PROTEIN PHOSPHORYLATION IN HUMAN PERIPHERAL LYMPHOCYTES - STIMULATION BY PHYTOHEMAGGLUTININ AND N^6 MONOBUTYRYL CYCLIC AMP*

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SUMMARY: Protein phosphorylation was studied in human peripheral lymphocytes. The cells were preincubated with $[^{32}PO_4^{=}]$, exposed to phytohemagglutinin (PHA), N^6 monobutyryl cAMP (MBcAMP) or 8 bromo cGMP (BcGMP), homogenized and analyzed by SDS-polyacrylamide gel electrophoresis. Both PHA and MBcAMP produced early increases in the $[^{32}P]$content of multiple proteins in the 30,000-100,000 molecular weight range. After further incubation with PHA there was a shift in the phosphorylation response to smaller molecular weight proteins. BcGMP (1 mM-10 pM) had no effect on protein phosphorylation. These results suggest a role for cAMP in the early action of PHA on human peripheral lymphocytes.

Early increases in intracellular cyclic AMP and cyclic GMP have been demonstrated in human peripheral lymphocytes incubated with the mitogenic lectin phytohemagglutinin (PHA) (1,2, reviewed in 3,4). However, attempts to mimic lymphocyte activation with agents which are known to elevate cyclic AMP or cyclic GMP or with analogs of cyclic AMP or cyclic GMP have been only partially successful (5,6). Since exogenous cyclic nucleotides may fail to penetrate into lymphocytes or may reach the wrong compartments of the cell these observations do not exclude an important role for cyclic nucleotides in lymphocyte activation. Since most or all of the effects of cyclic AMP (and quite possibly cyclic GMP) appear to be exerted through phosphorylation or dephosphorylation of intracellular proteins (7,8), the demonstration of early changes in protein phosphorylation in lectin stimulated cells would be presumptive evidence for cyclic nucleotide involvement in the response. In the present study human purified peripheral lymphocytes have been preincubated with $[^{32}PO_4^{=}]$, exposed to PHA, BcGMP or MBcAMP, homogenized and fractionated by SDS-polyacrylamide gel electrophoresis. Evidence has been obtained for rapid alterations in protein phosphorylation in cells incubated

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with PHA and MBcAMP, suggesting that cAMP is directly involved in some of the early biochemical changes in PHA stimulated cells.

MATERIALS AND METHODS

Preparation of Lymphocytes: Blood was obtained from normal human volunteers and the lymphocytes purified by dextran sedimentation and Ficoll-Hypaque density gradient centrifugation as previously described (9). The purified lymphocyte preparation was suspended in 0.15 M NaCl, 0.01 M $\left[PO_4^{=}\right]$ pH 7.4 (PBS) and centrifuged at 200 times gravity for 7 minutes to remove contaminating platelets. The resulting preparations contained 95-98 lymphocytes per 100 nucleated cells and less than 3 platelets per lymphocyte.

Incubation of Lymphocytes: Lymphocytes were suspended in phosphate free buffer (Buffer A) which contains 0.15 M NaCl, 5 mM Mg acetate, 3 mM $CaCl_2$, 5 mM KCl, 2 mM glutamine, 1.8 mM glucose and 10 mM TRIS, pH 7.4, at 100 x 10^6 cells/ml and incubated for 60 minutes at 37°C. The cells were washed once in buffer A and resuspended in the same medium containing 0.5 mCi/ml $\left[^{32}P\right]$ phosphate acid (Mallinckrodt, buffered to pH 7.4 immediately prior to use). After incubation for another 60 minutes at 37°C the suspension was washed once and resuspended in buffer A at 20 x 10^6 cells/ml; 0.1 volume of 0.1 M NaCl or stimulator in 0.1 M NaCl was added and the cells were incubated at 37°C for varying periods of time. After incubation the cell suspension was cooled to 0°, and the cells pelleted at 400 x g for 10 minutes in the cold. Preparation of the cells for electrophoresis is described in the legend to Figure 1.

RESULTS: SDS electrophoresis of 400 x g supernatants from homogenized lymphocytes revealed a complex pattern with more than 30 identifiable protein bands (Fig. 1). Incubation of lymphocytes with 20 µg/ml of PHA (HA-16, Burroughs Welcome), MBcAMP (Sigma), or 0.1 µM BcAMP (INC) caused no alteration in the pattern of proteins as determined by gel electrophoresis.

The incubation of cells with 20 µg/ml of PHA for 5 minutes at 37°C caused a substantial increase in the overall level of protein phosphoryla-

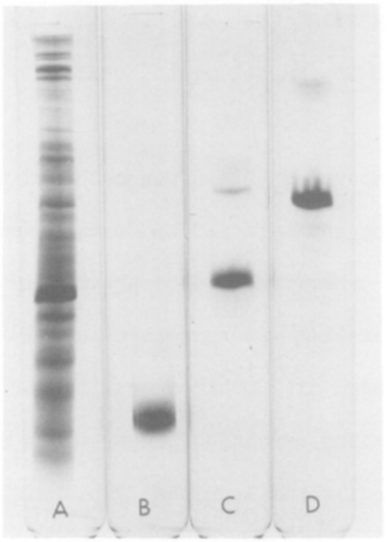
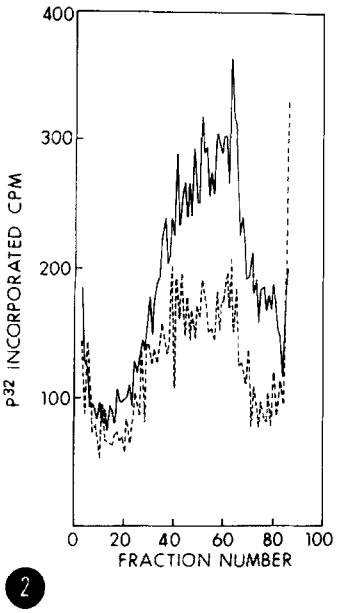
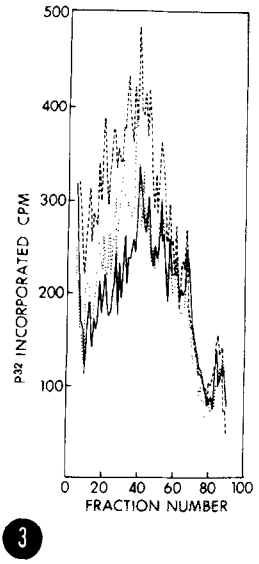


Figure 1: Electrophoretogram of lymphocyte homogenates. Purified human peripheral lymphocytes were incubated as described in the text. After incubation and centrifugation the cells were resuspended in hypotonic buffer (buffer A from which NaCl had been omitted) at $250X10^6$ cells/ml and disrupted by homogenization (200 strokes of a tight fitting Dounce homogenizer). The homogenate was centrifuged at 400 x g for 4 minutes at 4°C to remove nuclei and occasional unbroken cells. A sample of the supernatant was removed for determination of protein and the remainder was analyzed by SDS-polyacrylamide gel electrophoresis in 5.6% gels as described by Fairbanks et al. (10) except that the incubation with SDS and 2-mercaptoethanol was for 5 minutes at 100°C. Approximately 250 µg of protein was added to each gel. Gels were run until the dye marker had reached 85 mm. The gels were fixed overnight in 25% 2-propanol, 10% acetic acid, stained in 0.05% Coomassie blue in 10% acetic acid for 6 hours and destained in 10% acetic acid in a defusion destainer (Bio Rad) overnight. Cytochrome C (Sigma), egg albumin (Grand Island) and bovine serum albumin (Miles) were run on identical gels as molecular weight markers. A) Lymphocytes incubated with 0.1 M NaCl, B) Cytochrome C, C) Egg Albumin, D) Bovine serum albumin.

tion (Fig. 2). Less marked changes were seen at 10 minutes and no change at all after 30 minutes. The increases in radioactivity occurred in a broad area of the gel involving proteins with molecular weights ranging from approximately 10,000 to 70,000. There was no discernable selective increase in the radioactivity of individual bands in this area of the gel.

Increases in protein phosphorylation were also seen in cells incubated with PHA for 3 minutes at 37°C but a different phosphorylation pattern was obtained (Fig. 3). In this instance the majority of the increase was seen in higher molecular weight proteins, ranging from approximately 30,000 to





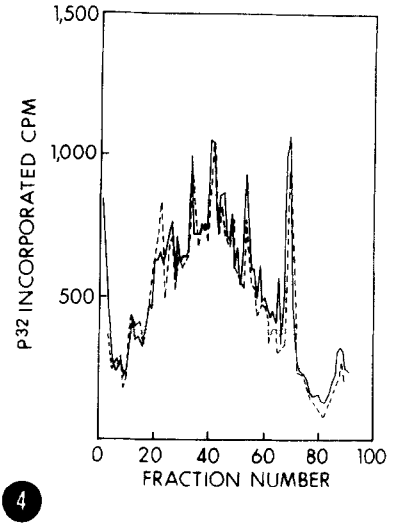


Figure 2: Electrophoretogram of lymphocytes incubated with 0.1 M NaCl ---------- or 20 μg/ml PHA in 0.1 M NaCl ———— for 5 minutes. Lymphocytes were incubated and prepared for SDS-polyacrylamide electrophoresis as described in the text and Figure 1. The gels were frozen, sliced into 1 mm sections in a Bio Rad gel slicer and counted by liquid scintillation.

Figure 3: Electrophoretogram of human peripheral lymphocytes incubated with 0.1 M NaCl ————, 20 μg/ml PHA ·····, or 10 μM MBcAMP for 3 minutes. Lymphocytes were incubated and prepared for SDS-polyacrylamide gel electrophoresis as described in the text and Figure 1.

Figure 4: Electrophoretogram of human peripheral lymphocytes incubated with 0.1 M NaCl ———— or 0.1 μM BcGMP ---------- for 3 minutes. Lymphocytes were incubated and prepared for SDS-polyacrylamide gel electrophoresis as described in the text and Figure 1.

100,000. Increases in phosphorylation were also obtained at 2 and 200 μg PHA/ml (not shown).

MBcAMP (10 μM) mimicked the increase in protein phosphorylation seen at 3 minutes in PHA treated cells (Fig. 3). In the experiment shown the absolute level of protein phosphorylation was higher with MBcAMP than with PHA but the two patterns were indistinguishable. The increase in protein phosphorylation induced by MBcAMP was transient. There was no increase in protein phosphorylation above control levels after 5 minutes or longer. The effect of MBcAMP was dose related with increases in phosphorylation at 1-1000 μM but not at lower concentrations.

BcGMP in concentrations ranging from 1 mM to 10 pM and time periods ranging from 3 to 30 minutes produced no increase in protein phosphorylation above controls (Fig. 4) (a total of 8 experiments).

Alterations in protein phosphorylation could be due either to increases in the activity of protein kinases or to increases in the specific activity of the acid soluble $[^{32}P]$ pool. Table 1 demonstrates that the specific activity of the $[^{32}P]$ pool remained essentially constant in cells prelabeled with $[^{32}P]$, washed in the usual way and incubated for an additional 10 minutes in the presence and absence of PHA or MBcAMP. Similar results were seen in cells incubated with BcGMP.

DISCUSSION

A major mechanism for the rapid modulation of cellular function is through the insertion or removal of esterified orthophosphate groups on phosphorylated enzymes (7). Both reactions are enzymatically mediated and partially controlled by cyclic nucleotides, particularly cAMP. In the present study rapid increases in protein phosphorylation have been demon-

TABLE I

Acid Soluble $[^{32}P]$ CPM/10^6 lymphocytes X 10^{-3}

Addition	Time (Minutes)					
	0	2	4	6	8	10
0.1 M NaCl (Control)	664±17	673±17	678±38	576±29	595±22	565±24
PHA-E 20 µg/ml	706±37	694±20	647±15	651±13	628±4	670±29
MBcAMP $1X10^{-5}$ M	683±13	685±10	655±12	635±18	645±12	600±16

Legend Table I: Purified human peripheral lymphocytes were incubated as described in the text. Stimulating agents or control solutions were added at time = 0 and the cells were incubated at 37°C. At the times indicated 50 µl of cell suspension was removed and the cells washed through fetal calf serum gradients 4 times. 1.0 ml of cold 6% TCA was added to the final cell pellet. The cells were vortexed, kept at 4°C for 30 minutes and centrifuged at 2200 x g for 30 minutes. The supernatant was removed and $[^{32}P]$ determined by liquid scintillation spectrometry. Values given are mean ± SEM for triplicate observations.

strated in lymphocytes incubated with PHA. The changes which occurred were transient with no alteration in the phosphorylation pattern being observed after 30 minutes of incubation. This is similar to what has been observed in synaptosomes (11) and toad bladder (12) membranes where evanescent changes in protein phosphorylation occur in association with hormone stimulation. It is of interest that the increase in protein phosphorylation seen in PHA stimulated lymphocytes involves a group of proteins which fractionate over a broad molecular weight range, in contrast to most other protein phosphorylation systems where the changes typically involve one or several selected proteins (11,12). Most other biochemical alterations in activated lymphocytes seem to follow a similar pattern, with lymphocytes doing things either better or faster with a remarkable lack of specificity (3,4).

The changes in protein phosphorylation seen at 3 minutes in PHA stimulated lymphocytes were mimicked almost entirely by 1-1000 μM MBcAMP suggesting that cAMP is directly involved in the early response to PHA. Whether PHA also produces non-cAMP dependent protein phosphorylation remains to be established although this possibility is suggested by the failure of MBcAMP to reproduce the phosphorylation response seen with PHA at 5 minutes. On the other hand MBcAMP may be rapidly degraded or more effective than cAMP in stimulating protein phosphatases resulting in the establishment of a more rapid equilibrium between phosphorylation and dephosphorylation.

In contrast to MBcAMP no increase in protein phosphorylation was seen with BcGMP over a broad dose response range and at times ranging from 3 to 30 minutes. This might indicate that PHA induced protein phosphorylation is not linked to the increases in cyclic GMP which have been described in human peripheral lymphocytes. However, in the one mammalian system in which specific phosphorylation has been shown to be modulated by cyclic GMP the phenomenon was extremely short lived--seen at approximately 20 seconds--and could only be demonstrated when the temperature of the reaction was reduced (13). In view of the design of the experiments presented in this communica-

tion we cannot exclude very early increases in protein phosphorylation in response to cyclic GMP or BcGMP.

The intracellular location of the phosphorylated proteins remains to be elucidated. In preliminary experiments several of the phosphorylated bands have appeared to correspond to bands seen in SDS-polyacrylamide gel electrophoresis of purified plasma membranes. However, increased phosphorylation has also been seen in other areas of the gel. Thus, changes may be occurring both in the plasma membrane and the cytosol. If this is true, phosphorylation of protein would be one of the earliest biochemical events in the cytoplasm of activated lymphocytes.

Although the results presented here provide support for the concept that cyclic AMP mediates at least a portion of the early events in lymphocyte activation they by no means prove that the phosphorylation is an obligatory step in the ultimate development of mitogenesis. It will be necessary to study other lectin and non-lectin mitogens such as con A, periodate, heavy metals, and antiimmunoglobulins, comparing their effects on protein phosphorylation with those of structurally related substances which do not produce mitogenesis.

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