DISTRIBUTION IN RAT TISSUES OF MODULATOR-BINDING PROTEIN OF PARTICULATE NATURE

Studies with ^{3}H-modulator protein

Kenji SOBUE, Yoshihiko MURAMOTO, Reiko YAMAZAKI[+] and Shiro KAKIUCHI

Institute of Higher Nervous Activity, Osaka University Medical School, Kita-ku, Osaka 530 and [+]*Nakamiya Mental Hospital, Hirakata, Osaka 573, Japan*

Received 19 June 1979

1. Introduction

Studies on Ca^{2+}-activatable cyclic nucleotide phosphodiesterase [1] led to the discovery of a protein modulator that is required for the activation of this enzyme by Ca^{2+} [2–4]. Modulator protein is identical to the activator protein originally discovered by Cheung [5,6]. Later, this protein has been shown to cause the Ca^{2+}-dependent activation of several enzymes that include phosphodiesterase [1–6], adenylate cyclase [7], a protein kinase from muscles [8–11], phosphorylase *b* kinase [12], actomyosin ATPase [13,14], membranous ATPase from erythrocytes [15,16] and nerve synapses [17]. Thus, modulator protein appears to be an intracellular mediator of actions of Ca^{2+}.

Besides the above enzymes, modulator protein has been shown to associate, in a Ca^{2+}-dependent fashion, with cellular proteins whose functions are yet to be identified [18–25]. It is expected that these modulator-binding proteins may represent Ca^{2+}/modulator protein-regulated enzymes (proteins) [19], or subunits of them [22]. While these studies [18–25] were carried out with the supernatant fraction of tissues, work in our laboratory [26] revealed the presence of modulator-binding protein of particulate nature in brain which, in the presence of Ca^{2+}, can associate ~1/3 of the soluble modulator present in this tissue.

The present work shows the distribution of this particulate modulator-binding component in rat tissues. This paper also describes the labeling of modulator protein with tritium without deteriorating its biological activities and application of this ^{3}H-modulator protein to the determination of the Ca^{2+}-dependent binding of modulator protein with membranous protein. This technique proves to be useful in studying enzymes or proteins whose functions are regulated by Ca^{2+}/modulator protein system.

2. Materials and methods

2.1. *Materials and methods in general*

[^{3}H]Acetic anhydride (spec. act. 500 mCi/mmol) was purchased from Radiochemical Centre, Amersham. Modulator protein and modulator-deficient phosphodiesterase were prepared from bovine brain as in [9]. Protein and modulator protein were determined by a micro-biuret method [27] and a method using brain phosphodiesterase [26], respectively. Disc gel electrophoresis was carried out by the methods of Davis [28] and Laemmli [29]. The latter contained 0.1% SDS.

2.2. *Determination of Ca^{2+}-dependent binding of ^{3}H-modulator protein with particulate fraction*

^{3}H-Modulator protein was prepared by acetylation using [^{3}H]acetic anhydride [30]. To a mixture of 0.8 ml 10 mM Tris–HCl (pH 7.5), 1 mM $MgSO_4$, 100 mM NaCl and modulator protein (6.6 mg/ml) and 0.1 ml 3 M Tris acetate (pH 7.0), 25 mCi [^{3}H]acetic anhydride was added. The tube was placed in an ice bath. The reaction proceeded for 60 min with gentle stirring. Then the mixture was applied to a Sephadex G-75 column (1 × 35 cm) which had been equilibrated

with 60 mM Tris–HCl (pH 7.5), 0.5 mM $MgSO_4$. The column was eluted with the same medium and fractions were collected. The first radioactive peak, corresponding to ^{3}H-modulator protein, was collected. It was concentrated through Amicon UM-10, then stored in aliquots at –25°C until use. ^{3}H-Modulator protein (7.5 μg) was incubated with particulate protein (~150 μg/tube) for 25 min at 30°C in 0.15 ml medium A (see below) that contained Ca^{2+}. Medium A consisted of 20 mM Tris–HCl (pH 7.5), 0.5 mM $MgSO_4$, 5 mM 2-mercaptoethanol, 100 mM NaCl, 0.1 mM $CaCl_2$ and bovine serum albumin (0.2 mg/ml). At the end of the incubation, 0.1 ml of the reaction mixture was rapidly filtered through Whatman GF/C glass microfibre papers pre-washed with medium A containing 3 mg/ml bovine serum albumin. The filter paper was washed by 5 ml medium A containing 1 mg/ml bovine serum albumin. Bound ^{3}H-modulator protein was retained on the filter paper and its radioactivity was determined in a liquid scintillation spectrometer. Control assays, in which Ca^{2+} in medium A was replaced by 1 mM EGTA, were run with each experiment, and values were subtracted from corresponding experimental values. This subtracted value, representing the Ca^{2+}-dependent binding of ^{3}H-modulator protein, agreed with that of the bound ^{3}H-modulator displaceable by a 100-fold excess of unlabeled modulator protein. The control value accounted for < 5% of the corresponding experimental value. The result of this filtration method agreed well with that obtained by the centrifugation method employed in [26]. It was confirmed that the amount of ^{3}H-modulator added to the reaction mixture was in a supramaximal level for the binding.

3. Results

^{3}H-Modulator protein prepared was homogeneous upon disc gel electrophoreses with (figure not shown) and without (fig.1) the presence of sodium dodecylsulfate. It coincided with the unmodified protein upon both electrophoreses. Specific activity of the labeled protein was ~500 mCi/mmol, indicating that 1 mol [^{3}H]acetyl was incorporated/mol modulator protein. Presence or absence (+EGTA) of Ca^{2+} during the acetylation reaction did not influence the yield of the label per mole of the protein or biological activities of the labeled protein. Alterations of the reaction time in a range of 10–120 min did not influence the yield of labeling.

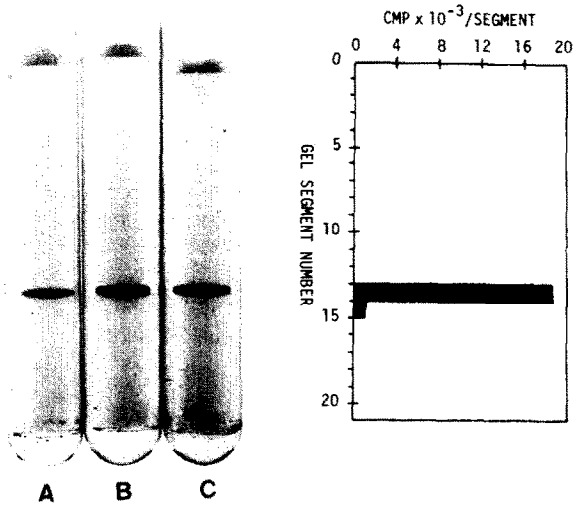

Fig.1. Disc gel electrophoresis of ^{3}H-labeled modulator protein. Electrophoresis was carried out by the method in [28]. After the electrophoresis, the gel was cut into 2 mm thick slices, and each slice was determined for its radioactivity. (A) unmodified modulator protein (2 μg); (B) a mixture of unmodified (2 μg) and ^{3}H-labeled (2.5 μg) modulator proteins; (C) ^{3}H-modulator protein (2.5 μg). Increasing the protein concentration to 18 μg/gel was examined with essentially the same result (radioactivity was not determined).

^{3}H-Modulator protein showed a Ca^{2+}-dependent activation of modulator-deficient phosphodiesterase with a dose–response curve shape identical to that of the unmodified protein (fig.2). However, the curve

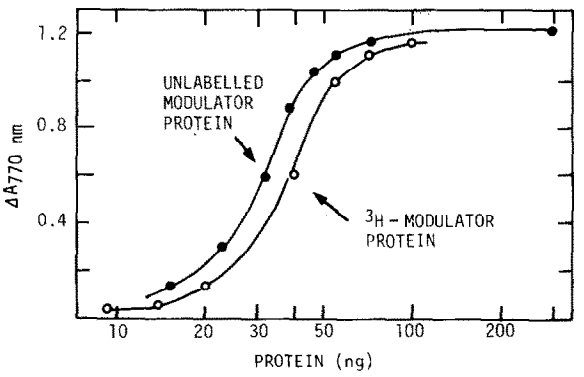

Fig.2. Activation of modulator-deficient brain phosphodiesterase by unmodified and ^{3}H-labeled modulator proteins. Conditions were as described in [9].

with ^{3}H-modulator protein shifted slightly towards the right: a half-maximum activation was attained with 32 and 40 ng of the unmodified and modified proteins, respectively.

With this ^{3}H-modulator protein, we developed a determination method for Ca^{2+}-dependent binding of modulator protein to the membranes (see section 2.2). Figure 3 shows a ubiquitous distribution of this binding component in rat tissues. Treatment of the particulate fraction with 0.6 M KCl decreased the value to some extent (hatched bars of fig.3). This is due to the removal of soluble (extrinsic) proteins or contractile proteins from the membranes. The latter was especially the case for heart and skeletal muscles, where the large decrease of the value resulted from the KCl treatment. Among tissues, cerebrum showed the highest activity, followed by adrenal and cerebellum. Table 1 shows the subcellular distribution of this binding activity in cerebrum tissue. The highest specific activity was found in fractions (P_2, B) containing synaptosomes or, after the disruption of the synaptosomes, in a fraction (P_{3-4}) where synaptic plasma membranes are concentrated.

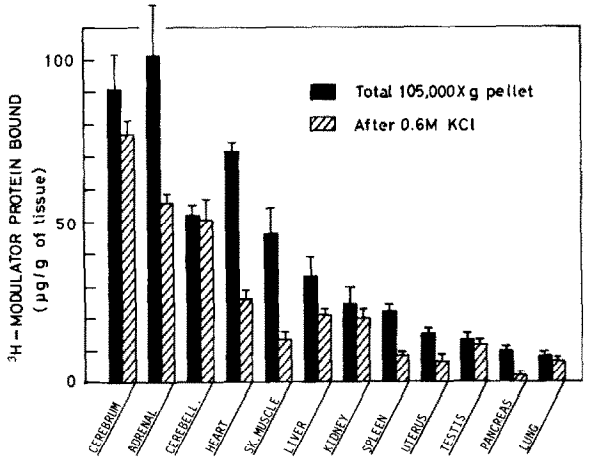

Fig.3. Distribution of Ca^{2+}-dependent binding activity of particulate nature for ^{3}H-labeled modulator protein. Tissues were rapidly removed from male Sprague Dawley rats killed by decapitation. Tissues were minced by scissors then homogenized with 9 vol. medium B consisting of 10 mM Tris–HCl (pH 7.5), 5 mM 2-mercaptoethanol, 1 mM EGTA and 1 mM $MgSO_4$. The homogenate was then centrifuged at 105 000 × *g* for 60 min. The resulting pellet was suspended in 10 original volumes of medium B and the mixture was centrifuged as above. This procedure was repeated once more and the final pellet, designated as total 105 000 × *g* pellet, was obtained. A portion of the latter pellet was dispersed with 20 original volumes of a medium consisting of 0.6 M KCl, 10 mM Tris–HCl (pH 7.5), 5 mM 2-mercaptoethanol and 1 mM EGTA. The suspension was kept in ice for 120 min and then centrifuged at 105 000 × *g* for 60 min. The resulting pellet, designated as After 0.6 M KCl, was obtained. Binding of ^{3}H-modulator protein to these particulate fractions was assayed as described in section 2.2. Bars indicated means ± SEM of 4 preparations.

4. Discussion

This work has introduced a method for the determination of the Ca^{2+}-dependent binding of modular protein with the membranes using ^{3}H-labeled modulator protein. The validity of this assay is supported by the following evidence*:

(i) Binding of ^{3}H-modulator protein is reversible by addition of either EGTA or an excess of unlabeled native modulator protein and the amounts of ^{3}H-modulator displayed by EGTA (= Ca^{2+}-dependent binding) and unlabeled modulator agreed with each other.

(ii) The binding is specific to modulator protein since excess additions of other proteins including troponin-C could not displace the bound ^{3}H-modulator.

(iii) Kinetic behavior of the binding of both labeled and unmodified modulator proteins agreed well with each other.

Labeled modulator protein first became available by ^{125}I-iodination [22,23]. This iodination reaction, however, yielded several species of product as discussed [23] and led to a 50% substantial loss [23], or 23–75% loss [22], in the ability of the protein to activate phosphodiesterase depending upon the iodination condition. The acetylation reaction used here yielded a homogeneous product as judged from its behavior on electrophoresis and its constant specific activity value: 1 mol [^{3}H]acetyl/mol protein was obtained with reaction times of 10–120 min. Moreover, its biological activity (~80% of that of unmodified modulator), in terms of the ability to activate phosphodiesterase, was consistent in several preparations. Although we did not determine the position of

* Sobue, K., Yamazaki, R. and Kakiuchi, S. (1979) manuscript in preparation

Table 1
Subcellular distribution of Ca^{2+}-dependent binding activity for ^{3}H-modulator protein in cerebrum tissue

Fraction	^{3}H-Modulator protein bound (μg ^{3}H-modulator protein .mg prot.$^{-1}$)	$(Na^+ + K^+)$-ATPase (μmol .mg prot.$^{-1}$.h^{-1})	Succinic dehydrogenase (A .mg prot.$^{-1}$. 30 min^{-1})
P_1	1.64 ± 0.16		
P_2	2.16 ± 0.17	9.9 ± 0.8	0.38 ± 0.03
P_3	1.61 ± 0.21	6.1 ± 0.7	0.01 ± 0.00
A	1.66 ± 0.10	3.2 ± 0.4	0.05 ± 0.01
B	2.44 ± 0.10	10.9 ± 1.3	0.22 ± 0.03
C	0.56 ± 0.05	4.0 ± 0.3	0.91 ± 0.07
P_{1-2}	2.38 ± 0.26	7.3 ± 0.9	0.02 ± 0.00
P_{3-4}	4.90 ± 0.08	14.1 ± 1.2	0.10 ± 0.01
P_{5-6}	3.82 ± 0.52	9.0 ± 1.1	0.63 ± 0.05

Cerebral cortices of male Sprague Dawley rats were homogenized and fractionated as in [17]. In short, primary fractions (P_1, P_2, P_3) and submitochondrial fractions (A, B, C) were obtained, and fraction B, after the hypotonic treatment to disrupt synaptosomes, yielded 3 subfractions, P_{1-2}, P_{3-4} and P_{5-6}. $(Na^+ + K^+)$-ATPase [32] and succinic dehydrogenase [33] activities were determined as marker enzymes for synaptic plasma membranes and mitochondria, respectively. Each value is mean ± SEM of 3 expt

amino acid residue acetylated, one of the 7 lysine residues [31] of this protein, possibly its N-terminal one, may be the site. Nevertheless, the availability of the ^{3}H-modulator protein will add another useful tool for the study of modulator-binding components since this labeled compound was shown to bind these components in a manner indistinguishable from the unmodified modulator (see above).

Cerebrum membranes showed the highest binding activity: the membranes derived from 1 g of this tissue bound ~80 μg of ^{3}H-modulator. This activity is comparable to, or even exceeding, that of the modulator-binding protein [19] found in the supernatant fraction since, according to [21], the latter activity accounted for < 20% of the total modulator protein: taking the amount of the total soluble modulator protein in brain tissue as 300–400 μg/g, the latter activity is represented by 60–80 μg modulator protein/g. Besides cerebrum, adrenal and cerebellum contained high activities. It is of interest that all these 3 are excitable tissues, performing secretion or release of hormones or transmitter substances. The result that the modulator-binding activity was concentrated in the fraction rich in synaptic membranes (table 1) is compatible with the above view.

References

[1] Kakiuchi, S. and Yamazaki, R. (1970) Proc. Jap. Acad. 46, 387–392.
[2] Kakiuchi, S., Yamazaki, R. and Nakajima, H. (1970) Proc. Jap. Acad. 46, 587–592.
[3] Teo, T. S. and Wang, J. H. (1973) J. Biol. Chem. 248, 5950–5955.
[4] Kakiuchi, S., Yamazaki, R., Teshima, Y. and Uenishi, K. (1973) Proc. Natl. Acad. Sci. USA 70, 3526–3530.
[5] Cheung, W. Y. (1970) Biochem. Biophys. Res. Commun. 38, 533–538.
[6] Cheung, W. Y. (1971) J. Biol. Chem. 246, 2859–2869.
[7] Brostrom, C. O., Huang, Y. C., Breckenridge, B. M. and Wolff, D. J. (1975) Proc. Natl. Acad. Sci. USA 72, 64–68.
[8] Dabrowska, R., Sherry, J. M. F., Aromatorio, D. K. and Hartshorne, D. J. (1978) Biochemistry 17, 253–258.
[9] Yagi, K., Yazawa, M., Kakiuchi, S., Oshima, M. and Uenishi, K. (1978) J. Biol. Chem. 253, 1338–1340.
[10] Waisman, D. M., Singh, T. J. and Wang, J. (1978) J. Biol. Chem. 253, 3387–3390.

[11] Barylko, B., Kuznicki, J. and Drabikowski, W. (1978) FEBS Lett. 90, 301–304.
[12] Cohen, P., Burchell, A., Foulkes, J. G., Cohen, P. T. W., Vanaman, T. C. and Nairn, A. A. (1978) FEBS Lett. 92, 287–293.
[13] Amphlett, G. W., Vanaman, T. C. and Perry, S. V. (1976) FEBS Lett. 72, 163–168.
[14] Dedman, J. R., Potter, J. D. and Means, A. R. (1977) J. Biol. Chem. 252, 2437–2440.
[15] Gopinath, R. M. and Vincenzi, F. F. (1977) Biochem. Biophys. Res. Commun. 77, 1203–1209.
[16] Jarrett, H. W. and Penniston, J. T. (1977) Biochem. Biophys. Res. Commun. 77, 1210–1216.
[17] Sobue, K., Ichida, S., Yoshida, H., Yamazaki, R. and Kakiuchi, S. (1979) FEBS Lett. 99, 199–202.
[18] Wang, J. H. and Desai, R. (1976) Biochem. Biophys. Res. Commun. 72, 926–932.
[19] Wang, J. H. and Desai, R. (1977) J. Biol. Chem. 252, 4175–4184.
[20] Klee, C. B. and Krinks, M. H. (1978) Biochemistry 17, 120–126.
[21] Sharma, R. K., Wirch, E. and Wang, J. H. (1978) J. Biol. Chem. 253, 3575–3580.
[22] La Porte, D. C. and Storm, D. R. (1978) J. Biol. Chem. 253, 3374–3377.
[23] Richman, P. G. and Klee, C. B. (1978) J. Biol. Chem. 253, 6323–6326.
[24] Wallace, R. W., Lynch, T. J., Tallant, E. A. and Cheung, W. Y. (1979) J. Biol. Chem. 254, 377–382.
[25] Grand, R. J. A., Perry, S. V. and Weeks, R. (1979) Biochem. J. 177, 521–529.
[26] Teshima, Y. and Kakiuchi, S. (1978) J. Cyclic Nucl. Res. 4, 219–231.
[27] Itzhaki, R. F. and Gill, D. M. (1964) Anal. Biochem. 9, 401–410.
[28] Davis, B. J. (1964) Ann. NY Acad. Sci. 121, 404–427.
[29] Laemmli, U. K. (1970) Nature 227, 680–685.
[30] Kagawa, Y. (1967) Methods Enzymol. 10, 526–528.
[31] Dedman, J. R., Jackson, R. L., Schreiber, W. E. and Means, A. R. (1978) J. Biol. Chem. 253, 343–346.
[32] Saito, K., Uchida, S. and Yoshida, H. (1972) Jap. J. Pharmacol. 22, 787–798.
[33] Kirshner, N., Kirshner, A. G. and Kamin, D. L. (1966) Biochim. Biophys. Acta 113, 332–338.