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Phosphorylated Proteins of Mitogen Stimulated-Rat Peripheral Blood Lymphocytes[1]

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ABSTRACT

This study was done to classify the proteins involved in the specific phosphorylation using the rat peripheral blood lymphocytes (rPBL) stimulated with mitogens, phorbol 12-myristate 13-acetate (PMA) and concanavalin A (Con A). The lymphocytes were incubated with ^{32}P-orthophosphate before PMA or Con A stimulation. The migration patterns of the phosphorylated proteins of mitogen-treated rPBL in two dimensional electrophoretic fields were analyzed after autoradiography. The stimulation of the lymphocytes with PMA and Con A increased the phosphorylation of thirteen protein fractions. The phosphorylation intensities of the protein spots differ to the treatments of the cells with specific kinase inhibitors, H-7 and W-7. These protein fractions were grouped into 3 classes, namely, PKC-mediated, CaM kinase-mediated, and other kinase mediated proteins. The effect of the duration of the stimulation on the phosphorylated behaviors occurred concurrently, not sequentially, although each individual protein fraction had a different time for the peak phosphorylation during the stimulation period upto 30 minutes. The phosphoproteins found in the cytosolic soluble fraction were phosphorylated prior to those in the pellet, whose phosphorylations were sustained at a high level for over 10 minutes. The above results suggest that the early events in lymphocyte activation involve 3 different sets of proteins which are phosphorylated by CaM kinase, PKC and other kinases, and those kinases do not work sequentially, but rather, independently or cooperatively.

Key Words: Phosphoprotein, Protein kinase, T-lymphocyte

INTRODUCTION

Protein phosphorylation is a principal mechanism by which a cell can modulate the biological activity of a particular protein (Greengard, 1978; Cohen, 1985; Elderman *et al.*, 1987). The phosphorylation of proteins is a reversible modification regulated by protein kinases and phosphoprotein phosphatases (Krebs, 1986). This phosphorylation event can either increase or decrease the function of a protein, depending on the particular protein and the actual site being phosphorylated.

The covalent attachment of a phosphoryl group (PO_4^{-2}) with its high charge density onto a protein induces a conformational change in the protein (Ehrlich and Kornecki, 1987). Many proteins are phosphorylated at multiple sites by distinct protein kinases with differing effects on biological activity (Kishimoto *et al.*, 1985; Nishizuka, 1986). Phosphoprotein phosphatases catalyze the removal of phosphate groups from these phosphoproteins (Cohen, 1989), causing the protein to return to its former functional state. Both protein kinases

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and phosphatases are under regulatory control, and hence, its functional activity. The profound effect of phosphorylation on protein function is utilized by the cell to regulate coordinately in a number of diverse biological processes including metabolism, secretion, gene expression, electrical excitability and contractility.

Since the reaction of protein phosphorylation/dephosphorylation is neither a process of synthesizing new proteins nor decomposing existing proteins, it is possible to regulate biological processes immediately and transiently. Protein phosphorylation plays an especially prominent role in the transduction and amplification of extracellular signals into intracellular responses.

Hormones, neurotransmitters and growth factors bind to cell surface receptors initiating a cascade of biological events that generates intracellular second messengers. These second messengers activate specific protein kinases, which continue the transduction pathway by phosphorylating various cellular proteins. There are a number of protein kinases in the living cells (Hunter, 1987). Among many protein kinases the role of cAMP dependent kinases (A-kinase), cGMP dependent kinase (G-kinase), protein kinase C and calcium/calmodulin dependent kinase (CaM kinase) are notable.

Through the technical development in molecular biology, much has been revealed concerning protein kinases, thus considerable changes have been made in the field of protein phosphorylation in the last 10 years. But, nevertheless, the complex network of protein kinases and phosphatases remains much to be seen. Therefore, in this study, we were trying to find out the phosphoproteins responsible for the early events in lymphocyte activation by mitogens-PMA and Con A.

MATERIALS AND METHODS

T-lymphocyte isolation

Sprague-Dawley rats were lightly anesthetized with ether. The blood was withdrawn from the abdominal aorta with a heparinized syringe, and mixed with the same volume of RPMI medium. Lymphocytes were separated by Ficoll-Hypaque density gradient centrifugation. The lymphocytes were then loaded on a nylon wool column. After 30 minutes of incubation at 37℃, unbound T-lymphocytes were collected from the column.

Protein phosphorylation in intact T-lymphocyte

T-lymphocytes were washed twice with RPMI medium and once with phosphate-free medium. The cells were suspended in a density of 10^6 cells/ml in phosphate-free media. Two hundred μCi of carrier-free ^{32}P orthophosphoric acid was added per ml of cell suspension, and incubated at 37℃ for 2 hours in the CO_2 incubator.

Mitogen-induced alteration of protein phosphorylation and the effect of kinase inhibitor

T-lymphocytes labelled with ^{32}P orthophosphoric acid were stimulated with PMA 100 ng/ml or Con A 25 μg/ml for 10 minutes. The kinase inhibitors H-7 and W-7 were used as 10^{-5} M solution 30 minutes prior to the PMA or Con A stimulation.

Two-dimensional gel electrophoresis

Intact cells after ^{32}P-labelling and drug treatments were directly dissolved in a sample buffer (9 M urea, 2% Ampholine pH 3.5-10, 5% 2-mercaptoethanol, 2% Nonidet P-40). The cell homogenates were precipitated with 2 volumes of cold ethanol and the proteins were dissolved in the sample buffer. Two-dimensional gel electrophoresis was done by non-equilibrium pH gradient electrophoresis (pH 3.5-10) followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (O'Farrell *et al.*, 1977). After electrophoresis, the gels were stained with Coomassie brilliant blue and dried. Phosphoproteins were identified by autoradiography at −70℃.

Cellular fractionation of phosphoproteins

After 10 minutes' stimulation with PMA (100 ng/ml), the cells were homogenized in hypotonic RSB buffer (0.01 M NaCl, 0.01 M Tris, pH 7.5, 8 mM $MgCl_2$), containing protease inhibitors. After conjugation, the supernatant was separated into pellet and supernatant by ultracentrifugation (150,000 xg, 90 min).

Time course of phosphorylation during mitogen stimulation

^{32}P-labelled cells were stimulated with PMA or

Con A during 1 min, 3 min, 5 min, 10 min and 30 min. Then the reaction was stopped by quick cooling, and the phosphorylation pattern was examined by two-dimensional gel electrophoresis and autoradiography.

RESULTS

Protein fractionation pattern on two-dimensional gel electrophoresis

Lymphocyte protein from rat peripheral blood on two-dimensional gel electrophoresis was stained with Coomassie brilliant blue. More than 100 protein spots were detected between the molecular weight of 30 kDa and 70 kDa and between the isoelectric point of 5 and 7 (Fig. 1A).

Phosphoprotein fractionation on autoradiograph after two-dimensional gel electrophoresis

^{32}P-labelled lymphocyte proteins were factionated by two-dimensional gel electrophoresis. Then the gel was autoradiographed at −70°C. Among more than 100 protein fractions, about 35 protein spots appeared on the autoradiograph. Of phosphoproteins, molecular weight (M. Wt.) 44 kDa, isoelectric point (pI) 6.8 protein and M. Wt. 18 kDa, pI 5.4 protein were noticed (Fig. 1B).

Changes of phosphoprotein pattern after mitogen stimulation

After PMA 100 ng/ml stimulation, 14 kDa/pI 5.9, 24/7.1, 24/7.2, 28/6.8, 29/6.9 proteins were newly phosphorylated and the phosphorylation of 18 kDa/pI 5.4, 25/7.3, 26/6.1, 44/6.8, 74/6.2, 58/6.2 and 54/5.2 proteins were increased (Fig. 2B, Table 1). After Con A stimulation (25 μg/ml), 28 kDa/pI 6.8 protein was newly phosphorylated and the phosphorylation of 18 kDa/pI 5.4, 26/6.1, 28/7.0 44/6.8, 58/6.2 74/6.2, 54/5.2 proteins were increased (Fig. 2C, Table 1).

Changes of phosphorylation by kinase inhibitor

By H-7 pretreatment (10^{-5}M), the phosphorylation of 24/7.1, 24/7.2, 26/7.1, 74/6.2 proteins which showed the increase of phosphorylation by

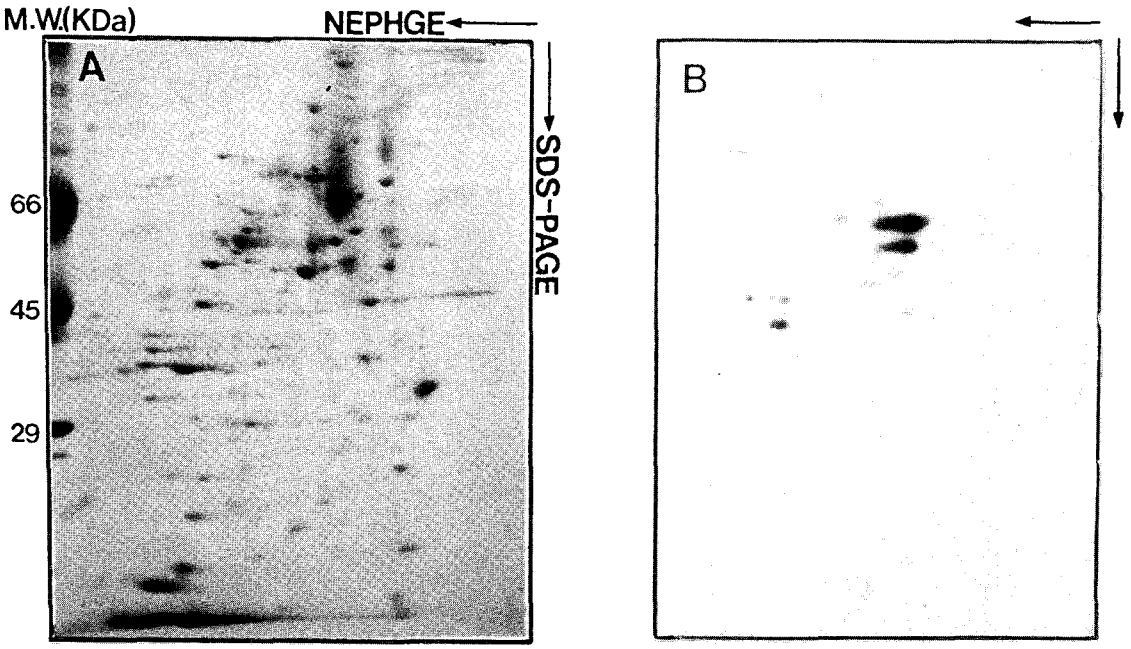

Fig. 1. Two-dimensional NEPHGE/SDS-PAGE separation of the proteins of rPBL. A: Coomassie brilliant blue stained gel. B: Autoradiograph of the gel.

PMA stimulation and, 26/6.1. 74/6.2 proteins which showed the increase of phosphorylation by Con A stimulation were seemingly decreased. (Fig. 3B, 4B). By W-7 pretreatment (10^{-5} M), the phosphorylation of 14/5.9, 28/6.8, 29/6.8, 29/6.9, 44/6.8, 58/6.2 proteins, which showed the increase of

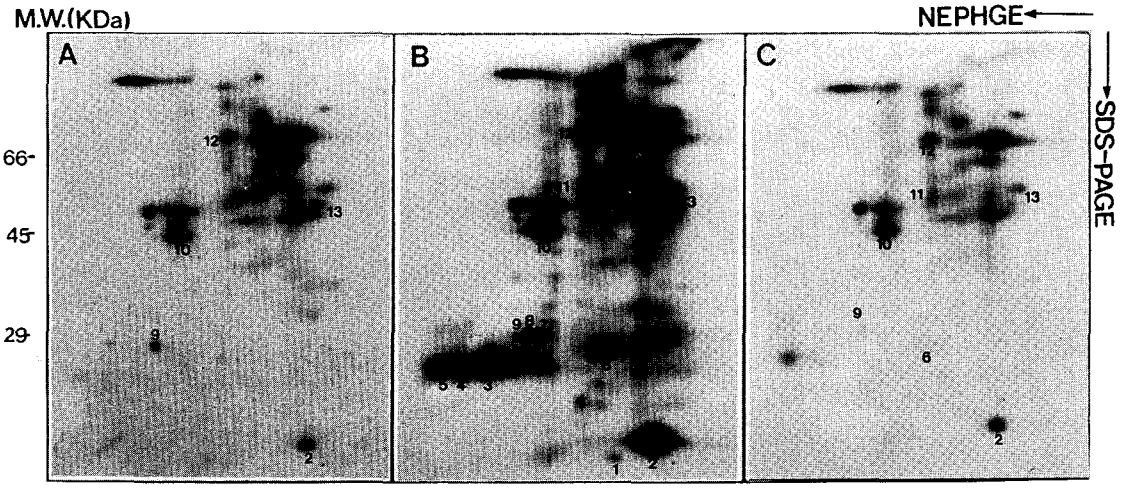

Fig. 2. Changes of phosphoprotein pattern after PMA and Con-A treatment. A: Phosphoprotein pattern in control group. B: Changes of phosphorylation after PMA (100 mg/ml) treatment. After stimulation, 14 kDa/pI 5.9, 24/7.1, 24/7.2, 28/6.8 and 29/6.9 proteins were newly phosphorylated and the phosphorylation of 18 kDa/pI 5.4, 25/7.3, 26/6.1, 44/6.8, 58/6.2, 74/6.2 and 54/5.2 proteins were increased. C: Changes of phosphorylation after Con-A (25 mg/ml) treatment. After Con-A stimulation, 28/6.8 protein was newly phosphorylated and the phosphorylation of 18/5.4, 26/6.1, 44/6.8, 58/6.2 and 54/5.2 proteins were increased.

Table 1. Sensitivity of Con A or PMA-stimulated phosphoproteins to kinase inhibitor pretreatment and their cellular location

No.	M.W./pI	con A			PMA			Inhibited by	location
		Alone	H-7	W-7	Alone	H-7	W-7		
3	24/7.1				+	↓		H-7	S
4	24/7.2				+	↓		H-7	S
6	26/6.1	↑	↓		↑	↓		H-7	S
12	74/6.2	↑	↓		↑	↓		H-7	P
1	14/5.9				+		↓	W-7	
7	28/6.8	+			+		↓	W-7	S
8	29/6.9				+		↓	W-7	S
9	28/7.0	↑		↓				W-7	S
10	44/6.8	↑		↓	↑		↓	W-7	S
11	58/6.2	↑			↑		↓	W-7	P
2	18/5.4	↑			↑			Neither	P
5	25/7.3				↑			Neither	S
13	54/5.2	↑			↑			Neither	P

S; Soluble fraction, P; Pellet

↑; Increased phosphorylation, ↓; Decreased phosphorylation, +; New phosphoprotein

phosphorylation by PMA stimulation and, 28/7.0 and 44/6.8 proteins which showed the increase of phosphorylation by Con A stimulation were decrease (Fig. 3C, 4C).

Cellular fractionation of phosphoproteins

Most of the proteins identified avove were

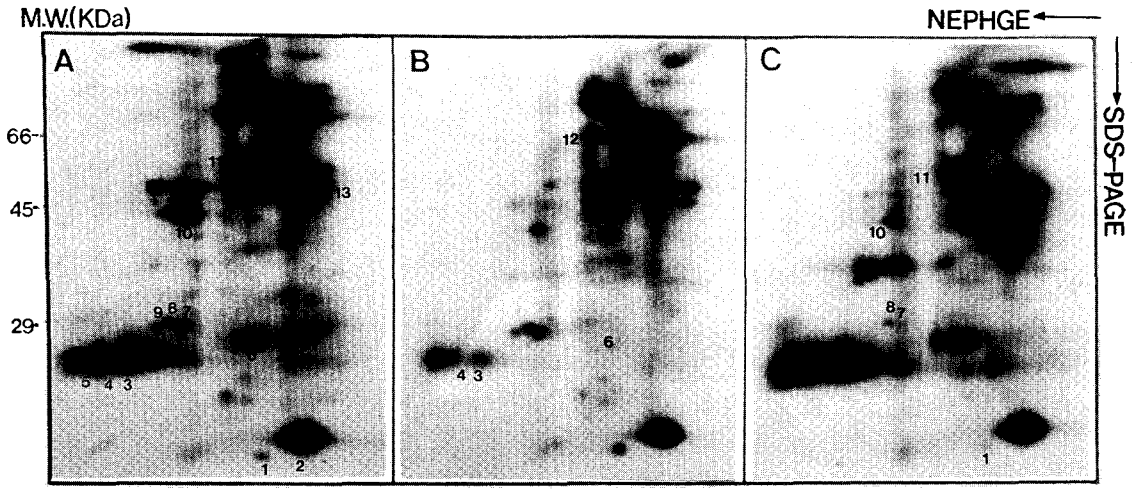

Fig. 3. Changes of PMA-induced phosphorylation by kinase inhibitors. A: Phosphoproteins of lymphocytes by PMA-stimulation. B: Changes of phosphorylation by H-7 (10^{-5} M) C: Changes of phosphorylation by W-7 (10^{-5} M). By H-7, phosphorylations of 24/7.1, 24/7.2, 26/6.1 and 74/6.2 proteins were decreased and by W-7, phosphorylations of 14/5.9, 28/6.8, 29/6.9, 28/7.0, 44/6.8 and 58/6.2 proteins were decreased.

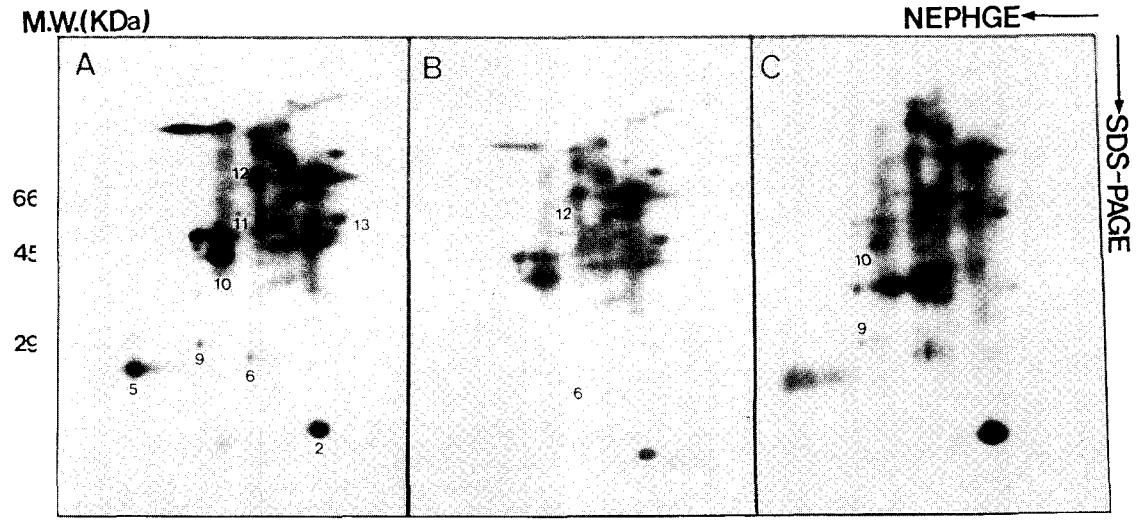

Fig. 4. Changes of Con-A-induced phosphorylation by kinase inhibitors. A: Phosphoproteins of lymphocytes by Con-A stimulation. B: Changes of phosphorylation by H-7 (10^{-5} M). C: Changes of phosphorylation by W-7 (10^{-5} M). By H-7, phosphorylations of 26/6.1 and 74/6.2 proteins were decreased and W-7, phosphorylations of 28/7.0 and 44/6.8 proteins were decreased.

found in the soluble fraction in the presence of Nonidet P-40, 0.5% (Fig. 5B). Only 18/5.4, 58/6.2 and 74/6.2 protein were found in the pellet and their phosphorylations lasted for more than 30 minute (Fig. 5C, Table 2).

Time course of phosphorylation during PMA-stimulation

We observed the changes of phosphorylation to

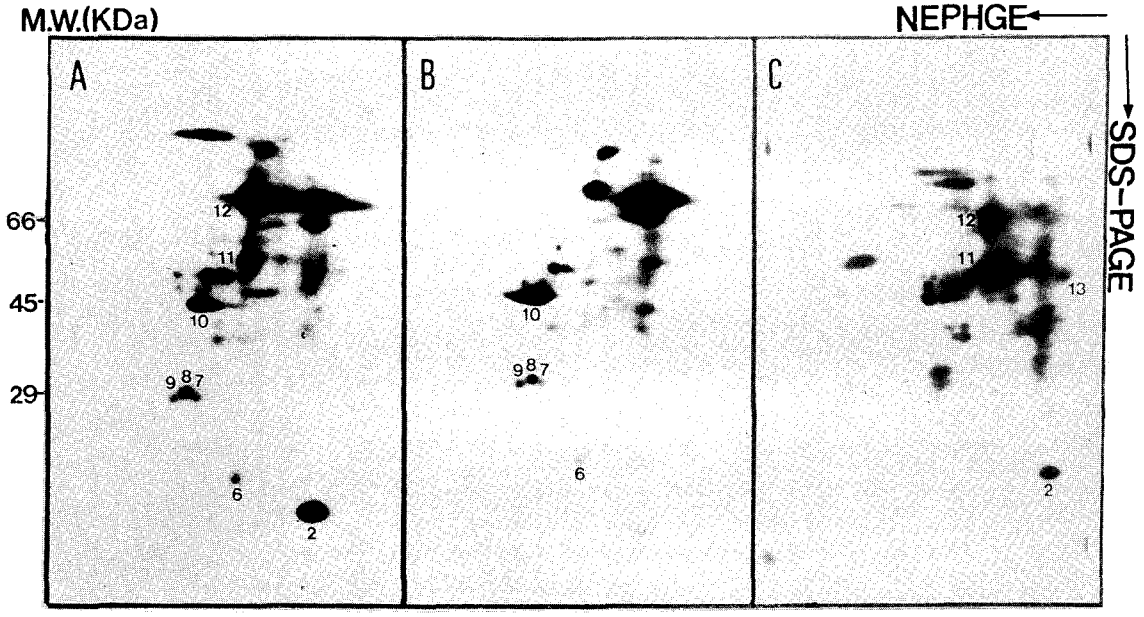

Fig. 5. Cellular localization of phosphoproteins. A: Phosphoproteins in homogenates of PMA-stimulated lymphocytes. B: Phosphoproteins in soluble fraction of homogenates in the presence of 0.5% Nonidet P-40. C: Phosphoproteins in the pellet.

Table 2. Time course of PMA-stimulated phosphorylation

No.	M.W./pI	Inhibited by	Location	Time after PMA stimulation		
				1 min	3 min	10 min
3	24/7.1	H-7	S	++	+	+
4	24/7.2	H-7	S	++	+	−
6	26/6.1	H-7	S	++	++	−
12	74/6.2	H-7	P	++	+++	+
7	28/6.8	W-7	S	+	++	−
8	29/6.9	W-7	S	+	−	−
9	28/7.0	W-7	S	++	++	+
10	44/6.8	W-7	S	++++	++	++
11	58/6.2	W-7	P	++	+++	++
2	18/5.4	Neither	P	+++	++++	++++
5	25/7.3	Neither	S	++++	+++	+
13	54/5.2	Neither	P	+	++	+++

S; Soluble fraction P; Pellet

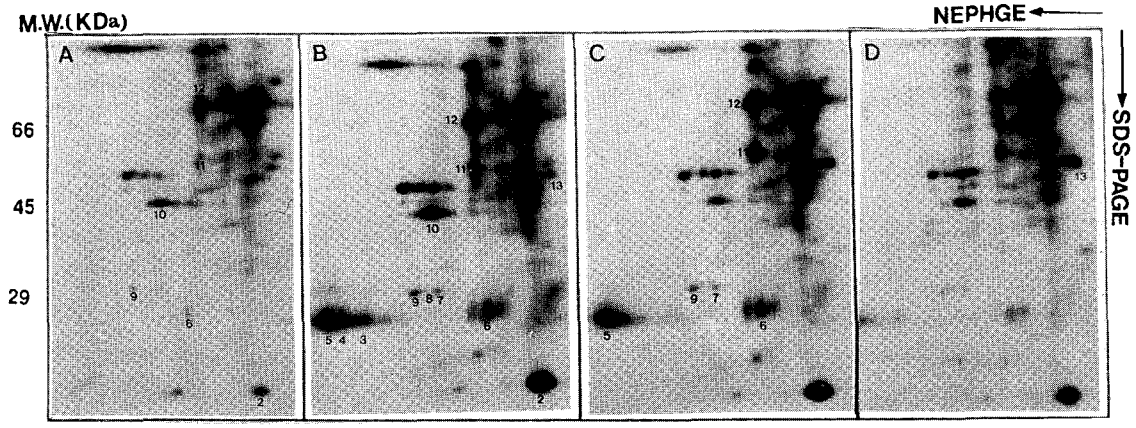

Fig. 6. Time course of phosphorylation during PMA stimulation. A: Autoradiograph of control. B: 1 minute stimulation. C: 3 minutes stimulation. D: 10 minutes stimulation.

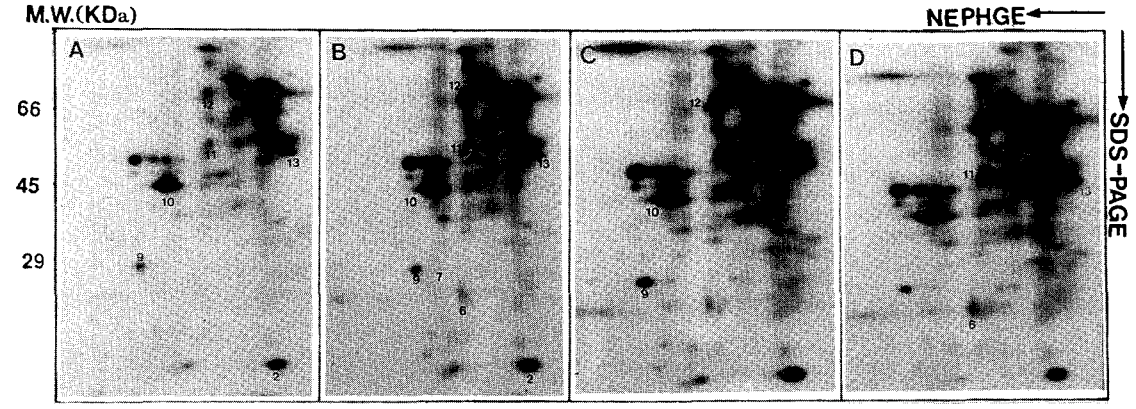

Fig. 7. Time course of phosphorylation during Con-A stimulation. A: Autoradiograph of control. B: 1 minute stimulation. C: 3 minutes stimulation. D: 10 minutes stimulation.

Table 3. Time course of Con A-stimulated phosphorylation

No.	M.W./pI	Inhibited by	Location	Time after Con-A stimulation		
				1 min	5 min	10 min
6	26/6.1	H-7	S	+	+	++
12	74/6.2	H-7	P	+	+++	++
7	28/6.8	W-7	S	+	+	+
9	28/7.0	W-7	S	++	+++	++
10	44/6.8	W-7	S	++	+++	++
11	58/6.2	W-7	P	++	++	+++
2	18/5.4	Neither	P	++	++	++
13	54/5.2	Neither	P	+	++	+++

S; Soluble fraction P; Pellet

the activation time. PMA (100 ng/ml) increased phosphorylation of most phosphoproteins within 1 minute after stimulation. The phosphorylation of 28/6.8, 74/6.2, 58/6.2 protein was sharply decreased after more than 3 minutes' stimulation. To the contrary, the phosphorylation of 54/5.2 protein lasted over 10 minutes. And 18/5.4 protein showed little difference to the stimulation time (Fig. 6, Table 2).

Time course of phosphorylation during Con A stimulation

Similiar to the PMA stimulation, increased phosphorylation appeared with 1 minutes' stimulation with Con-A. But, the maximal phosphorylation was observed at 5 minutes after Con-A stimulation (Fig. 7, Table 3).

DISCUSSION

The reversible phosphorylation of specific cellular proteins is an important biochemical reaction for the functional regulation, which is conserved in all mammalian cells. Mitogens trigger reorganization of phospholipid molecules in the cytoplasmic membrane, which then activate the protein kinases (Fisher *et al.*, 1971; Maino *et al.*, 1975). The activated kinases phosphorylate certain proteins to generate physiologically diverse responses on the various cellular stimuli. T cells, which take important roles in immune responses initiate an ordered, complex sequence of intracellular events when they are activated either by antigen or by mitogen. Since receptors peculiar to the immune system utilize signal transduction pathways common in other more extensively studied systems, it has been possible to apply techniques and concepts previously developed in other systems, to the investigation of B and T cell signaling.

In this study, using T-lymphocytes obtained from rat abdominal aorta, we tried to classify the proteins involved in the specific phosphorylation of lymphocytes stimulated with mitogens-phorbol 12-myristate 13-acetate (PMA) and concanavalin A (Con A). On autoradiogrsaph, more than 30 proteins were phosphorylated. Con A 25 μg/ml stimulated the phosphorylation of seven proteins. PMA 100 ng/ml-stimulation shows a similiar phosphorylation pattern.

To assess the substrats specificity of the phosphoproteins to various protein kinases, we observed the changes of phosphorylation to the kinases inhibitor pretreatment. H-7 treatment prior to mitogen stimulation decreased the phosphorylation intensity of 24/7.1, 24/7.2, 26/6.1 and 74/6.2 proteins. On the other hand, W-7 treatment of the phosphorylation intensity of 14/5.9, 28/6.8, 29/6.9, 28/7.0, 44/6.8 and 58/6.2 proteins. But 18/5.4, 25/7.3 and 54/5.2 proteins show little difference to the inhibitor treatment. From the above result, we may categorize the phosphoproteins into 3 groups according to their reaction to the inhibitor: the first group, whose phosphorylation is inhibited by H-7, the second group whose phosphorylation is inhibited by W-7, and the third group whose phosphorylation is neither inhibited by H-7 nor by W-7.

The early reaction occurring by Con A or PMA stimulation is mediated by PKC activation and calcium increase (Ku*et al.*, 1981). And H-7 and W-7 are the inhibitors of PKC and CaM kinase, respectively (Hidaka and Tanaka, 1983) Therefore, it may suggest that the proteins whose phosphorylation are inhibited by H-7 are the substrate proteins for PKC, and the proteins whose phosphorylation inhibited by W-7 are the ones for CaM kinase.

Then, the question arises how PMA, direct activator of PKC (Kikkawa *et al.*, 1983) can stimulate the above three kinds of proteins. It can be explained in the following two ways. First, since phosphorylation/dephosphorylation is a cascade of biochemical reaction, the proteins phosphorylated by PKC can activate other kinases. As a result, the reaction initiated by PMA can phosphorylate several kinds of proteins. Secondly, many proteins are phosphorylated at multiple sites by distinct protein kinases (Kishimoto *et al.*, 1985; Nishizuka, 1986) and PMA has many effects besides PKC activation: for example, guanylate cyclase activation (Coffey, 1986), increase of surface receptors for interleukin II (Farrer and Ruscetti, 1986) etc. So it is probable that those are the result of other actions of PMA occurring simultaneously with PKC activation.

To elucidate whether the results are due to the cascade activation of PKC or due to the other

actions of PMA concurring with PKC activation, we observed the time course of phosphorylation during mitogen stimulation. With PMA 100 ng/ml, most of the proteins are phosphorylated within 1 minute. 28 kDa/pI 6.8, 7.4/6.2 proteins show decreased phosphorylation after 3 minutes. But 54/5.2 protein shows sustained phosphorylation over 10 minutes. With Con A 25 μg/ml, most of the proteins show phosphorylation approximately within 5 minutes.

The grouping of phosphoproteins based on the changes according to the kinase inhibitor treatment, has little relation with the time course of phosphorylation. This suggests that the phosphorylation of those groups of phosphoproteins occur as a result of other actions of PMA concurring, not as a result of the cascade reaction of phosphorylation.

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=국문초록=

분열유발인자에 의한 흰쥐 림프구 단백의 인산화

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주 일 로·고 성 수·안 영 수

흰쥐 말초 T림프구에 분열유발 물질인 PMA와 Con A를 투여하여 인산화되는 단백을 확인하고, PKC 억제제인 H-7, CaM kinase 억제제인 W-7을 전처치한 후의 인산화 변동과 시간경과에 따른 인산화 변동을 관찰하였다. 그 결과 흰쥐 T림프구를 PMA로 자극하면 5개의 인산화 단백이 새로이 나타나고 7개 단백의 인산화가 증가 되었으며, Con A자극으로는 1개의 단백이 새로이 인산화 되고 7개 단백의 인산화가 증가되었다. PMA 및 Con A자극으로 인산화 되는 13개 단백은 kinase억제제 전처치에 의하여 3군으로 각각 구분되며, H-7 전처치로 24 kDa/pI 7.1, 24/7.2, 26/6.1, 74/6.2 단백의, W-7전처치로 14 kDa/pI5.9, 28/6.8, 29/6.9, 28/7.0, 44/6.8, 58/6.2 단백의 인산화가 현저히 감소 되었으며, 18 kDa/pI 5.4, 25/7.3 및 54/5.2단백은 두 억제제에 의해 영향을 받지 않았다. 이들 인산화 단백은 대부분 세포의 soluble fraction에서 확인되며 자극후 반응 초기에 인산화 된 후 인산화가 감소하나, 침전물에서 관찰되는 소수의 인산화 단백은 지속적인 인산화를 보였다. 한편 Kinase 억제제 처리에 의하여 구분된 3군에 속하는 단백들의 시간에 따른 인산화 양상을 관찰한 결과 각 군에 따른 인산화 양상에 상호 연관성이 없었다. 이상의 실험결과로 보아 림프구 활성의 초기 단계에서 인산화 되는 단백에는 PKC, CaM kinase 및 다른 kinase에 의해 인산화 되는 3종류의 단백이 존재하며, 3종류의 kinase의 활성은 단계적인 활성이 아니라 독립적 또는 상호 협동적으로 작용하여 림프구 활성을 유발시키는 것으로 생각된다.