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INHIBITORY EFFECT OF A LETHAL TOXIC FRAGMENT OF STAPHYLOCOCCAL α-TOXIN ON CYCLIC AMP-DEPENDENT PROTEIN KINASE ACTIVITY

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Summary

The effect of a lethal toxic fragment of staphylococcal α-toxin on the activity of adenosine 3′,5′-monophosphate(cyclic AMP)-dependent protein kinase was examined.

1. The lethal toxic fragment produced a dose-dependent decrease in both the binding of cyclic AMP to the regulatory subunit and phosphorylation activity of cyclic AMP-dependent protein kinase obtained from rabbit skeletal muscles upto a plateau at a 50% inhibitory effect. The decrease in the activity of protein kinase observed with low doses of the lethal toxic fragment (0.1 μM) resulted from a competitive inhibition, probably by its interaction with the cyclic AMP-binding site in the regulatory subunit molecule.

2. The effects of a lethal toxic fragment and epinephrine on the cyclic AMP level and protein kinase activity were investigated in the perfused rabbit heart slices. The lethal toxic fragment attenuated the stimulation of cyclic AMP-dependent protein kinase activity ratio by epinephrine.

3. It is suggested that the specific action of a lethal toxic fragment on the cellular membrane enzymes may be attributable to the inhibition of the cyclic AMP-dependent protein kinase activity.

Introduction

The α-toxin is probably the most studied of the staphylococcal membrane-damaging toxins. Yet its mechanism of action at the molecular level is still not fully understood. Much of the earlier work can be criticized on the grounds that the α-toxin preparations employed were contaminated with other mem-

brane-damaging toxins, especially with δ-toxin. For instance, Cassidy et al. [1] reported that three activities previously attributed to α-toxin, viz., disruption of mitochondrial membrane fraction, lysis of bacterial protoplasts and inhibition of the ($Na^+ + K^+$)-dependent ATPase of guinea pig kidney are not in fact properties of α-toxin. Since we now have an isolated lethal toxic fragment from the crystalline α-toxin, which is hemolytic, dermonecrotic and lethal [2,3], it was interest to examine the biological properties of the lethal toxic fragment preparation. In this paper, we investigated the effects of α-toxin and its lethal toxic fragment on the activities of the cellular membrane-bound enzymes and we demonstrated that a lethal toxic fragment inhibited the activity of a soluble adenosine 3′,5′-monophosphate(cyclic AMP)-dependent protein kinase by its interaction with the cyclic AMP-binding site in the protein kinase molecule.

Materials and Methods

Materials

Cyclic AMP, ATP, cyclic GMP, GTP, bovine serum albumin, histone II (calf thymus), protamine, cyclic AMP-dependent protein kinase inhibitor (bovine heart), trichloroacetic acid, sodium tungstate and L-epinephrine were purchased from Sigma Chemical Co. St. Louis. [γ-^{32}P]ATP (24 Ci/mmol), cyclic [^{3}H]AMP (16 Ci/mmol) and carrier-free $Na^{125}I$ (17 Ci/mg) were purchased from New England Nuclear Corp., Boston. All other chemicals were of analytical grade.

Crystalline α-toxin and a lethal toxic fragment

Staphylococcal α-toxin from strain Wood 46 was highly purified and crystallized as described previously [2]. A lethal toxic fragment was isolated from the crystallized α-toxin under mild conditions of tryptic digestion [3].

Preparation of cyclic AMP-dependent protein kinase and its subunit

1 kg rabbit skeletal muscle was homogeneized with 3 vols. 5 mM potassium phosphate buffer (pH 7.0), 4 mM EDTA for 2 min in a Waring Blendor. The homogenate was centrifuged in the cold at 27 000 × *g* for 30 min. The clear supernatant was precipitated with 500 g/l $(NH_4)_2SO_4$. The precipitate by centrifugation was resuspended in 5 mM phosphate buffer (pH 7.0) and dialyzed overnight. The dialyzed solution applied to a column of DEAE-cellulose according to the procedure of Gilman [4]. The second major peak (DEAE-II) was pooled, concentrated by precipitation with 65% $(NH_4)_2SO_4$ solution and dialyzed. The dialyzed holoenzyme was then applied to a column of DEAE-Sephadex A-50, equilibrated and eluted with a linear gradient of 50—300 mM potassium phosphate (pH 7,0). A major peak of binding and phosphorylation activity was collected, dialyzed against 5 mM phosphate buffer (pH 7.0). Such a holoenzyme preparation had an enzymic activity of 21 pmol $^{32}P_i \cdot \mu g^{-1}$ protein $\cdot$ min^{-1}, and bound 0.7 pmol cyclic AMP/μg protein under standard assay conditions. Protein was measured by the method of Lowry et al. [5] using bovine serum albumin as standard. The regulatory and catalytic subunits were prepared from the holoenzyme by fractionation on DEAE-cellulose in the presence of cyclic AMP by the procedure of Rubin et al. [6]. The catalytic subunit eluted by washing qith 5 mM potassium phosphate buffer (pH

7.0), 25 mM cyclic AMP and the fractions were pooled, concentrated by vacuum dialysis to at least 5 mg/ml protein and stored at −85° C. The regulatory subunit was eluted with 50 mM potassium phosphate buffer (pH 7.0), 0.5 M NaCl. The pooled fractions containing regulatory subunit were concentrated by vacuum dialysis and removal of cyclic AMP bound to the regulatory subunit was facilitated by incubation with 5 mM cyclic GMP at 25° C for 30 min followed by dialysis for 48 h against four 1-liter changes of 50 mM potassium phosphate buffer (pH 7.5) at 4° C and applied to an upward flow column (1.5 × 60 cm) of Sephacryl S-200 (Pharmacia). The column was eluted with 5 mM potassium phosphate buffer (pH 7.0) and the pooled fractions containing cyclic AMP binding activity were concentrated as indicated above. The regulatory subunit migrated as a single band in polyacrylamide gel electrophoresis containing dodecylsulphate corresponding to a molecular weight of 48 000 [7,8].

Binding assay of cyclic AMP

Cyclic AMP binding to a specific binding protein kinase was assayed according to the method of Gilman [4]. The standard binding reaction was conducted in a volume of 0.2 ml in 50 mM sodium acetate buffer (pH 4.0), and 0.1 μM cyclic [^{3}H]AMP. Reactions were initiated by addition of 5 μg binding protein kinase and were incubated for 100 min at 0° C. The mixture then were diluted to 1 ml with cold 20 mM potassium phosphate buffer (pH 6.0). About 5 min later they were passed through a 24-mm Millipore filter (0.45 μm). The filter was then washed with 10 ml of the same buffer and placed in a counting vial with 5 ml of Aquasol II (New England Nuclear, Boston) in which the filter readily dissolves and then were counted in a scintillation counter.

Cyclic AMP-dependent protein kinase activity

Cyclic AMP-dependent protein kinase from rabbit muscle was assayed according to the method of Kuo and Greengard [9]. The standard assay contained in a final volume of 0.2 ml, 50 mM sodium acetate buffer (pH 6.0), 40 μg histone II, 5 μM [γ-^{32}P]ATP containing about $1.4 \cdot 10^6$ cpm, 10 mM magnesium acetate, and 4 μg protein kinase in the absence or presence of 5 μM cyclic AMP. Incubations were carried out at 30° C for 10 min in a shaking water bath. The reaction was terminated by addition of 1 ml of 10% trichloroacetic acid containing 0.25% sodium tungstate, pH 2.0 and 0.2 ml of 0.5% bovine serum albumin was added as a carrier protein. The mixture was allowed to stand at 0° C for 5 min and the degree of histone phosphorylation was measured by the method of Miyamoto et al. [10]. A unit of protein kinase is defined as the pmol of $^{32}P_i$ transferred to the histone per min at 30° C.

Preparation of ^{125}I-labeled lethal toxic fragment

^{125}I-labeled lethal toxic fragment was carried out by the method of Hunter using chloramine-T as an oxidant [11]. Iodinated toxic protein retained 90% of its original lethal toxicity for mouse and a specific radioactivity of the toxic fragment had $2 \cdot 10^6$ cpm/μg protein. The radioactivity was 92% precipitable by rabbit antilethal toxic serum.

Results

Effect of a lethal toxic fragment on cyclic [^{3}H]AMP binding to protein kinase and its regulatory subunit

The effects of a lethal toxic fragment and nucleotides on cyclic AMP binding to the protein kinase and its regulatory subunit are shown in Table I. Additions of non-labeled cyclic AMP and cyclic GMP had only a 50% inhibitory effect at 0.5 μM and 100 μM, respectively, and 50 μM ATP had virtually no effect. 0.1 μM of both α-toxin and its lethal toxic fragment had a 50% inhibitory effect of the maximum binding of cyclic [^{3}H]AMP to the protein kinase or its regulatory subunit. Rabbit antiserum raised against purified α-toxin reversed the reaction completely at a concentration equivalent to that of the toxic fragment employed. Since both α-toxin (p*I* 7.9) and lethal toxic fragment (p*I* 8.2) are basic proteins, ribonuclease A and egg yolk lysozyme known as basic proteins were investigated, there were not effective. These findings seem to mean that electrostatic interaction of the acidic protein kinase with basic protein is not necessary to inhibit cyclic AMP-binding activity but it depends on the protein structure itself.

The binding of the ^{125}I-labeled lethal toxic fragment to the protein kinase (holoenzyme), which under nondenaturing conditions, the molecular weight is approximately 170 000 [12], was measured by a procedure of Sepharose-4B

TABLE I

INHIBITORY EFFECT OF A LETHAL TOXIC FRAGMENT ON CYCLIC [^{3}H]AMP BINDING TO PROTEIN KINASE AND ITS REGULATORY SUBUNIT

The standard assay for cyclic [^{3}H]AMP binding activity of cyclic AMP dependent protein kinase and the regulatory subunit was used.

Addition to assay mixture	Concentration	Cyclic [^{3}H]AMP bound * (pmol/μg protein)
Protein kinase alone	25 μg/ml	0.71 (100%)
α-Toxin	0.1 μM	0.36 (50)
α-Toxin	1.0 μM	0.36 (50)
Lethal toxic fragment	0.01 μM	0.62 (87)
	0.05 μM	0.48 (68)
	0.1 μM	0.36 (50)
	0.5 μM	0.35 (49)
	1.0 μM	0.35 (49)
	5.0 μM	0.36 (50)
Lethal toxic fragment (1 μM) + antiserum	40 μg/ml	0.71 (100)
Ribonuclease A	50 μg/ml	0.68 (95)
Lysozyme	50 μg/ml	0.72 (101)
Cyclic AMP	0.5 μM	0.36 (50)
Cyclic GMP	100 μM	0.36 (50)
GTP	50 μM	0.64 (90)
ATP	50 μM	0.70 (98)
Regulatory subunit alone	5 μg/ml	1.26 (100%)
α-Toxin	1.0 μM	0.62 (49)
Lethal toxic fragment	0.05 μM	0.74 (58)
	0.1 μM	0.62 (49)
	0.5 μM	0.63 (49)

* The data presented are the means of six experiments.

gel filtration. As can be seen in Fig. 1, the complex of enzyme-toxin subunit was eluted as a single peak containing ^{125}I-labeled toxic protein, corresponded to a molar ratio proportion of 2 : 1. Furthermore, ^{125}I-labeled lethal toxic fragment bound to the regulatory subunit by a procedure of isoelectric focusing in polyacrylamide gels (data not shown).

Inhibitory mechanism of cyclic AMP binding activity of protein kinase by the lethal toxic fragment

The effect of the letal toxic fragment on the binding of cyclic AMP to protein kinase was studied further to ascertain the nature of the inhibition. The relationship between the initial rate, v, of the enzyme activity of cyclic AMP binding and cyclic AMP concentration [S] was determined. The double reciprocal plot of $1/v$ against $1/[S]$ showed a linear relationship (Fig. 2). As can be seen in Fig. 2, the protein kinase had a high affinity of cyclic AMP binding constant of 2.7 nM, which value is in good agreement with that of Gilma [4] and the three lines obtained in the presence of 0.05 μM to 5 μM lethal toxic fragment intercepted the ordinate at the same value of $1/v$ without addition of the lethal toxic fragment. This indicates that the inhibition of the initial rate of

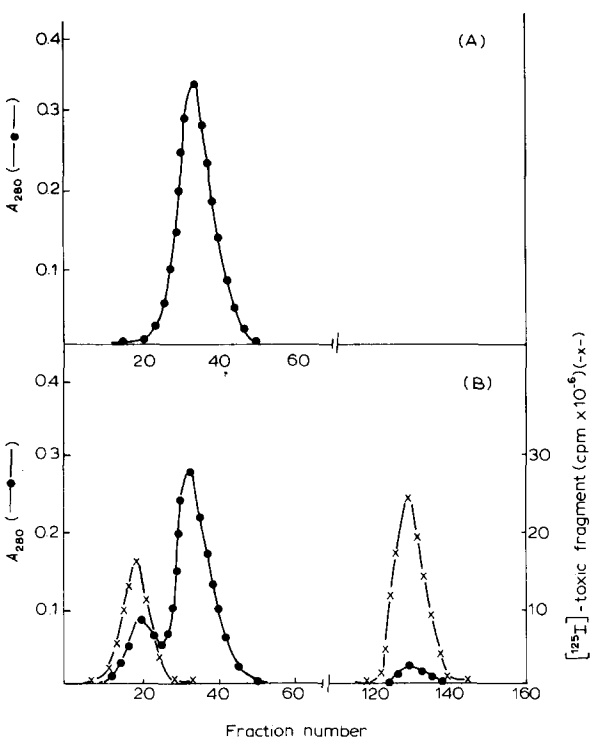

Fig. 1. Gel filtration of a complex of cyclic AMP-dependent protein kinase-lethal toxic fragment. Gel filtration of the protein kinase (holoenzyme) and ^{125}I-labeled lethal toxic fragment was on a Sepharose 4B column (75 X 2.4 cm) in 0.05 M potassium phosphate buffer (pH 7.0). Fraction volume was 2.0 ml. Holoenzyme alone (5 mg of protein) (A), and a mixture of the holoenzyme (5 mg) and ^{125}I-labeled lethal toxic fragment (1 mg, $1.2 \cdot 10^6$ cpm/μg) which was incubated at 30°C for 10 min (B).

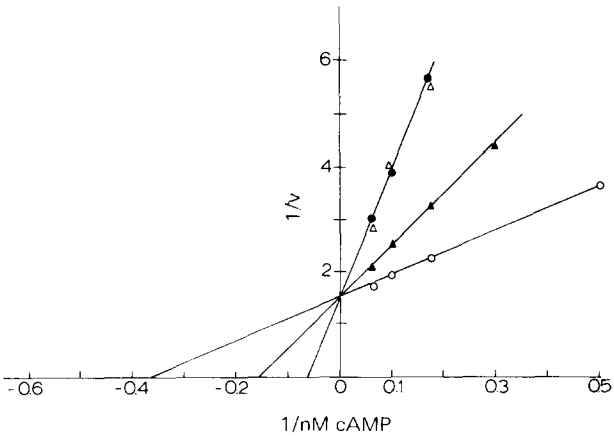

Fig. 2. Lineweaver-Burk plot showing competitive inhibition of substrate kinetics by a lethal toxic fragment of staphylococcal α-toxin. Assays were carried out by the method of Gilman [4] in the presence of cyclic [^{3}H]AMP (2—10 nM) and the indicated concentrations of lethal toxic fragment. Velocity (v) is express in pmol cyclic AMP per μg protein per 100 min incubation. The data presented are the means of three experiments. Concentrations of lethal toxic fragment: 0.05 μM (▲), 1 μM (●), 5 μM (△), and absence of lethal toxic fragment (○).

cyclic AMP binding to the protein kinase by the lethal toxic fragment is of a competitive nature.

Effect of a lethal toxic fragment on the phosphorylation activity of cyclic AMP-dependent protein kinase and its catalytic subunit

The histone II phosphorylation was stimulated by the addition of cyclic AMP to the protein kinase and the maximal amount of $^{32}P_i$ incorporated was 21 pmol of phosphate transferred to the histone protein (Table II). Less than 0.1 μg/ml of the purified cyclic AMP-dependent protein kinase inhibitor from bovine heart inhibited strongly the histone phosphorylation resulting from the protein inhibitor specifically binding to the dissociated catalytic subunits [12], while this enzyme inhibitor gave no significant inhibition of the binding of cyclic [^{3}H]AMP to the protein kinase. Addition of 0.1 μM of both α-toxin and its lethal toxic fragment showed a 50% inhibition of the maximal rate of the phosphorylated reactions in the presence of the holoenzyme and cyclic AMP, while no inhibition of the histone phosphorylation was observed with the catalytic subunit (Table II).

Effect of a lethal toxic fragment on cyclic AMP-dependent protein kinase activity in rabbit heart tissues

In our preliminary experiment of the distribution of ^{125}I-labeled lethal toxic fragment in the tissues of mice after intravenous injection, we found that even 24-h later the label from heart, small intestine, brain, kidney and lung continually remained at a high level, while a rapid disappearance of the label from the other tissues paralleled its rate of disappearance from the blood stream. In the present paper, the effects of the lethal toxic fragment, acetylcholine, and epinephrine on cyclic AMP and cyclic GMP levels and the cyclic AMP-dependent protein kinase activity ratio were investigated in rabbit heart. A 2 kg New

TABLE II

EFFECT OF A LETHAL TOXIC FRAGMENT ON THE PHOSPHORYLATION ACTIVITY ON PROTEIN KINASE AND ITS CATALYTIC SUBUNIT

The standard assay for the cyclic AMP-dependent protein kinase activity and the catalytic subunit activity was used. A unit of protein kinase activity is defined as the pmol of $^{32}P_i$ transferred to histone II per at 30°C. The data presented are means of six experiments.

Addition to assay mixture	Concentration	Protein kinase activity (units)	
Protein kinase alone	20 μg/ml	21.4	(100%)
Minus cyclic AMP *		1.1 *	
α-Toxin	0.05 μM	12.2	(57)
	0.1 μM	11.0	(51)
Lethal toxic fragment	0.05 μM	12.7	(59)
	0.1 μM	10.9	(50)
	0.5 μM	11.0	(51)
Protein kinase inhibitor	0.1 μg/ml	3.2	(15)
Cyclic GMP	5.0 μM	19.5	(91)
GTP	50 μM	19.2	(90)
ATP	50 μM	285.2 **	(1330)
Catalytic subunit alone	20 μg/ml	62.6	(100%)
Minus cyclic AMP		61.9	(99)
α-Toxin	0.1 μM	61.8	(99)
Lethal toxic fragment	0.05 μM	63.3	(101)
	0.5 μM	60.8	(97)
Protein kinase inhibitor	0.1 μg/ml	5.6	(9)

* Values of protein kinase (holoenzyme) experiments have been corrected for 1.1 units of activity where this occurred in the absence of added cyclic AMP.

** Corrected for the decrease in specific activity which resulted from addition of nonradioactive ATP.

Zealand rabbit was used. Sodium heparin (1500 units/kg) was injected intravenously 15 min before killing. The animal was anesthetized with sodium pentabarbital (100 mg/kg). The heart was quickly excised, immersed in cold Hanks' balanced medium (pH 7.4) until beating ceased, attached via the aorta to the perfusion cannula and perfused at 37° C with recirculating Ringer's solution. The perfused heart was cut into slices approximately 1 mm thick using a tissue slicer. About 10 mg of sliced tissue were placed in a 10 ml conical tube containing 1 ml of each reaction mixture. After incubation, the incubated tissue was homogenated and centrifuged. The supernatant was processed for assay as described under Materials and Methods. As shown in Table III, epinephrine (0.1 μM), a cardioactive agent, produced increases in cyclic AMP and in the cyclic AMP-dependent protein kinase activity ratio. Acetylcholine (5 μM), while producing a 3-fold increase in cyclic GMP, marginally suppressed epinephrine stimulation of the cyclic AMP and the protein kinase activity ratio. The lethal toxic fragment (0.1 μM) reduced the protein kinase activity ratio approximately 40% compared to that of the control and also suppressed epinephrine stimulation of the protein kinase activity ratio, although the toxic fragment slightly elevated the cyclic AMP levels resulting from the inhibition of binding of cyclic AMP to the protein kinase.

TABLE III

EFFECTS OF LETHAL TOXIC FRAGMENT, EPINEPHRINE AND ACETYLCHOLINE ON CYCLIC AMP CONTENT, CYCLIC AMP-DEPENDENT PROTEIN KINASE ACTIVITY RATIO AND CYCLIC GMP CONTENT IN RABBIT HEART SLICES

Rabbit heart slices were incubated with each reaction mixture containing (1) no additions, (2) 0.1 μM lethal toxic fragment, (3) 0.1 μM epinephrine, (4) 5 μM acetylcholine, (5) 0.1 μM epinephrine plus 5 μM acetylcholine, (6) 0.1 μM lethal toxic fragment plus 5 μM acetylcholine, (7) 0.1 μM lethal toxic fragment plus 0.1 μM epinephrine. The incubated slices were homogenated and centrifuged. Each value represents the mean of five heart slices.

Treatment	Cyclic AMP (nmol/g)	Protein kinase activity ratio (—cyclic AMP/ +cyclic AMP)	Cyclic GMP (pmol/g)
(1) Control	0.63 (0.44—0.82)	0.17 ± 0.05	82 (69— 93)
(2) Lethal toxic fragment	0.74 (0.55—0.93)	0.10 ± 0.02	83 (70— 96)
(3) Epinephrine	1.20 (0.94—1.46)	0.38 ± 0.02	120 (103—137)
(4) Acetylcholine	0.55 (0.48—0.62)	0.15 ± 0.04	299 (278—320)
(5) Epinephrine + acetylcholine	0.89 (0.67—1.10)	0.24 ± 0.04	364 (332—396)
(6) Lethal toxic fragment + acetylcholine	0.58 (0.50—0.66)	0.09 ± 0.02	290 (262—318)
(7) Lethal toxic fragment + epinephrine	1.10 (0.88—1.32)	0.18 ± 0.03	120 (101—139)

Discussion

With respect to the interaction of α-toxin with biological membranes, hemolytic and cytolytic activities and probably also the lethal effect of α-toxin are closely associated with biological membrane damage [13]. However, no evidence is available indicating a specificity of this toxin for identifiable substrate in the cell membrane or enzymic activity. Among the enzymes associated with rabbit erythrocyte membranes, $(Na^+ + K^+)$- and Mg^{2+}-ATPase, adenylate cyclase and cyclic AMP phosphodiesterase activities had no effects from the lethal toxic fragment. While, the $(Ca^{2+} + Mg^{2+})$-dependent ATPase activity of the rabbit erythrocyte membrane was stimulated to a considerable extent by lethal toxic fragment concentration (data not shown).

It is now well accepted that cyclic AMP-dependent protein kinase contains a dimeric regulatory subunit and two catalytic subunits which dissociate when cyclic AMP binds to the regulatory subunit (2 mol cyclic AMP/mol of subunit monomer) [14] and the protein kinase inhibitor specifically binds to the dissociated catalytic subunits [15], whereas, the letahl toxic fragment had no affect on the catalytic subunit activity. Concerning the effective concentrations of the lethal toxic fragment on the cyclic AMP-dependent protein kinase, we found that the lethal toxic fragment produced a dose-dependent decrease in both the binding of cyclic AMP activity to the regulatory subunit and phosphorylation activity of the protein kinase until reaching a plateau at a 50% inhibitory effect. On studying the kinetics obtained from linear double-reciprocal plots, this inhibition was found to be competitive in nature (Fig. 2). Since a complex of enzyme-toxin protein which appeared in the gel filtration corresponded to a molar ratio proportion of 2 : 1 (Fig. 1) and cyclic [^{3}H]AMP does not bind the α-toxin or its lethal toxic fragment, the mechanism proposed for the inhibitory effect of the lethal toxic fragment on binding of cyclic AMP to the protein

kinase is the interaction of the lethal toxic fragment with one of two cyclic AMP-binding sites in the regulatory subunit molecule.

The stimulation of protein kinase activity by cyclic AMP has been shown to mediate the effects of cardioactive agents on both heart metabolism and function [16]. An attempt to relate the demonstrated effect to a known physiological action of the toxin was shown: the lethal toxic fragment attenuated the stimulation of the cyclic AMP-dependent protein kinase activity ratio in the isolated heart by addition of epinephrine (Table III), since epinephrine stimulates contractile force and antagonizes the action of acetylcholine which decreases cardiac contractile force and produces an increase in the cyclic GMP level [17]. Further experiments attempting to clarify the action of the toxin on the function of central nervous cells through cyclic nucleotides pathway are in progress.

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References

1 Cassidy, P., Six, H.R. and Harshman, S. (1974) Biochim. Biophys. Acta 332, 413—423
2 Watanabe, M. and Kato, I. (1976) in Animal, Plant and Microbial Toxins (Ohsaka, A., Hayashi, K. and Sawai, Y. eds.), Vol. 1, pp. 437—454, Plenum Publishing Corp., New York
3 Watanabe, M. and Kato, I. (1978) Biochim. Biophys. Acta 535, 388—400
4 Gilman, A.G. (1970) Proc. Natl. Acad. Sci. U.S. 67, 305—312
5 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
6 Rubin, C.S., Erlichman, J. and Rosen, O.M. (1972) J. Biol. Chem. 247, 36—44
7 Corbin, J.D., Keely, S.L. and Rosen, O.M. (1975) J. Biol. Chem. 250, 218—225
8 Hofmann, F., Beavo, J.A., Bechtel, P.J. and Krebs, E.G. (1975) J. Biol. Chem. 250, 7795—7801
9 Kuo, J.F. and Greengard, P. (1970) J. Biol. Chem. 245, 4067—4073
10 Miyamoto, E., Kuo, J.F. and Greengard, P. (1969) J. Biol. Chem. 244, 6395—6402
11 Hunter, W.M. (1967) in Handbook of Experimental Immunology (Weir, D.M., ed.), pp. 608—654, Blackwell Publishing Company, London
12 Beavo, J.A., Bechtel, P.J. and Krebs, E.G. (1975) in Advances in Cyclic Nucleotide Research (Drummond, G.I., Greengard, P. and Robison, G.A., eds.), Vol. 5, pp. 241—251, Raven Press, New York
13 McCartney, A.C. and Arbuthnott, J.P. (1978) in Bacterial Toxins and Cell Membranes (Jelaszewicz, J. and Wadström, T., eds.), pp. 89—123, Academic Press, New York
14 Corbin, J.D., Sugden, P.H., West, L., Flockhart, D.A., Lincoln, T.M. and McCarthy, D. (1978) J. Biol. Chem. 253, 3997—4003
15 Demaille, J.G., Peters, K.A. and Fischer, E.H. (1977) Biochemistry 16, 3080—3086
16 Dobson, Jr., J.G. (1978) Am. J. Physiol. 234, H638—H645
17 George, W.J., Wilkerson, R.D. and Kadowitz, P.J. (1970) Proc. Natl. Acad. Sci. U.S. 66, 398—403