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MODULATOR BINDING PROTEINS OF TESTIS

Kenji Sobue and Shiro Kakiuchi

Institute of Higher Nervous Activity,
Osaka University Medical School,
Kita-ku, Osaka, Japan 530

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SUMMARY. Two types of modulator (calmodulin ; CDR)-binding protein were demonstrated in the cytosol of rat testis. One was heat-stable and had an estimated molecular weight of 88,000. The other one was heat-labile and had an estimated molecular weight of 410,000. Assay method for such cytosolic modulator-binding protein was developed by using [^{3}H] modulator protein in combination with a polyethylene glycol precipitation method.

Ca^{2+}-dependent modulator protein (calmodulin ; CDR) was discovered as a protein required for the Ca^{2+}-dependent activation of phosphodiesterase (1, 2). Independently, Cheung (3) reported on an activator protein for phosphodiesterase. The identity of both proteins was established subsequently (4). Recently, modulator protein has been shown to cause Ca^{2+}-dependent activation of several enzymes including phosphodiesterase (see above), brain adenylate cyclase (5), actomyosin ATPase (6, 7), erythrocyte membrane ATPase (8, 9), synaptic membrane ATPase (10), myosin light chain kinase from several tissues (11-13), skeletal muscle phosphorylase b kinase (14) and platelet phospholipase A2 (15). Modulator protein activates enzyme by forming a complex with it in the presence of Ca^{2+} (16).

Besides known enzymes, modulator protein was shown to associate, in the presence of Ca^{2+}, with some cellular proteins whose functions are yet to be identified. Thus, two modulator binding proteins have been purified from bovine brain cytosol (17-20), and modulator binding protein(s) of particulate nature

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was demonstrated in a variety of rat tissues (21, 22). While testis is known to be one of the richest source for modulator protein (23), its cellular proteins or components on which modulator protein exerts regulatory effects have been only poorly understood. Indeed, Beale et al, (24) failed to detect the requirement of modulator protein for the activation of the phosphodiesterase of Sertoli cell-enriched testis. We found that the level of modulator-binding protein of particulate nature is the lowest in testis among tissues examined (22). It therefore seems of considerable importance to search for testicular proteins or components which exhibit specific association with modulator protein. In the present communication we are able to demonstrate two of such proteins in the cytosol of rat testis.

MATERIALS AND METHODS. Modulator protein and [^{3}H] modulator protein were prepared as described in (12) and (22), respectively. The specific radioactivity of [^{3}H] modulator protein was 8.4 X 10^4 dpm/μg protein. Adult SD rat testes were detunicated and homogenized with 9 volumes of Tris·HCl (pH 7.5), 20 mM ; $MgSO_4$, 0.5 mM ; 2-mercaptoethanol, 5 mM ; and EGTA, 1 mM, using a polytron homogenizer. The homogenate was centrifuged at 105,000 X g for 60 min and the supernatant fraction was obtained. Phosphodiesterase activity was determined as described previously (25) using 2 mM cyclic AMP as substrate. Protein was determined by the method of Lowry et al, (26) with BSA as standard.

RESULTS AND DISCUSSION

Previously we showed a specific binding of ^{3}H-labeled modulator protein with particulate fractions of a variety of rat tissues (22). In the present study, this technique was applied to detect modulator binding proteins in the cytosol of testis. The tissue samples were incubated with [^{3}H] modulator protein and then bound and free forms of modulator protein were separated each other by two distinct methods : (a) precipitation of macromolecular-bound [^{3}H] modulator protein from solution with addi-

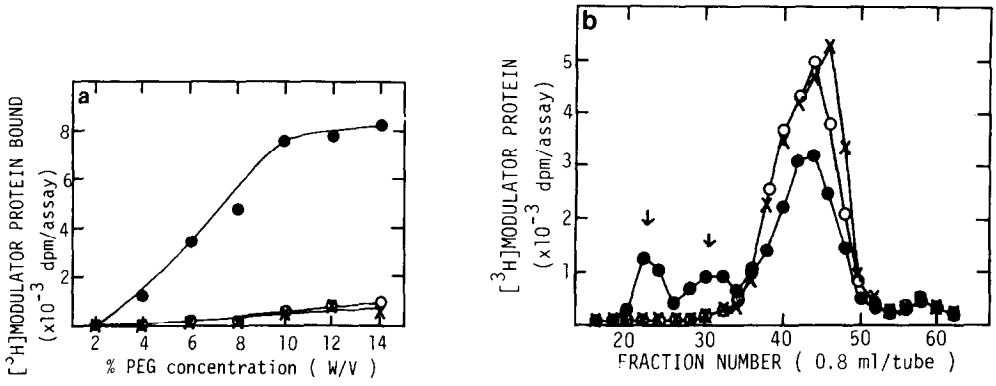

Fig. 1 Separation of macromolecular-bound modulator protein from free protein by two methods using polyethylene glycol (a) and a Sephadex G-200 column (b). In (a) (polyethylene glycol method), 225 µg protein of DEAE column fraction (see Fig. 2) was incubated for 25 min at 30° C in a reaction mixture containing Tris·HCl (pH 7.5), 20 mM ; $MgSO_4$, 0.5 mM ; 2-mercaptoethanol, 5 mM ; NaCl, 100 mM (Buffer A) ; bovine serum albumin, 0.1 mg/ml ; [^{3}H] modulator protein, 0,5 µg per tube, and other addtions (see below) in a total volume of 250 µl. Either 0.1 mM $CaCl_2$ (—●—), 0.1 mM $CaCl_2$ plus 50 µg of unmodified modulator protein (—○—), or 1 mM EGTA (—X—) was added. At the end of the incubation, 50 µl of chilled buffer A containing bovine γ-globulin, 1 mg/ml, was added and the mixture was stirred. Then 300 µl of polyethylene glycol (Carbowax 6000) dissolved in buffer A was added to make final concentrations indicated in the figure. The concentration of $CaCl_2$ in (—●—) and (—○—), or EGTA in (—X—) was maintained at 0.1 mM, or 1 mM, respectively, by appropriate additions of $CaCl_2$ or EGTA. The tubes were agitated vigorously and then placed in ice for 15 min. They were then centrifuged at 10,000 X g for 20 min. The supernatants being aspirated off, the residual pellets were washed once with corresponding solutions of polyethylene glycol. The precipitates thus obtained were dissolved in 0.1 % Triton X-100, 1 ml per tube, and redioactivity of the solutions was determined by a scintillation spectrometer. In (b) (gel filtration method), incubation was carried out as in (a) and then the reaction mixture was chromatographed on column (1.2 X 45 cm) of Sephadex G-200. Buffer A containing either 0.1 mM $CaCl_2$ (—●—, —○—) or 1 mM EGTA (—X—) was used for the equilibration and elution of the column. Column eluates were assayed for radioactivity.

tion of polyethylene glycol (27) (Fig. 1a) and, (b) Sephadex G-200 gel filtration of incubation mixtures (Fig. 1b). Results from both methods agreed each other, i.e., under the expermental conditions described in the legend to Fig. 1, polyethylene glycol (12∼%, w/v) precipitated 20 % of the radioactivity from the mixture (Fig. 1a), while elution profile of the gel filtration chromatography also showed that 20 % of the total radioactitivty was associated with two peaks of macromolecular-bound

[^{3}H] modulator protein (Fig. 1b ; see arrows for the bound forms of modulator protein). Results of Figs. 1a,b also show that the binding of testis cytosolic proteins with [^{3}H] modulator protein was Ca^{2+}-dependent and specific to modulator protein since addition of EGTA or a 100-fold excess of unlabeled modulator protein to incubation mixtures reduced the polyethylene glycol-precipitable redioactivity to less than 2 % of the total (Fig. 1a) and obliterate the peaks of macromolecular-bound [^{3}H] modulator protein from elution profile of the gel filtration chromatography (Fig. 1b). The third peak of Fig. 1b was not influenced by the addition of EGTA or an excess of unmodified modulator protein. This peak was identified as free [^{3}H] modulator protein from its estimated molecular weight value on gel filtration. In the follwing studies, polyethylene glycol method (at a final concentration

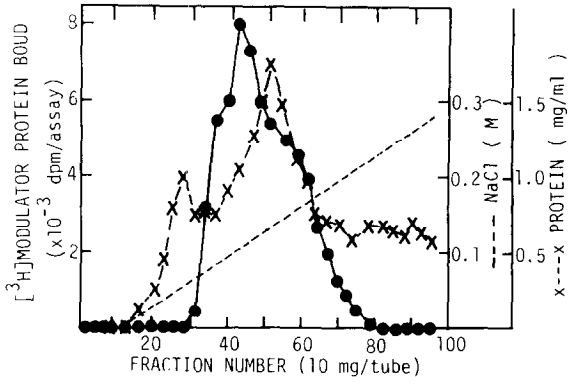

Fig. 2 DEAE-cellulose column chromatography. The supernatant fluid derived from 50 g rat testes was applied to a DEAE-cellulose column (2 X 25 cm). The column was washed with 100 ml of Tris·HCl (pH 7.5), 20 mM ; $MgSO_4$, 0.5 mM ; 2-mercaptoethanol, 5 mM ; and EGTA, 0.1 mM. It was then eluted with the above buffer containing a linear gradient of 0-275 mM NaCl. The eluate was monitored for the modulator-binding activity by the polyethylene glycol method (see Fig. 1a). The modulator-binding activity was recovered in a single broad peak consisting of tubes #32-65, corresponding to 70-200 mM NaCl. To this fraction $(NH_4)_2SO_4$ was added to a final concentration of 80 % saturation. The resultant precipitate, which was collected by centrifugation, was dissolved in 10 ml of buffer A containing 0.1 mM EGTA. The solution was dialyzed overnight against the same medium. Portions of the dialyzed solution, designated as DEAE column fraction, were subjected to studies of Figs. 1 and 3.

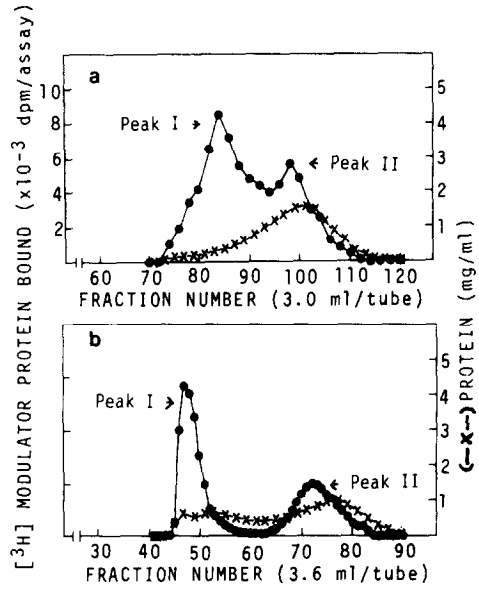

Fig. 3 Gel filtration column chromatographies using Sepharose 4B (a) and Sephadex G-200 (b). 3.5 ml each of the DEAE column fraction (see Fig. 2) was applied to a Sepharose 4B column (2.5 X 80 cm) (a) and a Sephadex G-200 column (2.5 X 90 cm) (b). The columns were eluted with medium A containing 0.1 mM EGTA, and the eluted fractions were assayed for the modulator-binding activity by the polyethylene glycol method. Peak I obtained from the Sepharose 4B column (tube 81 through 89) and peak II obtained from the Sephadex G-200 column (tube 67 through 76) were subjected to the study described in Table I.

of 12 %, w/v) was used to detect and determine the modulator-binding activity of testis samples. Each assay value was subtracted by the corresponding control to which an excess of unmodified modulator protein was added : the subtracted value represents specific binding displaceable by an excess of unmodified protein.

Fig. 2 shows a DEAE-cellulose column chromatography of the modulator-binding activity in the testis cytosol. One broad peak of this activity was obtained, which was eluted from the column by NaCl gradient at 70-200 mM (tube 32 through 65). Portions of this DEAE fraction were then subjected to gel filtration column chromatographies using Sepharose 4B (Fig. 3a) and Sephadex G-200 (Fig. 3b). Two peaks of [^{3}H] modulator-

TABLE I. PROPERTIES OF MODULATOR-BINDING PEAKS I AND II

Treatment	Binding activity (% of control)	
	I	II
None (control)	100	100
Boiling for 3 min	5.3 ± 0.7	96.5 ± 3.9
Trypsin	0.0 ± 0.0	4.0 ± 1.1
Trypsin + soybean trypsin inhibitor	101 ± 1.6	90.6 ± 1.7
Phospholipase C	96.1 ± 1.3	93.7 ± 3.6
Phospholopase D	102 ± 1.7	97.9 ± 2.8
DNase	97.1 ± 2.0	91.1 ± 1.7
RNase	98.1 ± 1.6	90.4 ± 2.6

Values are means ± S.E.M. of 4 determinations.

binding activity, designated as I and II with estimated molecular weights of 410,000 and 88,000, respectively, were detected on either elution profile. These results were consistent on two times experiments using independent batches of testes.

Table I shows properties of peaks I and II. Binding ability of peak I was abolished by boiling for 3 min, whereas peak II was thermostable. The activities in both peaks were susceptible to the digestion with trypsin, but resistant to treatments with other enzymes including phospholipases and nucleases. A phosphodiesterase activity with no Ca^{2+}/modulator protein-dependency was associated with peak I (1.7 ± 0.4 nmol/min·mg protein). Peak II had no phosphodiesterase activity.

This, we believe, is the first demonstration of modulator-binding proteins in the cytosol of testis. One is a heat-stable protein with an estimated molecular weight of 88,000. The other one is heat-labile and has an estimated molecular weight of 410,000. A modulator-binding protein similar to the former was found in the brain cytosol (17, 18). However, modulator-binding protein of the latter type has never been reported.

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