# MODULATION BY PHOSPHORYLATION OF INTERACTION BETWEEN CALMODULIN AND HISTONES

Yasushi IWASA, Takafumi IWASA, Kenji HIGASHI*, Kazuo MATSUI* and Eishichi MIYAMOTO
*Department of Pharmacology, Kumamoto University Medical School, Kumamoto 860, Japan*

Received 19 August 1981

## 1. Introduction

$Ca^{2+}$-Sensitive cyclic nucleotide phosphodiesterase (EC 3.1.4.17) and its $Ca^{2+}$-dependent modulator protein were first reported in [1–3]. An activator protein of phosphodiesterase in bovine brain was reported independently [4]. This protein, now called calmodulin, is thought to be a ubiquitous protein in eukaryotic cells and to play a pivotal role in regulation of $Ca^{2+}$-dependent cellular processes through activation of several $Ca^{2+}$-sensitive enzymes [5–8]. Some cellular proteins, other than $Ca^{2+}$-sensitive enzymes, which interact with calmodulin in a $Ca^{2+}$-dependent fashion have been demonstrated in various mammalian and avian cells and *Escherichia coli* [9–16]. However, physiological functions of these proteins are still under exploration. Cellular basic proteins such as histone, protamine and myelin basic protein have been shown to interact with calmodulin [17–21]. These basic proteins were suggested to bind to calmodulin in a $Ca^{2+}$-dependent manner [17,18]. However, in [19] this interaction was postulated to depend mainly on the high charge density between calmodulin and the basic proteins [19]. Here we show that the interaction of calmodulin with histones is a $Ca^{2+}$- and charge density-dependent reaction and that this interaction is modulated by phosphorylation of histones.

*Abbreviations:* EGTA, ethylene glycol bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid

* On leave from: Department of Obstetrics and Gynecology, Kumamoto University Medical School, Kumamoto 860, Japan

## 2. Materials and methods

Whole histone was prepared from calf thymus as in [22]. H1, H2A, H2B, H3 and H4 histones were purified as in [23]. Calmodulin, calmodulin-deficient phosphodiesterase and catalytic subunit of cyclic AMP-dependent protein kinase were purified from bovine brain by the methods in [24], [25] and [26], respectively. Calmodulin-coupled agarose affinity column was prepared as in [25] employing 18 mg homogenous calmodulin and 60 ml Sepharose 4B (Pharmacia). [γ-$^{32}$P]ATP was prepared as in [27]. *Crotalus atrox* venom (5'-nucleotidase), cyclic AMP and ATP were products of Sigma. Other chemicals were obtained from commercial sources.

Phosphodiesterase activity was assayed as in [28] with some modifications. The reaction mixture (0.5 ml) contained 40 μmol imidazole–HCl (pH 6.9), 1.5 μmol $MgCl_2$, 150 nmol dithiothreitol, 500 μg bovine serum albumin, 400 nmol cyclic AMP, 500 nmol $CaCl_2$ or 2.5 μmol EGTA, 51 μg bovine brain calmodulin-deficient phosphodiesterase and 0.36 μg calmodulin. Where indicated histones were added as specified. First incubation was done at 30°C. After 30 min, the reaction was stopped by immersing the tube in boiling water for 3 min. Then, 50 μg *Crotalus atrox* venom and 1.5 μmol $MnCl_2$ were added to the reaction mixture and second incubation was done at 30°C. After 20 min, the reaction was stopped by adding 100 μl 100% trichloroacetic acid. $P_i$ in the supernatant was determined colorimetrically.

Catalytic subunit of cyclic AMP-dependent protein kinase was assayed by measuring the amount of $^{32}$P incorporated into whole histone. The reaction mixture (0.2 ml) contained 4 μmol Tris–HCl (pH 7.5),

*Published by Elsevier/North-Holland Biomedical Press*
00145793/81/0000–0000/$02.75 

3.75 nmol [$\gamma$-$^{32}$P]ATP (9–11 X $10^4$ cpm/nmol) and protein kinase preparation. Incubation was done for 10 min at 30°C, and the reaction was terminated by the addition of 25% trichloroacetic acid. The acid-precipitable radioactivity was determined by membrane filter method in [29]. Protein was determined as in [30] with bovine serum albumin as a reference protein.

## 3. Results and discussion

Fig.1 shows that bovine brain phosphodiesterase was inhibited by H1, H2A, H2B, H3 and H4 histones. Increasing the amount of each histone progressively inhibited the activity of phosphodiesterase supported by $Ca^{2+}$ and calmodulin. H1 histones was the most potent inhibitor for the enzyme in these 5 histones tested. The activity of this enzyme in the presence of EGTA (i.e., basal activity) was not inhibited by any histone employed. High concentrations of each histone activated the basal activity very weakly. Addition of large amounts of calmodulin could abolish the inhibition of phosphodiesterase induced by histones, but $CaCl_2$ could not (not shown). It is likely, therefore, that histones interact with calmodulin but not $Ca^{2+}$, as reported in [19]. The mode of inhibition of

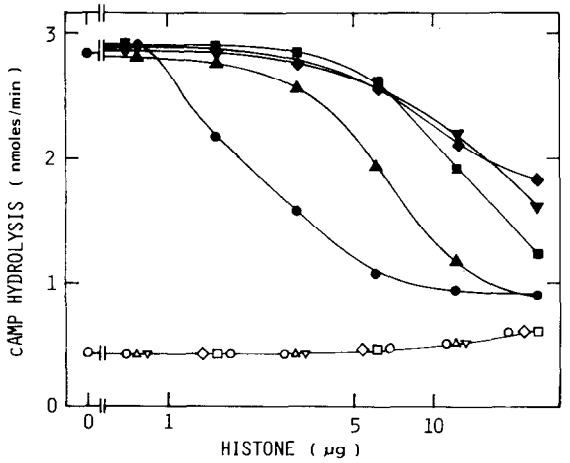


Fig.1. Inhibition of bovine brain cyclic nucleotide phosphodiesterase by histones. Phosphodiesterase was assayed under standard conditions in section 2 except that each histone was added to the reaction mixture as indicated. Phosphodiesterase activities with addition of H1 (○,●), H2A (□,■), H2B (△,▲), H3 (◇,◆) and H4 (▽,▼) histones are shown in the presence of 1 mM $CaCl_2$ (closed symbols) or 5 mM EGTA (open symbols).

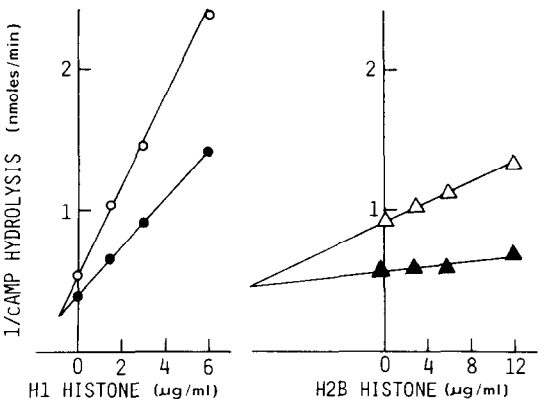


Fig.2. Kinetic analysis of the inhibition of phosphodiesterase induced by H1 and H2B histones with Dixon plot. Phosphodiesterase activity in the presence of 0.225 μg (●,▲) or 0.1125 μg (○,△) of calmodulin was assayed under standard conditions except that H1 or H2B histone was added to various concentrations as indicated.

phosphodiesterase by histones was analyzed by a Dixon plot [31]. As shown in fig.2, the analysis revealed that histones inhibited the enzyme activity in competition with apoenzyme for calmodulin. In [18] gel electrophoresis showed that H2B histone interacted with calmodulin in a $Ca^{2+}$-dependent fashion [18]. However, in [19] polylysine–agarose affinity column and turbidity measurement of calmodulin and basic protein mixture showed that this interaction did not require $Ca^{2+}$ but depended mainly on the high charge density present on both macromolecules [19]. To conclude this controversy, the experiment in fig.3 was done. H2B histone was not released from the calmodulin–agarose affinity column by washing with a NaCl- and $Ca^{2+}$-containing medium. After this washing, the histone was eluted by EGTA- and NaCl-containing medium (fig.3A). Conversely, when the column was washed initially with EGTA, H2B histone was not eluted. Then, the histone could be eluted by a medium containing both EGTA and NaCl (fig.3B). Namely, H2B histone could be released from the affinity column only by a medium containing both EGTA and NaCl. This result suggested that the interaction of calmodulin with histones was a $Ca^{2+}$- and charge density-dependent reaction. Some inhibitory effect of NaCl on phosphodiesterase was also found on the affinity column chromatography (fig.3). The chromatography in fig.3B gave better recovery of H2B histone than that in fig.3A. The rea-

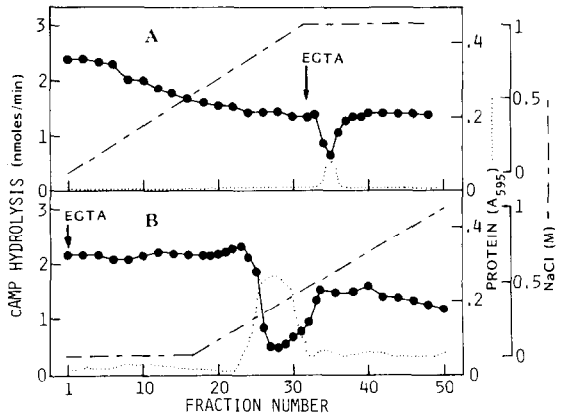


Fig.3. Investigation of the calmodulin–H2B histone interaction on calmodulin–agarose affinity column chromatography. Calmodulin-coupled agarose (Sepharose 4B) 10 ml, was equilibrated with buffer A (20 mM Tris–HCl at pH 7.5, 10 mM 2-mercaptoethanol, 1 mM imidazole, 1 mM magnesium acetate and 1 mM $CaCl_2$). The calmodulin-coupled agarose was incubated with 2 mg H2B histone for 16 h at 4°C in 40 ml buffer A with gentle stirring. The slurry was poured into a column (1.8 × 4.5 cm) and washed with 50 ml buffer A. (A) Following the washing with buffer A, the column was re-washed with a 100 ml linear concentration gradient of NaCl (0–1 M) in buffer A. Then H2B histone was eluted by buffer B (20 mM Tris–HCl at pH 7.5, 1 mM imidazole, 1 mM magnesium acetate, 10 mM 2-mercaptoethanol and 5 mM EGTA) containing 1 M NaCl. (B) After the calmodulin–agarose affinity column was prepared and washed with buffer A as above, the column was re-washed with 50 ml buffer B. Then, H2B histone was eluted by a linear concentration gradient of NaCl (0–1 M) in buffer B. Fractions of 3 ml each were collected. Phosphodiesterase was assayed with 0.35 ml aliquot of each fraction.

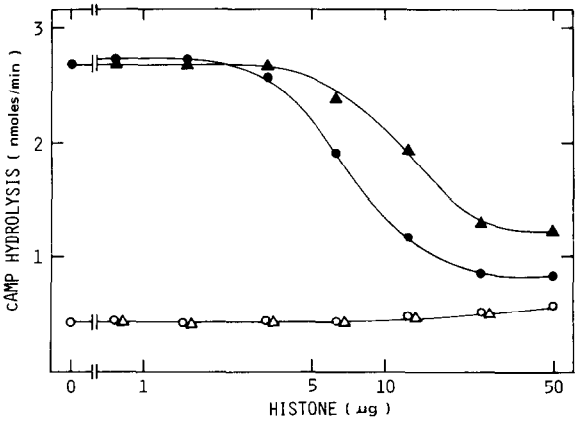


Fig.4. Effect of phosphorylation on the inhibition of phosphodiesterase by H2B histone. H2B histone (1 mg) was phosphorylated in a large scale (2 ml) of the protein kinase assay mixture in section 2 with 385 μg catalytic subunit of cyclic AMP-dependent protein kinase for 90 min at 30°C. The reaction was stopped by freezing. For the control, H2B histone was incubated for 60 min at 30°C in the reaction mixture for histone phosphorylation except that [$\gamma$-$^{32}$P]ATP was absent. Phosphodiesterase activities with addition of various amounts of non-phosphorylated (○,●) and phosphorylated (△,▲) H2B histones are shown in the presence of 1 mM $CaCl_2$ (closed symbols) or 5 mM EGTA (open symbols).

son is not yet clear. To explore the role of charge density present between calmodulin and histone in interaction of both proteins, the following experiments were done. Histones were phosphorylated by catalytic subunit of cyclic AMP-dependent protein kinase to reduce the degree of positive charge. After prolonged incubation (90 min) under the conditions in section 2, ~0.5, 0.2, 0.5, 0.2 and 0.2 mol phosphate were incorporated/mol H1, H2A, H2B, H3 and H4 histones, respectively. As shown in fig.4, phosphorylated histones could inhibit the phosphodiesterase in a dose-dependent manner as well as non-phosphorylated histones. However, the dose–response curve was shifted to the right by phosphorylation. When the mode of inhibition of the enzyme induced by phosphorylated histones was analyzed, the Dixon plot again showed a competition with apoenzyme for calmodulin (not shown). Phosphorylation of histones affected the interaction with calmodulin by increasing $K_i$-values (table 1). $K_i$-Values with H2A, H3 and H4 histones, which were phosphorylated by cyclic AMP-dependent protein kinase to a small extent, were affected slightly by phosphorylation. It was suggested that decreasing the degree of positive charge

Table 1
Effect of histone phosphorylation on $K_i$-values with histones

| Histone | $K_i$ (M) | |
|---|---|---|
| | Non-phosphorylated | Phosphorylated |
| H1 histone | $3.8 \times 10^{-8}$ | $5.2 \times 10^{-8}$ |
| H2A histone | $3.4 \times 10^{-7}$ | $3.7 \times 10^{-7}$ |
| H2B histone | $8.6 \times 10^{-7}$ | $1.1 \times 10^{-6}$ |
| H3 histone | $6.1 \times 10^{-7}$ | $6.1 \times 10^{-7}$ |
| H4 histone | $9.3 \times 10^{-7}$ | $9.5 \times 10^{-7}$ |

Each histone was phosphorylated as specified in fig.4. $K_i$-Values with histones were determined by the kinetic analysis (Dixon plot) as mentioned in fig.2. $M_r$-Values with H1, H2A, H2B, H3 and H4 histones are 21 000, 14 000, 14 000, 15 000 and 11 000, respectively [32]

with histones by incorporation of negatively-charged phosphates weakened the interaction with calmodulin. Therefore, it is likely that the highly acidic nature of calmodulin and highly basic nature of histones profoundly contribute to the interaction between these proteins. However, the above results do not necessarily mean that calmodulin–histone interaction is a non-physiological reaction as postulated [19], since a $Ca^{2+}$-dependent reaction is associated with the interaction (fig.3). The calmodulin–histone interaction is a complicated phenomenon, and further studies should be done to clarify the physiological significances of this interaction.

## 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] Kakiuchi, S. and Yamazaki, R. (1970) Biochem. Biophys. Res. Commun. 41, 1104–1110.
[4] Cheung, W. Y. (1970) Biochem. Biophys. Res. Commun. 38, 533–538.
[5] Wolf, D. J. and Brostrom, C. O. (1979) Adv. Cyclic Nucl. Res. 11, 27–88.
[6] Means, A. R. and Dedman, J. R. (1980) Nature 285, 73–77.
[7] Klee, C. B., Crouch, T. H. and Richman, P. G. (1980) Annu. Rev. Biochem. 49, 489–515.
[8] Cheung, W. Y. (1980) Science 207, 19–27.
[9] Dumler, I. L. and Etingof, R. N. (1976) Biochim. Biophys. Acta 429, 474–484.
[10] Sharma, R. K., Wirch, E. and Wang, J. H. (1978) J. Biol. Chem. 253, 3575–3580.
[11] Wallace, R. W., Lynch, T. J., Tallant, E. A. and Cheung, W. Y. (1978) J. Biol. Chem. 254, 377–382.
[12] Sobue, K., Muramoto, Y., Yamazaki, R. and Kakiuchi, S. (1979) FEBS Lett. 105, 105–109.
[13] Sobue, K. and Kakiuchi, S. (1980) Biochem. Biophys. Res. Commun. 93, 850–856.
[14] Maekawa, S. and Abe, T. (1980) Biochem. Biophys. Res. Commun. 97, 621–627.
[15] Glenney, J. R. jr and Weber, K. (1980) J. Biol. Chem. 255, 10551–10554.
[16] Iwasa, Y., Yonemitsu, K., Matsui, K., Fukunaga, K. and Miyamoto, E. (1981) FEBS Lett. 128, 311–314.
[17] Grand, R. J. A. and Perry, S. V. (1979) Biochem. J. 183, 285–295.
[18] Grand, R. J. A. and Perry, S. V. (1980) Biochem. J. 189, 227–240.
[19] Itano, T., Itano, R. and Penniston, J. T. (1980) Biochem. J. 189, 455–459.
[20] Wolff, D. J., Ross, J. M., Thompson, P. N., Brostrom, M. A. and Brostrom, C. O. (1981) J. Biol. Chem. 256, 1846–1860.
[21] Iwasa, Y., Iwasa, T., Matsui, K., Higashi, K. and Miyamoto, E. (1981) Life Sci. in press.
[22] Johns, E. W. (1967) Biochem. J. 104, 78–82.
[23] Van der Westhuyzen, D. R., Böhm, E. L. and Von Holt, C. (1974) Biochim. Biophys. Acta 359, 341–345.
[24] Teo, T. S., Wang, T. H. and Wang, J. H. (1973) J. Biol. Chem. 248, 588–595.
[25] Klee, C. B. and Krinks, M. H. (1978) Biochemistry 17, 120–126.
[26] Takai, Y., Kishimoto, A., Inoue, M. and Nishizuka, Y. (1977) J. Biol. Chem. 252, 7603–7609.
[27] Post, R. L. and Sen, A. K. (1967) Methods Enzymol. 10, 773–776.
[28] Iwasa, Y., Yonemitsu, K. and Miyamoto, E. (1981) FEBS Lett. 124, 207–209.
[29] Iwasa, Y., Takai, Y., Kikkawa, U. and Nishizuka, Y. (1980) Biochem. Biophys. Res. Commun. 96, 180–187.
[30] Bradford, M. M. (1976) Anal. Biochem. 72, 248–254.
[31] Dixon, M. (1953) Biochem. J. 55, 170–171.
[32] Elgin, S. C. and Weintraub, H. (1975) Annu. Rev. Biochem. 44, 725–774.