

Inhibition of Adenosine 3',5'-Monophosphate-Dependent Protein Kinase by Staphylococcal Alpha-Toxin

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Staphylococcal alpha-toxin inhibited the activity of cyclic adenosine 3',5'-monophosphate-dependent protein kinase by competitive inhibition, probably by its interaction with a cyclic adenosine 3',5'-monophosphate-binding site in the protein kinase molecule.

Crystalline staphylococcal alpha-toxin, a protein with a molecular weight of 36,000, has a lytic activity on rabbit erythrocytes (one hemolytic unit = 0.02 μ g of protein), a lethal toxicity in mice (50% lethal dose, 1 μ g of protein) (5-7, 11), and a capacity to introduce contraction of mammalian smooth muscle (1). Recently, we reported that a lethal toxic fragment (mouse 50% lethal dose, 8 μ g of protein) could be isolated from the alpha-toxin molecule under mild conditions of tryptic digestion (13). However, the specific mechanism of the action of alpha-toxin and its lethal toxic fragment is still unknown.

Cyclic adenosine 3',5'-monophosphate (cAMP)-dependent protein kinase is central to the mediation of the physiological effects of cAMP in mammalian tissues (8). In this study we present data indicating that the inhibition of the activity of cAMP-dependent protein kinase by staphylococcal alpha-toxin and its lethal toxic fragment is due to competition with a cAMP-binding site in the protein kinase molecule.

Crystalline staphylococcal alpha-toxin and the purified lethal toxic fragment were obtained by methods described previously (12, 13). cAMP-dependent protein kinase from rabbit muscle was prepared by the procedures described by Miyamoto et al. (9) and Gilman (4) by using chromatography on diethylaminoethyl-cellulose and Sephadex G-200. The purified protein kinase, a single protein of molecular weight 56,000, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, had a cAMP-binding activity and catalyzed the rate of phosphorylation with a variety of protein substrates, such as histone or protamine. Table 1 shows that both alpha-toxin and its lethal toxic fragment at 1 μ M inhibited cAMP binding and the activity of protein kinase until a plateau was reached at 50% inhibitory effect. Rabbit antiserum raised against purified alpha-toxin reversed the reaction completely at a concentration equivalent to that of the alpha-toxin employed.

Since both alpha-toxin (isoelectric point, 7.9) and the lethal toxic fragment (isoelectric point, 8.2) are basic proteins, ribonuclease A and egg yolk lysozyme (both basic proteins) were investigated, but they were not effective. However, histone II (Sigma Chemical Co.) and protamine (Sigma) stimulated the cAMP binding to the protein kinase (Table 1). These findings seem to indicate that electrostatic interaction of the acidic protein kinase with basic proteins is not necessary to inhibit cAMP binding and protein kinase activity but that the inhibition depends on the protein structure itself. The effect of nucleotides and related compounds on cAMP binding is shown in Table 1. These results were in reasonable agreement with those of Gilman (4). This protein kinase showed a high affinity for cAMP binding (binding constant, 2.7 nM; Fig. 1), and a cAMP-dependent protein kinase inhibitor (Sigma) from rabbit muscle increased the affinity of cAMP for the enzyme (binding constant, 1.6 nM; Fig. 1), whereas it inhibited markedly the protein kinase activity.

Reciprocal plots of cAMP binding against cAMP concentrations (Fig. 1) suggested that the inhibition of initial rate of cAMP binding by alpha-toxin (0.2 to 1 μ M) was competitive in nature.

The binding of the alpha-toxin to cAMP-dependent protein kinase was measured by isoelectric focusing in polyacrylamide gels by using the method of Righetti and Drysdale (10) and 125 I-labeled alpha-toxin. As Fig. 2 shows, an enzyme-toxin protein complex appeared in the gel as an intermediary band containing 125 I-labeled alpha-toxin. 125 I-labeled alpha-toxin bound to the enzyme corresponded to a molar proportion of 2:1 (data not shown). It is now well accepted that cAMP-dependent protein kinase contains a dimeric regulatory subunit and two catalytic subunits which dissociate when cAMP binds to the regulatory subunits (2 mol of cAMP per mol of subunit monomer) (2) and that the protein ki-

TABLE 1. Effect of staphylococcal alpha-toxin on cAMP binding and activity of cAMP-dependent protein kinase

Addition to assay mixture	Concn	Amt of [³ H]cAMP bound (pmol/μg of protein) ^a	Protein kinase activity (U) ^b
None		0.71 ^c (100) ^d	21.4 ^c (100) ^d
Alpha-toxin	0.5 μM	0.60 (85)	18.8 (88)
Alpha-toxin	1 μM	0.36 (50)	11.4 (53)
Alpha-toxin	5 μM	0.37 (52)	11.1 (52)
Alpha-toxin (1 μM) + antiserum	40 μg/ml	0.70 (99)	20.8 (97)
Lethal toxic fragment ^e	1 μM	0.35 (49)	10.0 (47)
Protein kinase inhibitor	50 μg/ml	0.89 (127)	4.1 (19)
Ribonuclease A	50 μg/ml	0.68 (95)	21.6 (101)
Lysozyme	50 μg/ml	0.72 (101)	21.8 (102)
Histone II	10 μg/ml	0.99 (133)	
Protamine	50 μg/ml	0.80 (113)	
Cyclic guanosine 5'-monophosphate	5 μM	0.35 (50)	19.5 (91)
Guanosine 5'-triphosphate	50 μM	0.64 (90)	19.2 (90)
Adenosine 5'-triphosphate	50 μM	0.70 (98)	285.2 ^f (1330)

^a The standard binding reaction mixture contained, in a final volume of 0.2 ml, 50 mM sodium acetate buffer (pH 4.0) and 0.1 μM [³H]cAMP (16 Ci/mmol). Reactions were initiated by the addition of 5 μg of protein kinase and were incubated for 100 min at 0°C. [³H]cAMP binding assay was performed by the method of Gilman (4).

^b The standard assay contained, in a final volume of 0.2 ml, 50 mM sodium acetate buffer (pH 6.0), 40 μg of histone II, 5 μM [³²P]adenosine 5'-triphosphate containing 1.4×10^6 cpm, 10 mM magnesium acetate, and 5 μg of protein kinase in the absence or presence of 5 μM cAMP. Incubations were carried out at 30°C for 10 min. Histone-bound ³²P was measured by the method of Miyamoto et al. (9). A unit of protein kinase is defined as 1 pmol of ³²P transferred to the histone per min at 30°C.

^c The data presented are the means of six experiments.

^d The numbers in parentheses are percentages compared with the value obtained when there was no addition to the assay mixture, which was defined as 100%.

^e The purified lethal toxic fragment has a molecular weight of 17,000 (13).

^f Corrected for the decrease in specific activity which resulted from addition of non-radioactive adenosine 5'-triphosphate.

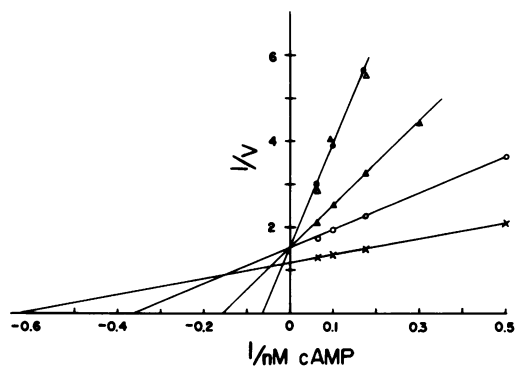


FIG. 1. Lineweaver-Burk plot showing competitive inhibition of substrate kinetics by staphylococcal alpha-toxin. Assays were carried out by the method of Gilman (4) as described in Table 1, footnote a, in the presence of [³H]cAMP (2 to 10 nM) and the indicated concentrations of alpha-toxin. Velocity (V) is expressed in picomoles of cAMP per microgram of protein per 100-min incubation. The data presented are the means of three experiments. Concentrations of alpha-toxin: \blacktriangle , 0.5 μM; \bullet , 1 μM; \triangle , 5 μM; \circ , no alpha-toxin. \times , 50 μg of protein kinase inhibitor per ml.

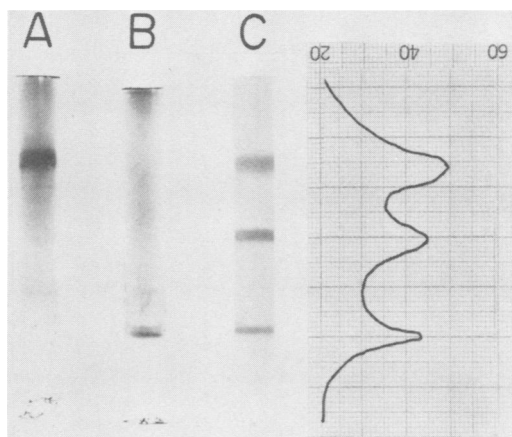


FIG. 2. Protein kinase-alpha-toxin complex in isoelectric focusing gel. Protein kinase and ¹²⁵I-labeled alpha-toxin (1.2×10^5 cpm/μg of protein) were mixed and incubated at 30°C for 10 min. Isoelectric focusing was performed with 2% ampholyte (LKB Instruments Inc.) at pH 3.5 to 10. After focusing, the gels were stained, scanned for absorbance at 570 nm, and measured for radioactivity. Lane A, protein kinase (10 μg); lane B, alpha-toxin (8 μg); and lane C, mixture of protein kinase (12 μg) and alpha-toxin (8 μg).

nase inhibitor specifically binds to the dissociated catalytic subunit (3). Since [^3H]cAMP does not bind the alpha-toxin or the lethal toxic fragment and the alpha-toxin does not change the activities of adenylate cyclase and cAMP phosphodiesterase (unpublished data), the mechanism proposed for the inhibitory effect of the alpha-toxin on binding of cAMP to the protein kinase is the interaction of the alpha-toxin with one of two cAMP-binding sites in the regulatory subunit molecule. On this basis, further experiments attempting to clarify the effect of staphylococcal alpha-toxin on the spastic paralysis of isolated smooth muscle through the cyclic nucleotide pathway are in progress.

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