytes are exposed to PHA (for a recent review see Reference 1), little attention has been focused on the effect that PHA may have on the lymphocyte plasma membrane. Recently, Fisher and Mueller (2) have reported that within minutes after PHA is added to lymphocytes there is an increase in the rate of ^{32}P incorporation into membrane phospholipids. However, the consequences of PHA binding to lymphocytes on the functional characteristics of the plasma membrane need to be further explored. Therefore we have investigated the effect of PHA on a membrane-mediated process, namely the uptake of α-aminoisobutyric acid, a nonutilizable amino acid (3).

METHODS

Bacto-phytohemagglutinin P was obtained from Difco Laboratories, Detroit, Mich. Dextran-grade H with a mol wt of approximately 180,000 was purchased from Pharmachem Corporation, Bethlehem, Pa. Ficoll (mol wt of approximately 400,000), crystallized bovine serum albumin, and puromycin dihydrochloride grade II were obtained from Sigma Chemical Company, St. Louis, Mo. Winthrop Laboratories, New York, was the source of sodium diatrizoate (Hypaque). Eagle's minimal essential medium for suspension

[1] *Abbreviations used in this paper:* PHA, phytohemagglutinin; AIB, α-aminoisobutyric acid.

cultures (4), fetal calf serum, and fetuin (Spiro Method) were purchased from Grand Island Biological Company. The fetuin was dialyzed against 0.9% NaCl prior to use. Cycloheximide came from Nutritional Biochemicals Corporation. New England Nuclear Corp., Boston, Mass. was the source of α-aminoisobutyric-1-^{14}C acid, uridine-5-^{3}H, and thymidine-methyl-^{3}H. Reagent grade ^{131}I was purchased from Mallinckrodt Chemical Works, St. Louis, Mo.

Preparation of PHA. Difco PHA-P was further purified by sequential SE-Sephadex G-50 column chromatography and Sephadex G-150 gel filtration according to the method of Weber, Nordman, and Gräsbeck (5). The erythroagglutinating PHA (Peak III of Weber et al.), which gave a single broad band on disc gel electrophoresis, was used in all incubation studies with lymphocyte cultures. The material is a potent mitogen. This PHA was iodinated with ^{131}I by the chloramine-T method of Hunter (6).

Preparation of lymphocytes. In order to obtain peripheral blood lymphocytes as free as possible from contamination with granulocytes, monocytes, erythrocytes, and platelets the following procedure was devised (Fig. 1). Blood donors were normal subjects on no medications and without recent coffee intake. Approximately 100–400 ml of blood were collected in 50 ml plastic syringes using sterile precautions in subsequent steps only in experiments of greater than 12 hr duration. The blood was defibrinated by continuous rotation for 5 min in a 500 ml Erlenmeyer flask containing a dozen 6 mm glass beads. This procedure eliminates platelets, which are taken up in the clot. ⅕ volume of saline containing 6 gm of dextran per 100 ml was added, and the blood was then allowed to settle for 1 hr at 37°C in 100 ml graduated cylinders (7). The serum-dextran layers, enriched for leukocytes, were removed, pooled, and mixed with 2 volumes of saline. 36 ml portions were layered over 9 ml of the following solution in a 50 ml conical glass centrifuge tube: 1.80 ml Hypaque 50% weight per volume, 6.35 ml Ficoll 9 *g* per 100 ml water, and 0.85 ml water (8, 9). Isopycnic centrifugation was performed at room temperature in an International PR-2 centrifuge (International Equip, Needham Heights, Mass.) at 500 *g* for 40 min, or in later experiments at 1500 g for 10 min with no decrease in yield of lymphocytes. The white fluffy interface layer between the dextran-serum phase and the Ficoll-Hypaque phase was removed by aspiration. This layer contained 75–92% lymphocytes, with 8–25% granulocytes and monocytes and 1–8% contamination with erythrocytes. The cells were then washed twice with saline by centrifugation at 500 *g* for 5 min and resuspended in 10 ml of the donor's fresh serum. The suspension was poured through a nylon fiber column packed in a 40 ml syringe and eluted with 100 ml minimal essential medium (10). The eluted cells were harvested by centrifugation and resuspended in the incubation medium (see below) at a cell concentration of 5×10^6–2×10^7 cells per ml. Differential counts showed 99–100% lymphocytes with the remaining nucleated cells consisting of monocytes or granulocytes. The ratio of RBC:WBC was 1:100 and platelets were not present. The recovery of lymphocytes was 30-50%. The entire procedure required 3–4 hr.

Lymphocyte incubation studies. For short term experiments lasting up to 12 hr, the incubation medium consisted of minimal essential medium containing albumin 3 g per 100 ml, glutamine 4 mM, penicillin 5,000 U per 100 ml, and streptomycin 5,000 μg per 100 ml. For experiments of longer duration, the albumin was replaced with 7.5% fetal calf serum. Lymphocytes suspended in incubation medium were distributed in 0.2 ml portions into 15 ml conical glass centrifuge tubes to give a final cell count of 10^6–4×10^6 cells per tube. The cell suspensions were incubated without agitation in an incubator at 37°C with an atmosphere of 5% CO_2 and 95% air. Upon completion of incubation with ^{14}C-AIB the tubes were chilled in ice and washed 3 times with 5 ml of cold saline containing 1 mM AIB by centrifugation for 3 min at 1200 g. Control studies indicated that this washing procedure resulted in the loss of less than 5% of the accumulated ^{14}C-AIB from the cells. The washed pellets were then dissolved overnight in 0.5 ml NCS solubilizer and counted in Bray's solution (11) in a Packard Model 3380 Liquid Scintillation Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). For studies of incorporation of isotopically-labeled precursors into DNA and RNA, 8 ml of 5% trichloroacetic acid were added to the washed pellets at 4°C, and the resulting cell precipitates were disrupted on a Biosonix sonicator (Bronwill Scientific, Inc., Rochester, N. Y.) at a probe intensity of 40 for 15 sec. The precipitates were collected by centrifugation, washed twice with cold 5% trichloroacetic acid, and then dissolved and counted as above.

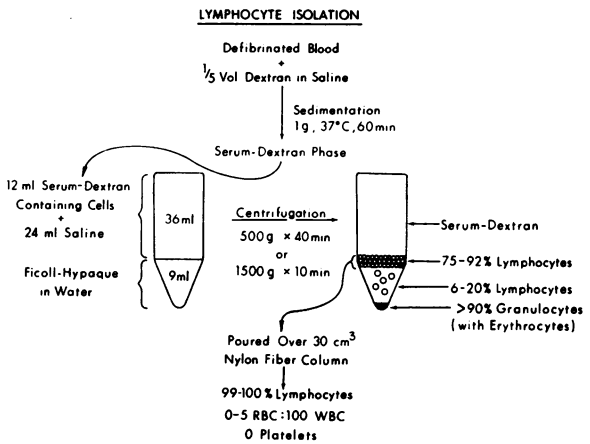

FIGURE 1 Outline summary of the method for lymphocyte purification.

For studies of ^{131}I-PHA binding to lymphocytes incubations were performed in Packard plastic counting tubes presoaked in 2 g % albumin for 30 min to reduce nonspecific binding of the PHA to the tubes. After appropriate manipulation and washing steps the cell pellets were counted directly in a Packard Autogamma Spectrometer Series 314E.

All of the experimental points represent the average of duplicate or triplicate cultures.

RESULTS

PHA response of lymphocytes. Since the exposure of lymphocytes to Ficoll and Hypaque is not customary in lymphocyte culture studies, cells obtained from the isopycnic banding step of purification were tested for their response to PHA using the standard parameters of increased DNA and RNA synthesis. These cells demonstrated the typical rise in incorporation of uridine-5-^{3}H into RNA at 24 hr, and of thymidine-methyl-^{3}H into DNA at 48–72 hr. Thus lymphocytes purified in this manner react in the usual pattern when stimulated

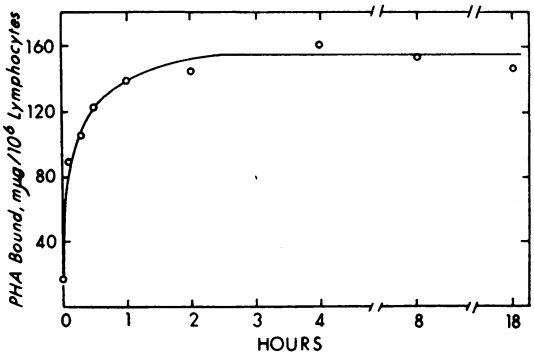

FIGURE 2 Uptake of PHA by purified lymphocytes as a function of time. Cultures containing 3.3×10^6 lymphocytes were incubated with 10 μg per ml ^{131}I-PHA, 9.4×10^5 cpm per μg. At the appropriate intervals paired cultures were harvested and washed, and the amount of ^{131}I-PHA bound to the cells was determined. The maximum uptake was 25% of the added ^{131}I-PHA.

with PHA (1). The lymphocytes were 98% viable as judged by the method of trypan blue dye exclusion.

PHA binding to lymphocytes. The time course of PHA binding to lymphocytes is shown in Fig. 2. By 7.5 min 50% of maximal binding had occurred and at approximately 60 min the binding reaction had reached a steady state. In other experiments it was determined that the ^{131}I-PHA binds to lymphocytes prepared in this manner with an apparent dissociation constant of 11 μg per ml, which is in good agreement with our previously published data (12).

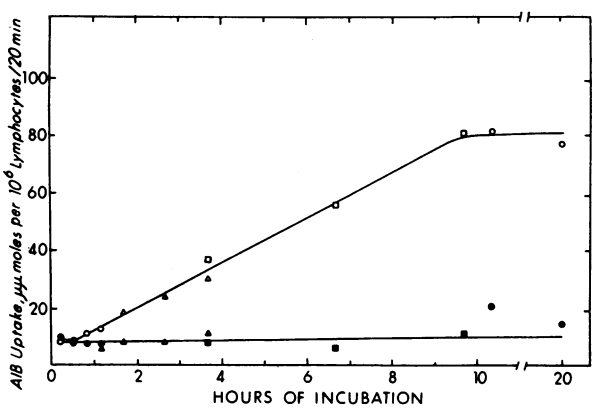

FIGURE 3 Initial rate of AIB uptake by lymphocytes after varying periods of incubation with and without 20 μg per ml PHA. After appropriate incubation periods, 0.19 mM ^{14}C-AIB (final concentration) was added for 20 min. The cultures were then chilled and harvested for counting as described. The open symbols indicate cultures with added PHA and the filled symbols indicate controls. The groups of ○ ●, △ ▲, □ ■ indicate experiments done on different days with different preparations of lymphocytes, and show minor variations.

Uptake of AIB. The uptake of AIB by lymphocytes proceeded at a linear rate for more than 20 min in cultures exposed to PHA and in control cultures. The effect of exposing lymphocytes to PHA for varying periods of time on the initial rate of AIB uptake is shown in Fig. 3. After a lag period of about 30 min, the cells incubated with PHA undergo a linear increase in the initial rate of AIB uptake, which reaches a plateau level after about 10 hr. In contrast, the initial rate of AIB uptake by control lymphocytes is constant over a 20-hr period. The extent of the PHA effect on AIB uptake is dependent on the concentration of PHA in the media (Fig. 4), with the half maximal effect occurring at a PHA concentration of about 4 μg per ml.

In addition to the aforementioned studies of the initial rate of AIB transport, experiments were performed to measure the accumulation of AIB by cells continuously exposed to AIB. The uptake of AIB by both control and PHA-treated cells did not reach equilibrium for about 6–8 hr (Fig. 5) with the final steady state level of AIB uptake being 3.7-fold higher in PHA-treated cells than in control cells. The steady state AIB ratio (intracellular concentration/extracellular concentration) can be calculated from these data by estimating that the average diameter of these small lymphocytes is 8 μ and that the average water content per cell is 70% of the cell volume. Using these assumed values, the calculated AIB concentration ratio is 2.2 for control cells and 8.1 for lymphocytes exposed to PHA.

In order to characterize the kinetics of AIB uptake in the presence and absence of PHA, the studies shown in Fig. 6 were performed. In Fig. 6A, it is evident that the rate of uptake of AIB follows typical saturation kinetics both in the presence and absence of PHA. When these data were plotted according to Lineweaver and Burk (13) (Fig. 6B) the apparent Michaelis constant for AIB uptake was 2 mM in both situations. However, in the presence of PHA the V_{max} for AIB uptake increased by

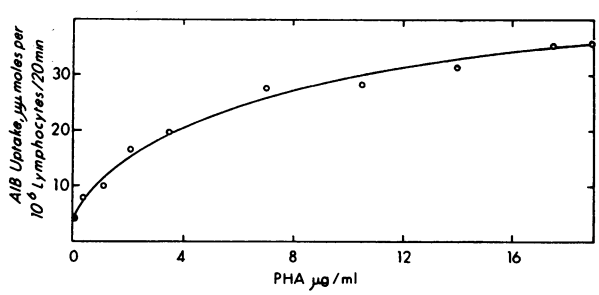

FIGURE 4 Initial rate of AIB uptake after incubation with varying concentrations of PHA. Cultures containing 1.6 $\times 10^6$ lymphocytes were incubated with PHA for 4 hr; 0.24 mM ^{14}C-AIB (final concentration) was then added for 20 min and the cells were harvested for counting as described.

7-fold, going from 0.1 to 0.7 mμmoles of AIB per 10^6 cells/15 min. Thus, PHA does not alter the affinity of the cell membrane transport sites for AIB; rather, it acts either by increasing the rate of transport by these sites or by increasing the total number of active sites for AIB transport.

Effect of protein synthesis inhibition on PHA effect. Lymphocyte cultures were preincubated with puromycin in doses that were shown in preliminary experiments to inhibit ^{14}C-amino acid incorporation into protein by more than 90%. Over a period of 2 hr, puromycin caused no alteration in the PHA-induced increase in AIB uptake (Fig. 7). Likewise, preincubation with cycloheximide at concentrations that inhibited protein synthesis in lymphocytes by more than 85% produced no reduction in the augmented uptake of AIB (Fig. 7). These experiments demonstrate that PHA-induced changes in this membrane transport function occur even when *de novo* protein is inhibited by 85–90%.

Effect of temperature on PHA effect. The uptake of AIB by lymphocytes as well as the effect of PHA on this uptake was demonstrated to be temperature dependent. Lymphocytes were incubated with PHA (25 μg per ml) for 3 hr at 0°C followed by the addition of 0.2 mM ^{14}C-AIB to cultures either maintained at 0°C or warmed to 37°C. Lymphocyte cultures maintained at 0°C did not take up AIB from the medium. Cultures warmed to 37°C just prior to the addition of AIB did show uptake, but there was no increased uptake by cultures previously exposed to PHA at 0°C. Thus control cultures maintained at 37°C throughout the experiment had a 6.1-fold increase in the rate of AIB uptake in the presence of PHA, whereas the cultures exposed to PHA at 0°C and then warmed to 37°C just prior to the AIB pulse showed only a 1.1-fold increase in the rate of AIB uptake.

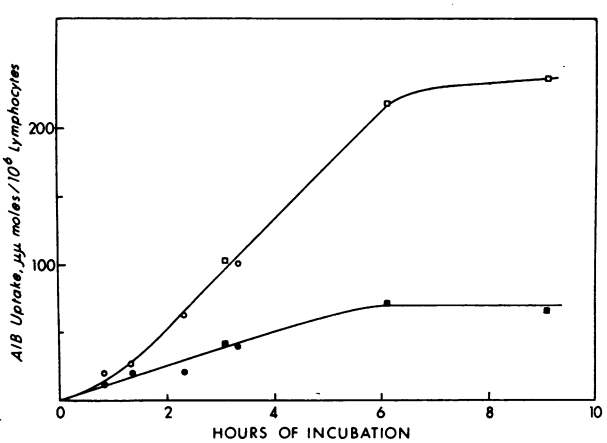

FIGURE 5 Accumulation of AIB by lymphocytes in the presence or absence of 20 μg per ml PHA. Cultures containing 1×10^6 lymphocytes were grown continuously in the presence of 0.19 mM ^{14}C-AIB. After the appropriate time interval, the cells were harvested for counting as described. ○, □ — in the presence of PHA; ●, ■ — without PHA.

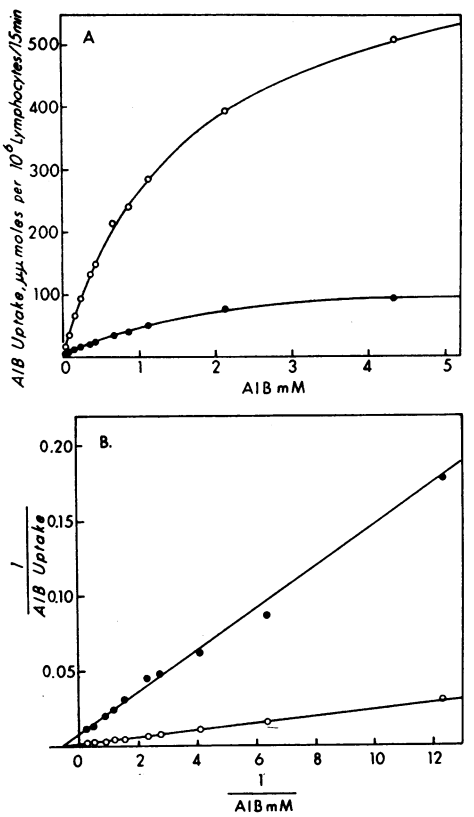

FIGURE 6 Uptake of AIB by control (●) and PHA stimulated (○) lymphocytes as a function of AIB concentration. Cultures containing 1.5×10^6 lymphocytes were incubated for 7 hr with or without 20 μg per ml PHA. The cells were then exposed to varying concentrations of ^{14}C-AIB for 15 min, and harvested for determination of isotope uptake as described. The data shown are from one of three similar experiments.

The mechanism of AIB transport was further characterized by incubating lymphocytes with unlabeled 1mM AIB prior to measurement of the rate of uptake of ^{14}C-AIB (Table I). The preincubation period produced no alteration in the rate of AIB uptake either in the presence or absence of PHA indicating an absence of exchange diffusion.[2]

Removal of PHA from lymphocytes. Having demonstrated that PHA can alter the membrane function of lymphocytes, we next investigated whether this effect would be sustained or reversed when the bound PHA was removed from the lymphocyte cell surface. In pre-

[2] The term "exchange diffusion" is used to indicate the exchange of labeled and unlabeled molecules of the same species across the cell membrane without concomitant net flow (14).

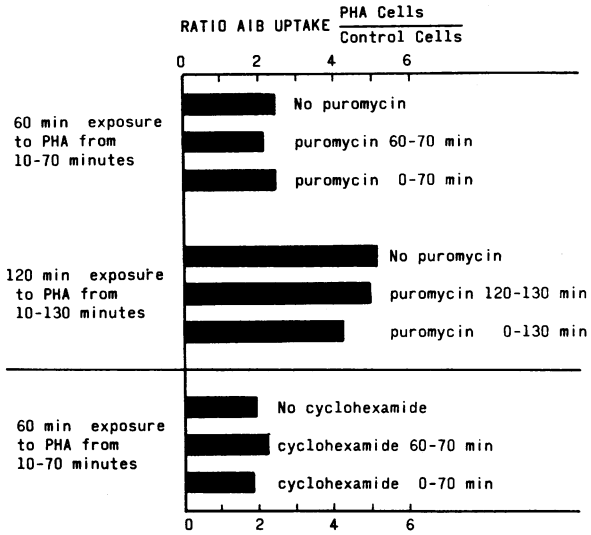

FIGURE 7 The effect of puromycin and cycloheximide on the PHA-induced increase in the rate of AIB uptake by lymphocytes. Inhibitors of protein synthesis were added initially to lymphocyte cultures at a concentration of 5×10^{-4}M for puromycin and 9×10^{-5}M for cycloheximide. After a period of 10 min incubation with inhibitor, PHA was added to some of the cultures at a concentration of 20 μg per ml. The rates of ^{14}C-AIB uptake were determined after 1 or 2 hr. The results are expressed as the ratio of AIB uptake by PHA-stimulated cells versus control cells which received no PHA. Under these conditions there were no significant differences in the rates of AIB uptake by control cultures when incubated in the presence or absence of inhibitors.

liminary studies we found that when lymphocyte cultures were incubated with ^{131}I-PHA for 60 min to allow maximal uptake and the cells were then harvested and washed with vigorous vortexing in minimal essential medium, only 4% of the bound PHA was removed. A second washing removed an additional 2%. If the washed

TABLE I

*Test for Exchange Diffusion Mechanism of AIB Uptake by Lymphocytes**

	AIB uptake‡		Ratio of uptake
	+PHA	−PHA	+PHA/−PHA
Preincubation with 1 mM AIB (unlabeled)	48	12	4.0
Preincubation without AIB	52	12	4.3

* Cultures containing 1×10^6 lymphocytes were incubated for 3 hr with additions of PHA (20 μg/ml) and nonradioactive AIB as indicated. The cultures were then sedimented by centrifugation, washed twice with minimal essential medium and resuspended in fresh incubation medium. ^{14}C-AIB 0.19 mM was added immediately, and after 20 min incubation th cells were harvested for counting as described in the methods.

‡ μμmoles ^{14}C-AIB/10^6 cells/20 min.

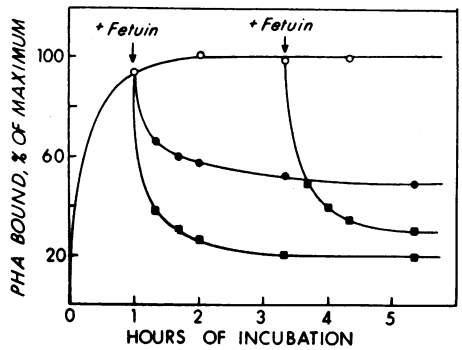

FIGURE 8 Release of ^{131}I-PHA bound to lymphocytes by addition of fetuin. Cultures containing 3×10^6 lymphocytes were incubated with 11 μg per ml ^{131}I-PHA (8×10^4 cpm per μg) in a volume of 0.4 ml. After 1 and 3 hr fetuin was added at the concentrations noted below and the incubations were allowed to proceed. At the indicated times paired cultures were harvested and washed, and the amount of ^{131}I-PHA bound to the cells was determined. ■ — 2 mg fetuin; ● — 0.5 mg fetuin; ○ — no fetuin.

lymphocytes were then incubated at 37°C, 20% more of the PHA was released into the medium over a 2–3 hr period. These findings show that the PHA is tightly bound to the cell membrane. In previous studies we had demonstrated that the carbohydrate side-chains of fetuin have a structure somewhat similar to those of the erythrocyte membrane receptor sites for PHA (15). Further, we had shown that fetuin is a potent inhibitor of PHA binding to erythrocytes. As shown in Fig. 8, fetuin was capable of rapidly removing bound PHA from lymphocytes, even after the PHA had been in contact with the cells for 3 hr. The absolute amount of PHA removed was dependent on the concentration of PHA and fetuin in the medium. At a PHA concentration of 10 μg per ml, it required 5 mg per ml fetuin to remove 80% of the bound PHA (Fig. 8).

Effect of PHA removal from lymphocytes on AIB uptake. In a series of experiments, lymphocytes were incubated with PHA for various periods of time and then fetuin was added to remove the bound PHA. The effect of fetuin addition on the rate of AIB uptake was then determined. As shown in Fig. 9, when fetuin was added to lymphocyte cultures which had been exposed to PHA for 2 hr, the increased rate of AIB uptake was arrested at the rate reached after the 2 hr of exposure to PHA. This rate of AIB uptake was then maintained for the next 3 hr following PHA removal. In contrast, the cultures continuously exposed to PHA for the entire 5 hr period of the experiment showed the usual continued rise in the rate of AIB uptake. In experiments with lower concentrations of fetuin, a lesser degree of inhibition was observed.

Fetuin blocks the PHA effect on AIB transport by its interaction with PHA rather than by exerting a non-

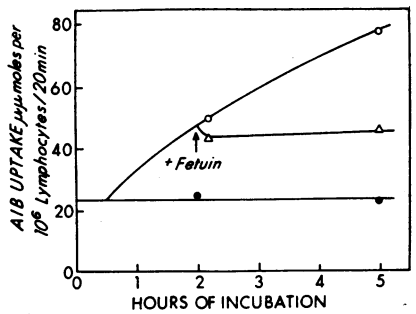

FIGURE 9 Effect of fetuin on the PHA-induced stimulation of AIB uptake. Cultures containing 5×10^6 lymphocytes in 0.2 ml medium were incubated in the presence or absence of PHA (10 μg per ml). After 2 hr incubation, 1 mg of fetuin was added to some of the cultures as noted. At the times indicated the cultures were incubated with 0.19 mM ^{14}C-AIB for 20 min and harvested as described. ● — no PHA; ○ — plus PHA; △ — plus PHA and (after 2 hr) fetuin.

TABLE II

*Reversal of Fetuin Effect with PHA**

PHA added at 0	PHA added at 1 hr	Fetuin added at 1 hr: 0 mg/ml	0.75 mg/ml	2.0 mg/ml	3.7 mg/ml
μg/ml		AIB uptake‡			
0	0	22	26	30	31
10	0	169	138	114	76
10	12	173	168	132	120
10	42	166	168	158	137

* Cultures containing 2×10^6 lymphocytes were incubated with or without 10 μg/ml PHA for 60 min. Fetuin and additional PHA at the varying concentrations shown were then added to some of the cultures, and the incubation was continued for 4 additional hr. Then 0.19 mM ^{14}C-AIB was added and the rate of uptake over a 20 min interval was determined as described.

‡ μμmoles per 10^6 lymphocytes/20 min.

specific toxic effect, as shown in Table II. Thus, when control lymphocyte cultures not exposed to PHA were incubated with fetuin for 4 hr they exhibited a slight stimulation in the rate of AIB uptake. If the fetuin had been toxic one would have expected the AIB uptake to be depressed. Furthermore, in the case of PHA-stimulated cultures, by increasing the concentration of PHA the inhibitory effect of fetuin was progressively overcome, showing the competitive nature of the reaction.

To prove that fetuin could be incubated with lymphocytes for long periods of time without causing toxic effects, the experiment shown in Table III was performed. The ability of control lymphocytes to synthesize DNA at the end of a 3-day incubation was not significantly affected by the addition of fetuin at a concentration of 3.75 mg per ml. Further, while fetuin was a potent inhibitor of the mitogenic response of lymphocytes induced by PHA, the inhibition could be overcome by increasing the concentration of PHA in the cultures. The fact that fetuin inhibits the PHA-induced mitogenic response provides evidence that its effect on AIB uptake is not an isolated phenomenon but rather related to its ability to remove PHA from the lymphocyte surface.[8] With fetuin concentrations greater than 5 mg/ml, a suppression of ^{3}H-thymidine incorporation into DNA in control cultures was observed.

DISCUSSION

We have demonstrated that PHA induces a profound alteration in at least one functional property of the lymphocyte plasma membrane, namely, the ability to transport AIB. Recently Quastel and Kaplan (16) have reported that PHA also induces an increase in K^+ uptake by lymphocytes. The uptake of K^+ was characterized by saturation kinetics with PHA causing an increase in the V_{max} of K^+ transport without affecting the Km, similar to our findings with AIB. As well as causing these effects on carrier-mediated active transport systems,

TABLE III

*Effect of Fetuin on the Incorporation of ^{3}H-Thymidine into DNA by Lymphocytes**

	^{3}H-thymidine incorporated (cpm per 4 hr/culture)	Inhibition‡ (%)
Control (no PHA)	2,850	
+0.75 mg fetuin	5,100	
+3.75 mg fetuin	2.350	
PHA (3.4 μg/ml)	42,240	
+0.75 mg fetuin	26,370	46
+3.75 mg fetuin	4,655	94
PHA (17 μg/ml)	70,140	
+0.75 mg fetuin	59,570	19
+3.75 mg fetuin	16,880	78

* Replicate 1 ml cultures containing 2.5×10^6 lymphocytes and the additions as noted were incubated for 68 hr. Three μc of ^{3}H-thymidine (6.7 c per mM) were then added to each culture for 4 hr and the uptake into DNA was determined as described in the Methods.

‡ The cpm incorporated by the control cultures (basal DNA synthesis) were subtracted from the cpm incorporated by PHA-treated cells before calculating the per cent inhibition.

[8] This action of fetuin should be kept in mind when studies using PHA are carried out in medium containing fetal calf serum which is rich in fetuin.

PHA also enhances the pinocytotic activity of lymphocyte membranes. Thus Hirschhorn, Brittinger, Hirschhorn, and Weissman (17) here shown that PHA enhances neutral red uptake within 20 min of PHA addition while Robineaux, Bona, Anteunis, Orme-Rosselli (18) here demonstrated increased pinocytosis of several compounds, including horseradish peroxidase. It is therefore apparent that PHA induces a variety of membrane alterations which could have significant effects on the metabolism of the cell.

Although the binding of PHA to cell surface receptor sites occurred fairly rapidly (50% maximal binding in 7.5 min), the membrane alteration in AIB transport evolved at a much slower rate. Thus, for the first 30 min after PHA addition no change was detected and then a linear increase in the rate of AIB uptake occurred over the next 8–10 hr. For this alteration in membrane function to occur, the normal rates of *de novo* protein synthesis were not necessary, as shown by the finding that puromycin and cycloheximide did not block the development of the PHA effect.

The alterations induced in the lymphocyte membrane by PHA are undoubtedly complex and the effects produced can be divided into those which occur immediately after PHA binding and those which develop more slowly. Thus Fisher and Mueller (2) have shown that within minutes after PHA addition to lymphocyte cultures there is an increase in the rate of ^{32}P incorporation into membrane phospholipids, primarily into phosphatidylinositol. Smith, Steiner, and Parker (19) have reported that within a few minutes of PHA binding to lymphocytes there is a rapid increase in the activity of a cell membrane associated enzyme, adenylcyclase. The rapidity of these changes suggest that they are a direct consequence of PHA binding to the plasma membrane. An immediate change of this sort could be caused by an alteration in a membrane component directly involved in the changed function. The alteration could be induced either by PHA binding directly to such a membrane component or by PHA binding elsewhere on the membrane and indirectly causing a conformational change in the structure of the component. In contrast, the PHA effect on AIB and K^+ transport has a delayed onset and requires a number of hours to evolve. This delayed type of change is best explained by the proposal that PHA induces a chemical "remodeling" of the plasma membrane, one consequence of which is an altered rate of AIB and K^+ transport. The alteration in membrane phospholipid metabolism which occurs after PHA addition could be an early reflection of this chemical remodeling.

The experiments utilizing fetuin to remove bound PHA from the cell membrane show that PHA must be continuously present on the cell surface for the full stimulatory effect on AIB transport to be reached. When the PHA is removed with fetuin, the rate of AIB uptake by these lymphocytes is immediately arrested at the level achieved during the time of incubation with PHA. The experiments demonstrating the arresting effect of removal of PHA were performed during the first 6 hr of incubation, when the PHA-induced increase in the rate of AIB uptake is still rising towards a peak level reached only after 8–10 hr. Further studies will be required in order to establish whether or not removal of PHA after 10 hr of incubation with lymphocytes will alter the increased rate of AIB uptake over the ensuing 2–3 days. This will entail changing from an incubation medium of minimal essential medium containing albumin to one fortified with serum. It is important to stress that these data showing a rapid increase in membrane-mediated AIB transport by lymphocytes do not distinguish between (*a*) a direct action of PHA upon the cell membrane, and (*b*) the possibility that PHA is continuously entering the cell at a slow rate where it then exerts its effect. Others have previously shown that a small percentage of PHA does enter lymphocytes although there is no direct evidence that this intracellular PHA is responsible for its biological activity (20–25). Pogo, Allfrey, and Mirsky (26) have reported that within less than 15 min of exposure to PHA there is an increased acetylation of lymphocyte histones, which they interpret as a sign of increased template activity of chromatin in RNA synthesis (26).

The studies on the kinetics of AIB uptake by control and PHA-treated lymphocytes demonstrate that PHA increases the V_{max} of AIB uptake without affecting the Km of the transport system. This change in the V_{max} could result from an increased rate of transport by each site, or from the induction of an increased number of sites for AIB transport. Previously Helmreich and Kipnis (14), studying the transport of AIB by guinea pig and rabbit lymph node cells, found that at low concentrations of AIB (up to 0.1 mM) uptake occurred by an active transport process exhibiting saturation kinetics with an apparent Michaelis constant of 0.11 mM. At higher concentrations of AIB, uptake occurred by a first order process with characteristics of diffusion. Our findings differ from these in that we do not detect significant diffusion of AIB into the cells at concentrations of AIB as high as 5 mM and further, the Km for AIB binding in our system is 2 mM. One major difference in these studies is that we have used human peripheral blood lymphocytes which were processed in a fashion quite different from that employed by Helmreich and Kipnis. The failure to demonstrate exchange diffusion in human lymphocytes is in agreement with the data of these investigators utilizing guinea pig and rabbit lymph

node cells. Studies of AIB transport by 3T3 cells (27) and renal tubule cells (28) have yielded Km's in a range similar to our value of 2 mM obtained for human lymphocytes. In the studies of Foster and Pardee (27) the rate of AIB uptake by polyoma virus-transformed 3T3 cells was found to vary with their growth conditions. Thus AIB was accumulated about 30% less rapidly by confluent than by nonconfluent 3T3 cells in tissue culture.

The effect of PHA on AIB uptake differs in several ways from its effect on uridine uptake, as previously described by Lucas (29) and by Hausen and Stein (30). These investigators found that the stimulation of uridine incorporation into RNA which steadily increased during the first 24 hr of exposure to PHA was accompanied by a progressive rise in the concentration of uridine nucleotides in the soluble intracellular pool. Further, the enhanced uptake of uridine was shown to occur concomitant with a rise in uridine kinase activity. When uridine kinase was selectively inhibited by adding cytidine to the media, the uptake of uridine into RNA was also inhibited (29). These authors suggested that the rate limiting step in uridine uptake may be the phosphorylation of uridine by uridine kinase rather than an actual change in active transport of uridine through the cell membrane. A similar phenomenon cannot account for the enhanced uptake of AIB by lymphocytes since it is a nonmetabolizable amino acid (3). Hausen and Stein also showed that PHA stimulates thymidine uptake into the soluble intracellular pool only after a lag period of 20–24 hr which correlates well with the time at which PHA induced DNA synthesis is first demonstrable. The fact that PHA stimulates the uptake of AIB, uridine and thymidine at very different times after its addition makes it unlikely that PHA is inducing a nonspecific increase in membrane permeability. Consistent with this proposal is our finding that the rate of uptake of inulin (mol wt about 5,000) is not affected by PHA.[4]

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