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Phosphorylation of 44-kilodalton Proteins in Peripheral T-lymphocyte of Rat

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ABSTRACT

Using T-lymphocytes obtained from rat peripheral blood, we found that the 44kD/pI6.8 protein was the major phosphoprotein of T-lymphocytes under basal condition, and that the 44kD/pI6.3 protein was a new phosphoprotein appeared in T-lymphocytes stimulated with β-agonist. The phosphorylation of the 44kD/pI6.3 protein was also induced by forskolin but inhibited by H-8 pretreatment. To clarify the character of the 44kD/pI6.3 protein, we used Con-A and kinase inhibitors, H-7 and W-7. Con-A stimulation induced phosphorylation of 44kD/pI 6.3 protein but that was inhibited by W-7 pretreatment. The phosphorylation of 44kD/pI6.3 protein was not induced by the PKC activator, PMA. Instead, the phosphorylation of 44kD/pI6.8 protein was reduced by H-7, a PKC inhibitor. From the above results,it can be concluded that the 44kD/pI6.3 protein can be a common substrate for A-kinase and CaM kinase. The two dimensional tryptic peptide mapping revealed that the 44kD/pI6.8 and 44KD/pI6.3 proteins are different.

Key Words: Phosphoprotein, T-lymphocyte, 44kD/pI6.3 protein

INTRODUCTION

Posttranslational modification of a protein is a mechanism to regulate the biological activity of the protein in cell (Wold, 1981). Of the many types of posttranslational modification of proteins, relatively few are readily reversible. The most common type of readily reversible protein modification is phosphorylation (Krebs, 1986). The phosphorylation-dephosphorylation of a given protein is known to be an almost universal mechanism for the regulation of protein functions (Greengard, 1978).

The reaction of protein phosphorylation and dephosphorylation is catalyzed by converter enzymes such as protein kinases and phosphoprotein phosphatases (Krebs, 1986). There are a number of protein kinases in living cells. However, from the pharmacological point of view, four kinds of protein kinases are currently recognized as being involved in signal transduction pathways. Those are cAMP dependent kinase (A-kinase), cGMP dependent kinase (G-kinase), protein kinase C (PKC) and calcium-calmodulin dependent kinase (CaM-kinase) (Krebs and Beavo, 1979).

Once a neurotransmitter, hormone or drug is bound to its receptor, the signal is transmitted through a transducer protein to the catalytic protein. Then activation of an enzyme gene-

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rates a secondary messenger which activates a specific kinase (Graziano and Gilman, 1987). It is generally accepted that the phosphoprotein which is modified by activated protein kinase is thought to be a final mediator responsible for the cellular responses to the ligand-binding to the receptor.

Although the signal transduction pathway has been intensively studied, there are still limited reports on the specific protein as a substrate for various kinases.

Therefore, in our laboratory, we have been trying to find the specific substrate for each kinase in T-lymphocyte. T-lymphocyte has β-adrenergic receptor which is linked to A-kinase, as well as PKC and CaM-kinase which is linked to phosphatidyl inositide metabolism. During the study, we found that the 44 kD/pI 6.8 protein was the major phosphoprotein of T-lymphocyte under basal condition and that 44kD/pI 6.3, was another, new phosphoprotein appeared in T-lymphocytes stimulated with β-agonist. In this study, therefore, we analyzed the changes of phosphorylation of these two proteins by stimulation or inhibition of various kinases in relation to the signal transduction pathways.

MATERIALS AND METHODS

T-lymphocyte isolation

Sprague-Dawley rats were lightly anesthetized with ether and 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-Hypague density gradient centrifugation (Tasaka *et al.,* 1981). The lymphocytes were then loaded on a nylon wool column. After 30 minutes incubation at 37°C, unbound T-lymphocytes were collected from the column.

Protein phosphorylations in intact T-lymphocyte

T-lymphocytes were washed twice with RPMI medium and once with phosphate-free medium. The cells were suspended to a density of 10^6 cells/ml in phosphate-free media. One Hundred μCi of carrier-free ^{32}p orthophosphoric acid was added per ml of cell suspension, and incubated at 37°C for 4 hours in the CO_2 incubator. Then cells were stimulated with a specific agonist for 10 min after an additional 30 min incubation under the presence or absence of the respective inhibitor for a given agonist.

Cellular fractionation

After protein phosphorylation, T-lymphocytes were suspended in 3 volumes of standard buffer solution (10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 1 mM dithiothreitol, 50 units/ml soybean trypsin inhibitor, 2 μg/ml pepstatin A, 0.1 mM PMSF), and destructed by ultrasonication for 1 min. The homogenates were centrifuged at 2,000 RPM for 20 min. The supernatant was taken and recentrifuged at 150,000 ×g for 30 min to separate cytosolic fraction (supernatant) from membrane fraction (pellet).

Solubilization of membrane protein

Radiolabeled T-lymphocytes were washed twice with NKM buffer (130mM NaCl, 50 mM KCl, 8 mM $MgCl_2$) in the presence of protease inhibitors (1 mM PMSF, 1 mM N-ethylmaleimide, 2 mM EDTA, 50units/ml soybean trypsin inhibitor) for 10min. Then the cells were suspended in hypotonic solution (10 mM Tris, pH 7.4, 10 mM NaCl, 8 mM $MgCl_2$). After 10 min of incubation in hypotonic solution, 0.5 % of Nonidet P-40 were added and homogenized in Dounce homogenizer, and the solution was centrifuged at 2,000 RPM for 10min. The supernatant was recentrifuged at 150,000xg for 30min to separate detergent-soluble fraction (supernatant) from insoluble fraction (pellet).

Two-dimensional gel electrophoresis

Intact cells after ^{32}P-labelling and drug treatments were directly dissolved in O'Farrel sample buffer (9M 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 O'Farrel sample buffer. Two-dimensional gel electrophoresis was done by non-equilibrium pH gradient electrophoresis

(NEPHGE, pH 3.5~10) followed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (O'Farrel *et al.,* 1977). After electrophoresis, the gels were stained with Coomassie brilliant blue and dried. Phosphoproteins were identified by autoradiography at −70°C.

Tryptic peptide mapping of protein

Two dimensional tryptic peptide mapping was done by the slight modified method of Elder *et al.* (1977). Proteins were sliced from the SDS-gel with a razor blade. The slices were placed in siliconized tubes and washed extensively with 10% methanol, and dried by lyophilization. The proteins were then radioiodinated with ^{125}I in the gel slice with a modification of the chloramine-T method. After iodination of proteins, the gel slices were placed in 0.5 ml of trypsin solution (50 μg /ml of trypsin dissolved in 0.05 M NH_4HCO_3, pH 8.0). The eluted tryptic peptides were dried and dissolved in 20 μl of buffer I (acetic acid: formic acid: water, 15:5:80, pH 1.9), and spotted on to a thin layer chromatography (TLC) plates. Then peptide maps were made by high voltage (1KV) electrophoresis in buffer I followed by ascending chromatography in buffer II (butanol: pyridine: acetic acid: water, 32.5:25:5:20), and the peptides were identified by autoradiography (Zweig, 1981).

Experimental Scheme

Fig. 1 is the scheme of this experiment. In order to examine the β-receptor coupled A-kinase system, we used isoproterenol (10^{-5} M), a well known β-receptor agonist and forskolin (10^{-6}~10^{-5} M) as a stimulator of adenylate cyclase. Propranolol (10^{-5} M) and H-8 (10^{-6}~10^{-5} M) were used for a β-receptor blockade and an A-kinase inhibitor, respectively.

For the study of phosphatidyl inositide coupled CaM-kinase and PKC, concanavalin-A (Con-A, 25 μg/ml) and phorbol 12-myristate 13-acetate (PMA, 100 ng/ml) were used to stimulate a membrane receptor and PKC, and W-7 (10^{-5} M) and H-7(10^{-5} M) were used as inhibitors of CaM-kinase and PKC, respectively.

Drugs and reagents

RPMI media and fetal bovine serum were purchased from Hyclone Laboratories, Inc

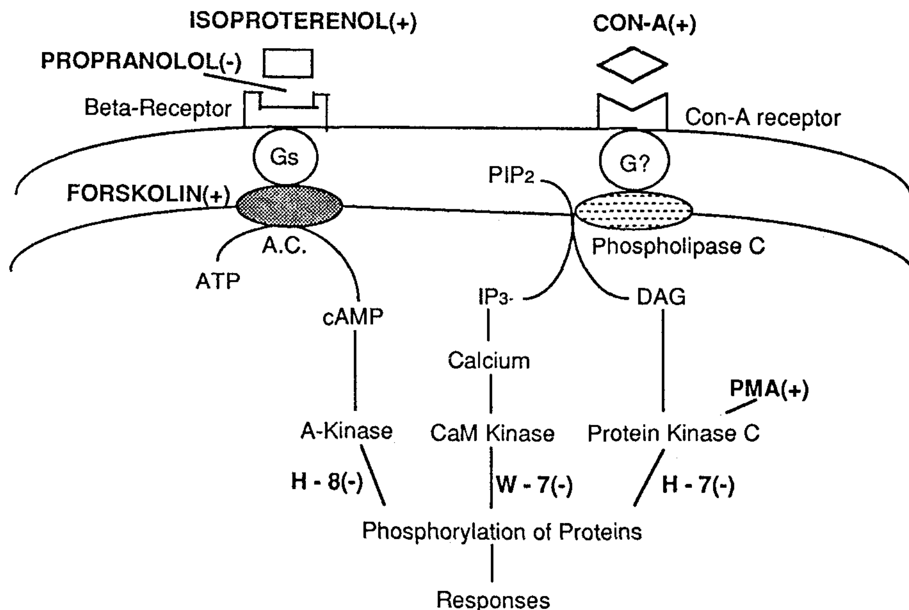

Fig. 1. Scheme of present experiment. To test the substrate for 3 kinases, various drugs (bold character) were used. (+) means the stimulator and (-) means inhibitor of each step.

(Logan, UT). Radioisotopes were purchased from Amersham, nylon wool column from Robbins Scientific Corp. (Sunnyvale, CA), Ampholine carrier ampholyte from LKB (Broma, Sweden), and N,N'-methylene-bis-acrylamide from Bio-Rad Lab. (Richmond, CA). H-8, H-7 and W-7 were purchased from Seikagaku America, Inc (St. Petersburg, FL). All the other drugs and reagents were purchased from Sigma Chemical Co. Inc (St. Louis, MO).

RESULT

Phosphoproteins of lymphocytes on the two dimensional gel electrophoresis

Two dimensional gel electrophoresis of intact, non-stimulated murine lymphocytes (10^6 cells) shows more than one hundred proteins by Coomassie brilliant blue staining (Fig. 2A). After an autoradiography of the gel, about 50 phosphoproteins were observed. The major phosphoprotein in murine lymphocyte was 44kD protein at isoelectric point of 6.8 (Fig. 2B). It was noticed that the amount of 44kD/pI 6.8 protein in Coomassie staining was relatively small (Fig. 2A, Arrow).

Changes of 44 kD/pI 6.3 protein phosphorylation by A-kinase pathway

When lymphocytes were stimulated by isoproterenol (10^{-6} M), an adrenergic β-receptor stimulant, a new phosphoprotein appeared on the acidic side of 44kD/pI 6.8. This phospho-

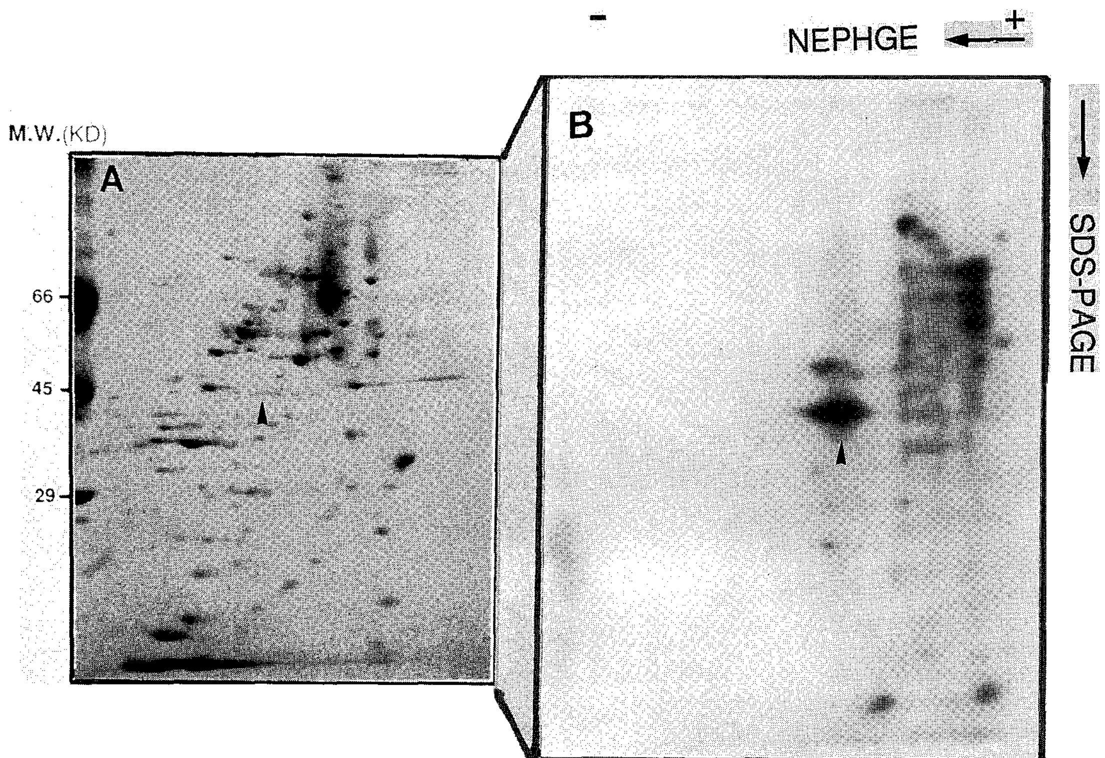

Fig. 2. Two dimensional non-equilibrium pH gradient electrophoresis (NEPHGE pH3.5-10)/sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation of the proteins of rat peripheral T-lymphocyte. Arrow heads indicate 44kD/pI 6.8 protein.
A: Coomassie brilliant blue stained gel.
B: Autoradiograph of the gel.

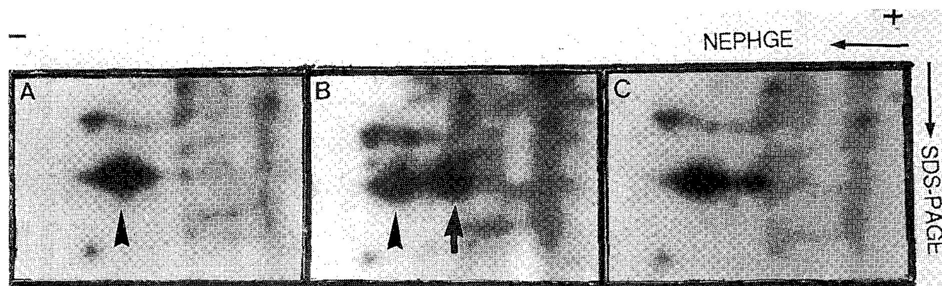

Fig. 3. Phosphorylation of 44kD/pI 6.3 protein by β-receptor agonist and antagonist.
A: Control.
B: Isoproterenol (10^{-5} M) treatment.
C: Propranolol(10^{-5} M) pretreatment followed by isoproterenol treatment. Arrow head indicates 44kD/pI 6.8 protein and arrow indicates 44kD/pI 6.3 protein. NEPHGE: Non-equilibrium pH gradient electrophoresis, SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

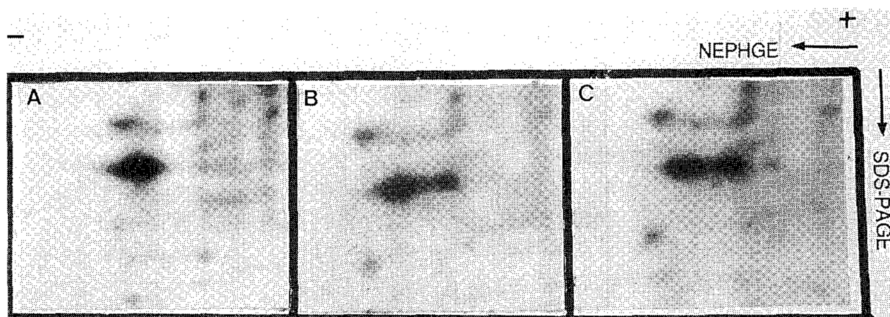

Fig. 4. Phosphorylation of 44kD/pI 6.3 protein by forskolin.
A: Control.
B: Forskolin 10^{-6} M.
C: Forskolin 10^{-5} M. Other legends are the same as Fig. 3.

protein had molecular weight of 44kD and isoelectric point of 6.3 (Fig. 3B). This isoproterenol-induced phosphorylation of 44kD/pI 6.3 protein was decreased by the pretreatment of propranolol, a β-receptor antagonist (Fig. 3C).

To test whether the phosphorylation of 44 kD/pI 6.3 was mediated by adenylate cyclase, we treated cells with forskolin which stimulates adenylate cyclase directly. Forskolin increased the phosphorylation of 44kD/pI 6.3 in a dose-dependent manner from 10^{-6} M to 10^{-5} M (Fig. 4).

To test whether the phosphorylation of 44kD/pI 6.3 was due to the action of A-kinase, we treated cells with the isoquinolinesulfonamide A-kinase inhibitor, H-8. Fig. 5 shows that isoproterenol-induced phosphorylation of 44 kD/pI 6.3 was decreased by H-8 pretreatment dose-dependently.

Changes of the 44kD protein phosphorylation by CaM-kinase and PKC pathways

When lymphocytes were stimulated with Con-A (25 μg/ml), phosphorylation of the 44 kD/pI 6.3 was also induced (Fig. 6A). However, PMA had no effect on the phosphorylation of the 44 kD/pI 6.3 (Fig. 6B). W-7 pretreatment inhibited the Con-A induced phosphorylation of 44 kD/pI 6.3. W-7 also decreased the phosphorylation of the 44 kD/pI6.8 protein (Fig. 6C). Interestingly, H-7 pretreatment also inhibited the phosphorylation of 44 kD/pI 6.3 protein

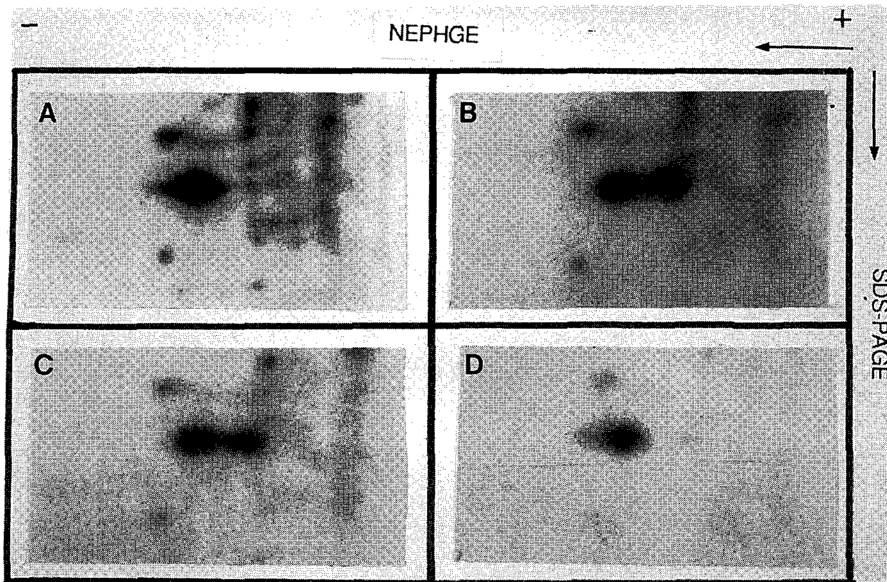

Fig. 5. Inhibition of 44kD/pI6.3 protein phosphorylation by H-8.
A: control.
B: Isoproterenol (10^{-5} M) treatment.
C: H-8(10^{-6} M) pretreatment followed by isoproterenol (10^{-5} M). D:H-8(10^{-5} M) pretreatment followed by isoproterenol. Other legends are the same as Fig. 3.

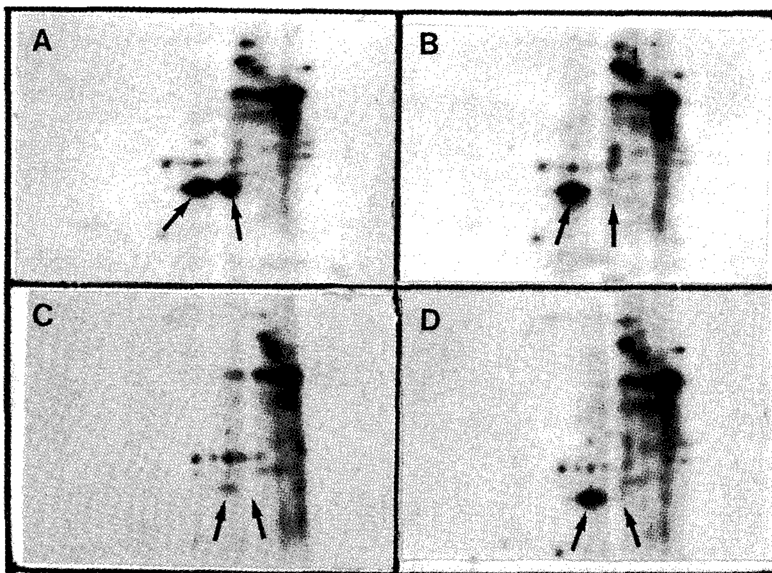

Fig. 6. Phosphorylation of 44 kD proteins by mitogen treatment.
A: Concanavalin-A (25 μg/ml) treatment.
B: PMA(100 ng/ml) treatment.
C: W-7(10^{-5} M) pretreatment followed by Con-A.
D: H-7(10^{-5} M) pretreatment followed by Con-A. Left arrows indicate 44 kD/pI 6.8 protein and right arrows for 44 kD/pI 6.3 protein.

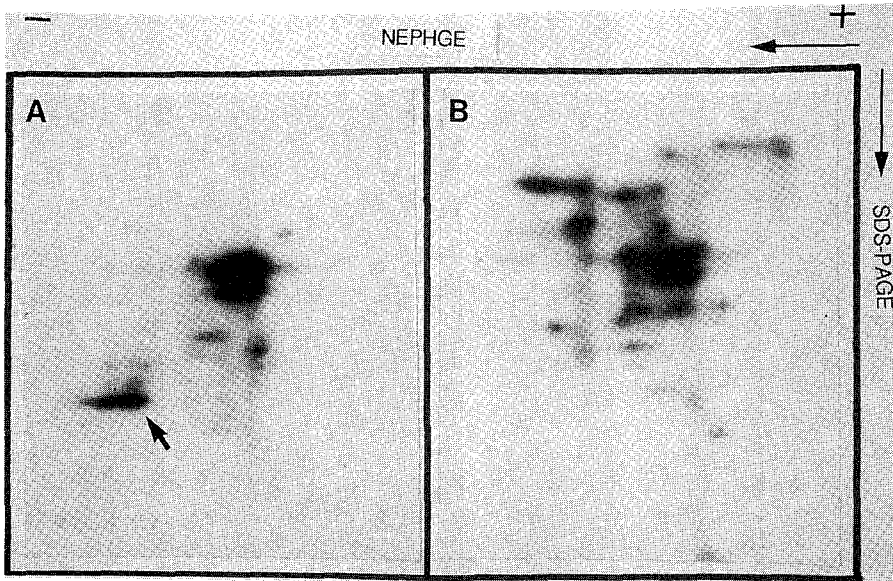

Fig. 7. Intracellular localization of 44 kD proteins after solubilization of T-lymphocyte with Nonidet P-40.
A. soluble fraction.
B: Pellet.

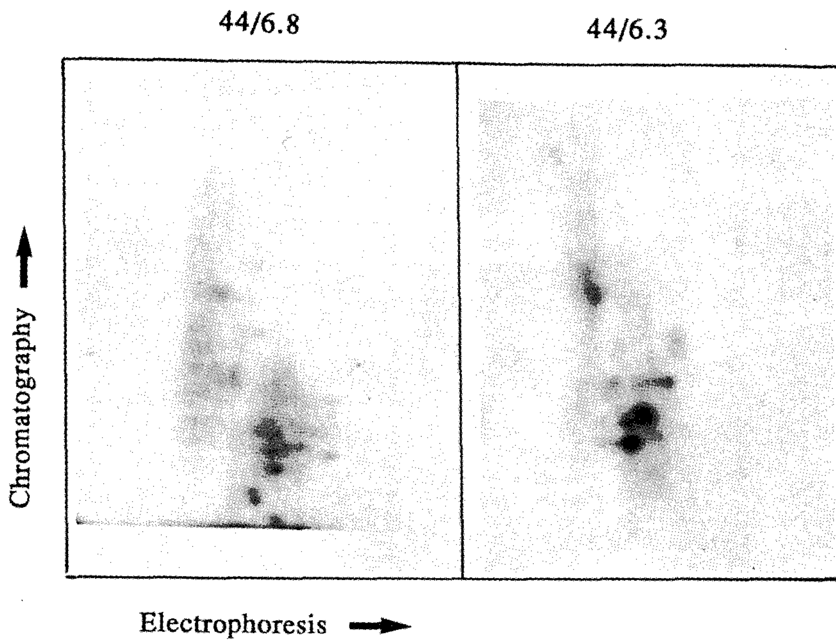

Fig. 8. Two-dimensional fractionation of tryptic peptides of the 44 kD proteins labeled with ^{125}I by chloram ine-T method.

(Fig. 6D).

Cellular localization of 44 kD proteins

When cells were fractionated into cytosolic and membrane fraction, the 44 kD/pI 6.8 and 44 kD/pI 6.3 proteins were observed both in cytosolic and membrane fractions. However, in the presence of non-ionic detergent (0.5% Nonidet P-40), both 44 kD proteins were only fell into a soluble fraction (Fig 7).

Peptide mapping of 44 kD protein

The 44 kD proteins had identical molecular weights and differed in charge. This suggested that the 44 kD/pI 6.3 protein could be in multiple phosphorylated form of 44 kD/pI 6.8 protein. Two dimensional tryptic peptide mapping was performed, therefore, to see whether two proteins are the same. As shown in Fig 8, the tryptic peptide mappings of two proteins were quite different.

DISCUSSION

In this study, we found that the phosphorylation of 44 kD/pI 6.3 protein was induced by β-receptor agonist-isoproterenol, and inhibited by the pretreatment of β-receptor antagonist in rat T-lymphocytes. The phosphorylation of 44 kD/pI 6.3 protein was also induced by forskolin a direct stimulator of adenylate cyclase, and inhibited by H-8 - an A-kinase inhibitor. This experiment demonstrated that the phosphorylation of 44 kD/pI 6.3 protein was induced by A-kinase which is linked to β-receptor and adenylate cyclase.

To test whether the 44 kD/pI 6.3 protein is the specific substrate for only A-kinase, the next experiment using drugs that activate or inhibit CaM-kinase and PKC was performed. Con-A which activates both kinases, also induced the phosphorylation of the 44 kD/pI 6. 3 protein. CaM-kinase inhibitor, W-7, inhibited the Con-A induced phosphorylation of 44 kD/ pI 6.3. Therefore, it was shown that the phosphorylation of the 44 kD/pI 6.3 was also induced by CaM-kinase.

PKC does not seem to phosphorylate the 44 kD/pI 6.3 protein, because the PKC activator, PMA, did not phosphorylate the protein. H-7 which Hidaka *et al.*, (1987) originally reported as a specific PKC inhibitor, is now known to inhibit strongly A-kinase and G-kinase as well as PKC. The fact that the phosphorylation of the 44 kD/pI6.3 protein was suppressed by H-7, could be explained as the A-kinase inhibitory action of H-7. Instead, the phosphorylation of the 44 kD/pI 6.8 protein was suppressed by W-7 indicates that this could be the specific substrate for CaM-kinase.

It is reported that cAMP, its lipophilic analogs or agents which stimulate increases in intracellular cAMP prevented subsequent events in lymphocyte activation (Hadden, 1977; Coffey and Hadden, 1985). Also, PMA, a selective mitogen for a subpopulation of human T lymphocytes (Toulaine *et al.*, 1977) actually caused a 50% decrease in adenylate cyclase activity of human lymphocytes (Coffey and Hadden, 1983). Then the question is how the 44 kD/pI6.3 protein can be a common substrate for A-kinase and CaM-kinase. One possible explanation is that Con-A can increase cAMP. The first report of mitogen-induced changes in lymphocyte cAMP was that of Smith *et al.*, (1971) who observed small increase in cAMP in human lymphocytes 2 min after exposure to phytohemagglutinin. This phenomenon was confirmed by some workers using Con-A (Watson, 1975; Coffey *et al.*, 1977). However, there are still contrary reports in that they were unable to detect early increase in cAMP using optimal concentrations of mitogens (Novogrodsky and Katchalski, 1970; Largen and Votta, 1983). Another possibility is derived from the reports that β-receptor stimulation or prostaglandin which increases cAMP, caused dual effects on the function of T-lymphocytes as well as natural killer cells (Kendall and Targan, 1980; Hellstrand *et al.*, 1985). Further experiments are needed to clarify the issue.

Since the report of Langan (1978) who demonstrated that several protein kinases may phosphorylate a common substrate at multiple sites, it is generally accepted that protein kinases phosphorylate their target proteins at discrete sites. Therefore, it is possible that the

phosphorylation sites for the respective kinases may be different, which has to be confirmed by phosphopeptide analysis.

Two 44kD phosphoproteins in this experiment differ only in their charge by 0.5 pI unit. This suggests that 44kD/pI 6.3 could be an additionally phosphorylated form of 44kD/pI 6.8. This possibility was denied by the two dimensional tryptic peptide mapping of two proteins, which showed that the two proteins were different.

From this experiment, it is hard to imagine the function or the role of the 44kD proteins and we need further study to characterize the proteins.

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=국문초록=

흰쥐 말초 혈액 림프구의 분자량 44 kD 단백의 인산화

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안영수 · 주일로 · 오도연[†] · 임승욱 · 박경선[††]

흰쥐 말초혈액에서 얻은 T 림프구를 아드레날린성 β-수용체 효현제 및 concanavalin A (Con-A)로 자극해 다음과 같은 결과를 얻었다. 자극이 없는 상태에서의 주 인산화 단백은 분자량 44kD, 등전점 6.8의 단백이었으며 효현제로 자극시키면 분자량 44kD, 등전점 6.3의 단백이 새로이 인산화되어 나타났다. 이 분자량 44kD, 등전점 6.3의 단백은 forskolin에 의해 역시 인산화되며 A-kinase 억제제인 H-8을 전처치하면 인산화의 억제가 나타났다. 또한 Con-A로 자극시키면 44 kD/pI 6.3 단백의 인산화가 증가되었으며 이 인산화의 증가는 CaM kinase 억제제인 W-7 전처치에 의해 억제되었다. H-7은 분자량 44 kD, 등전점 6.8 단백의 인산화를 감소 시켰다.

이상의 결과로 분자량 44 kD 등전점 6.3의 단백은 A-kinase와 CaM kinase 모두에 의해 인산화 되는 기질단백으로서 tryptic peptide map상에서 44 kD/pI 6.8 단백과 44 kD/pI 6.3 단백은 서로 다른 단백임을 알 수 있었다.