Two types of Ca^{2+}-dependent phosphorylation in rabbit leukocytes

Toshiya Hirayama and Iwao Kato

Department of Bacterial Infection, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo, Japan

Received 21 July 1982

Protein phosphorylation *Leukocytolytic toxin* *Protein kinase* *Calmodulin dependence*
Ca^{2+}-dependence *(Rabbit leukocyte)* (Pseudomona aeruginosa)

1. INTRODUCTION

Ca^{2+} as well as cyclic nucleotides is believed to function as a key regulatory agent in a variety of responses of leukocyte against both particulate and soluble stimuli. However, extracellular Ca^{2+} and influx of Ca^{2+} do not appear to be essential components of leukocyte activation since the cells are capable of responding in the absence of extracellular Ca^{2+} [1]. Intracellular Ca^{2+}, however, seems to be essential; superoxide anion generation and lysosomal enzyme release can be abolished by an inhibitor of intracellular Ca^{2+} mobilization (TMB-8) and inhibitors of calmodulin (trifluoperazine and W-7) [1–4]. Although the detailed function of intracellular Ca^{2+} had not yet been determined, protein phosphorylation is clearly the major general mechanism by which intracellular events respond to external physiological stimuli, and enzymes themselves or regulatory proteins are now known to be controlled by reversible phosphorylation [5].

One possible mechanism by which Ca^{2+} could regulate responses of leukocyte against stimuli is by activating protein kinase, which would phosphorylate specific proteins. Therefore, we investigated whether Ca^{2+} stimulates phosphorylation of specific proteins of the leukocyte. Here, we have found that phosphorylation of a 31 500-M_r protein is dependent on Ca^{2+} and calmodulin (CaM), and that a 28 000-M_r protein was phosphorylated in a Ca^{2+}- and CaM-independent manner. The addition of Ps-toxin, which is a leukocytolytic toxin from *Pseudomonas aeruginosa* requiring intracellular Ca^{2+} mobilization for leukocytolysis [6], stimulated the phosphorylation reaction to a greater extent than Ca^{2+} alone.

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N*-tetraacetic acid; SDS, sodium dodecyl sulfate; TMB-8, 8-(*N,N*,-diethylamino)octyl-3,4,5-trimethoxybenzoate; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphtalene sulfonamide

2. MATERIALS AND METHODS

2.1. *Preparation of leukocytolytic toxin (Ps-toxin)*

Pseudomonas aeruginosa 158 used here was known to produce leukocytolytic toxin in [7] and kindly supplied by Dr F. Lutz (Institute of Pharmacology and Toxicology, Justus-Liebig-University). The microorganisms were grown upon a Difco trypticase soy broth medium containing 0.5% glucose at 30°C for 12 h. The culture fluid was then centrifuged at 1100 × *g* for 15 min at 4°C, and the bacteria washed and suspended in 1.4 l phosphate buffer in [8]. The autolysis of the bacteria was carried out by the shaking incubation at 37°C for 48 h. The supernatant after centrifugation of the autolysate at 20 000 × *g* for 20 min was dialyzed against 0.05 M sodium phosphate buffer (pH 7.2). The dialysate was applied to a DEAE–Sephadex A-50 column (5 × 100 cm) equilibrated with 0.05 M sodium phosphate buffer (pH 7.2). After washing the column with the equilibrated buffer, fractions containing leukocytolytic activity were eluted with 0.5 M sodium phosphate buffer (pH 7.2) and were concentrated by an Amicon ultrafiltration apparatus equipped with a PM 10 membrane. The resulting solution was applied to a Sephadex G-100 column (5 × 100 cm) pre-equilibrated with 0.05 M sodium phosphate buffer

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$2.75

(pH 7.2). After elution of protein with the same buffer, fractions containing leukocytolytic activity were collected and concentrated. This solution, equilibrated with 0.05 M sodium phosphate buffer (pH 8.0), was applied to a zone electrophoresis on Pevikon C-870 using the same buffer at 20 mA for 24 h. Active fractions from this final purification step were collected, concentrated by membrane filtration, crystallized by means of dialysis against ammonium sulfate [9], and stored at −95°C. Leukocytolytic activity was assayed by the microscopic slide adhesion method as in [9] except for using Ca^{2+}-Dulbecco's phosphate-buffered saline (PBS; 0.01 M sodium–potassium phosphate buffer (pH 7.4), 0.15 M NaCl, 2.5 mM KCl and 1 mM $CaCl_2$).

2.2. *Isolation of rabbit leukocyte and protein determination*

Polymorphonuclear leukocytes (referred to here as leukocytes) were prepared from rabbit peripheral blood on Ficoll isokinetic gradients [10]. Estimation of protein was performed by the Lowry method [11], using bovine serum albumin as standard.

2.3. *Phosphorylation studies*

Each homogenate of leukocytes (3.2×10^6 cells) was prepared by sonication with 30 μl 0.01 M Tris–HCl buffered saline (pH 7.2) containing 5 mM $MgCl_2$, using a Bransonic 12 sonifier set for 1 min at 4°C, and was incubated for 5 min at 37°C in a 50 μl reaction mixture containing 5 μM [γ-^{32}P]ATP (34 Ci/mmol), without any addition or with 1 mM $CaCl_2$, 3.7 μM calmodulin (CaM), 0.23 μM Ps-toxin (M_r 42 500), 3.3–100 μM trifluoperazine (TFP), or EGTA, where indicated. The reaction was terminated by addition of 10 μl mixture containing 10% SDS and 10% 2-mercaptoethanol, 0.008% bromphenol blue, and 30% sucrose, and heated at 100°C for 5 min. For each sample, 20 μl portions were used for electrophoresis. Electrophoresis was performed by using a discontinuous SDS–polyacrylamide slab-gel system. The stacking gel contained 5% acrylamide, 0.125 M Tris–HCl buffer (pH 6.8) and 0.1% SDS. The resolving gel contained 14% acrylamide, 0.375 M Tris–HCl buffer (pH 8.8), and 0.1% SDS. The electrode buffer was made of 0.05 M Tris–0.37 M glycine and 0.1% SDS. After electrophoresis at 20 mA the gels were stained in 0.2% Coomassie brilliant blue, destained by diffusion in 5% ethanol and 7.5% acetic acid, and then dried. Autoradiography was carried out with Kodak X-O mat S film for 20 h using intensifying screens from Kodak. M_r standards used were hen egg albumin (45 000), soybean trypsin inhibitor (21 000), and myoglobin (18 000).

3. RESULTS AND DISCUSSION

Fig.1 shows the results of experiments where the phosphorylated proteins were separated by SDS–polyacrylamide slab-gel and autoradiography. In the presence of endogenous Ca^{2+}, incorporation of ^{32}P-radioactivity into some proteins was observed (fig.1, lane 1). Addition of Ca^{2+} (1 mM) caused an increase in phosphorylation of a single protein band with app. M_r 28 000 (fig.1, lane 2). In the presence of Ca^{2+}, 3.7 μM CaM resulted in in-

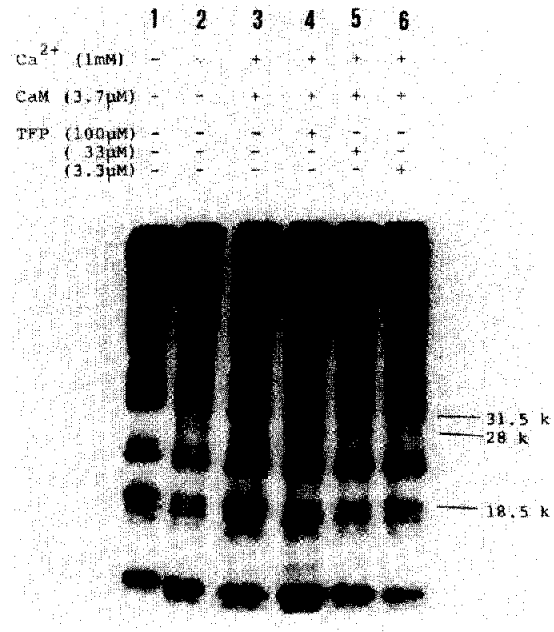

Fig.1. Effects of Ca^{2+}, CaM, and TFP on leukocyte protein phosphorylation. The autoradiogram illustrates phosphorylated proteins. Leukocyte homogenates were incubated for 5 min with 5 μM [γ-^{32}P]ATP and the indicated additions as in section 2. Lane 1 shows the phosphorylation pattern without 1 mM Ca^{2+} and other lanes with 1 mM Ca^{2+}. Other additions were: lane 3, 3.7 μM CaM; lanes 4–6, 3.7 μM CaM and 3.3–100 μM TFP.

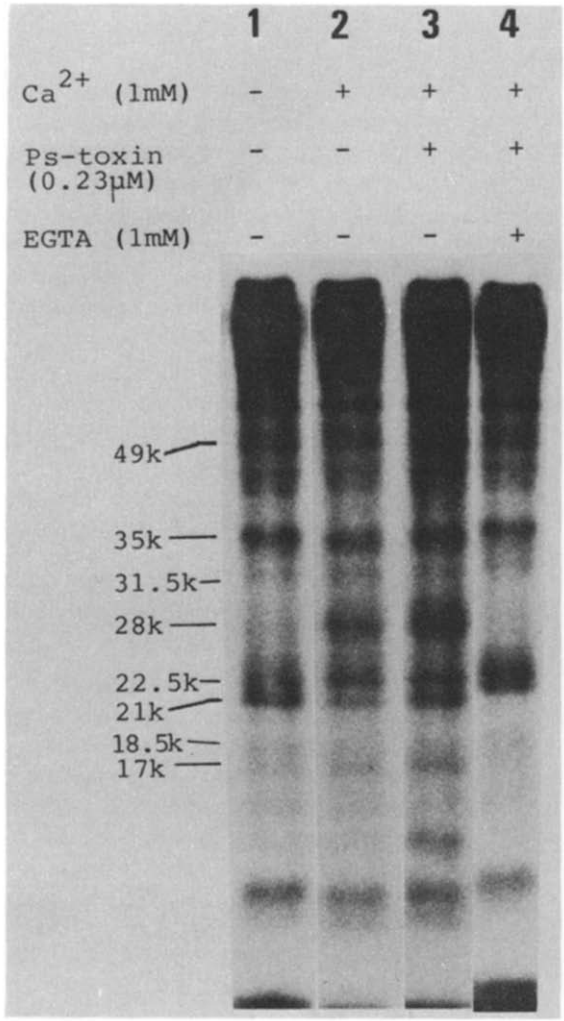

Fig.2. Autoradiogram showing effects of Ps-toxin and EGTA on phosphorylation of leukocyte protein. Leukocyte homogenates were incubated for 5 min with 5 μM [γ-^{32}P]ATP and the indicated additions as in section 2. Lane 1 shows the phosphorylation pattern without 1 mM Ca^{2+} and other lanes with 1 mM Ca^{2+}. Other additions were: lane 3, 0.23 μM Ps-toxin; lane 4, 0.23 μM Ps-toxin and 1 mM EGTA.

creased phosphorylation of 2 proteins with M_r 31 500 and 18 500 (fig.1, lane 3). In the presence of 1 mM Ca^{2+} and 3.7 μM CaM, the Ca^{2+}- and CaM-induced phosphorylation of both protein bands inhibited markedly in a dose-dependent manner by the addition of 3.3–100 μM TFP (fig.1, lanes 4–6). Whereas phosphorylation of the 28 000-M_r protein was greatly enhanced by 100 μM TFP (fig.1, lane 4). Furthermore, phosphorylation of the 28 000-M_r protein band was increased markedly in the presence of Ca^{2+} and Ps-toxin (0.23 μM) (fig.2). To quantify ^{32}P-incorporation, these portions of the gel were cut out and counted. The radioactivity in the 28 000-M_r region of the gel was 1.7-fold higher in the sample to which Ps-toxin was added than in the sample with Ca^{2+} alone and 45-fold higher than in the sample without Ps-toxin and Ca^{2+}. EGTA (1 mM) was found to drastically reverse the effect of Ca^{2+} and Ps-toxin (fig.2, lane 4). The addition of 10 μM cyclic AMP or 33 μM TFP into the reaction mixture did not affect on the phosphorylation of 28 000-M_r protein (not shown).

From the above results, it can be concluded that the rabbit leukocyte contains 2 types of Ca^{2+}-dependent protein kinase; one of them is a Ca^{2+}- and CaM-dependent protein kinase that phosphorylates 31 500- and 18 500-M_r proteins in the leukocyte homogenate and the other is a Ca^{2+}-dependent and CaM-independent protein kinase that phosphorylates the 28 000-M_r protein. However, Ca^{2+}-activated, phospholipid-dependent protein kinase (C-kinase) has been reported in various mammalian tissues [12–15] such as cerebral cytosol and pancreas of rat, and human platelet. In [16] TFP was demonstrated as a potent inhibitor of C-kinase with 24–50 μM. Here, TFP had no effect on the phosphorylation of the 28 000-M_r protein at 33 μM, whereas in fig.1 (lane 4) 100 μM TFP had a stimulatory effect on phosphorylation. It is thus probable that the Ca^{2+}-dependent and CaM-independent protein kinase differs from C-kinase. The presence of 2 types of Ca^{2+}-dependent protein kinase may provide insight into the role of Ca^{2+} in leukocyte functions.

ACKNOWLEDGEMENTS

We wish to thank Miss M. Nagamori for skillful technical assistance. This work was supported by grants 56440033 and 57770323/1982 from the Ministery of Education, Science and Culture of Japan.

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