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Effect of Hypocalcemic Protein P-MSY obtained from Bovine Parotid Gland on the Cyclic Adenosine-5′-monophosphate and Cyclic Guanosine-5′-monophosphate Contents of Murine Thymus and Spleen Cells[1)]

HAJIMU YAMAMOTO,* IKUKATSU SUZUKI, and AKIRA MIZUTANI[2)]

Faculty of Pharmaceutical Sciences, Nagoya City University, Tanabedori 3, Mizuho-ku, Nagoya 467, Japan

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The effect of P–MSY, a hypocalcemic protein from bovine parotid gland, on the levels of cyclic adenosine-5′-monophosphate (cAMP) and cyclic guanosine-5′-monophosphate (cGMP) in thymic and splenic cells of 4-week-old C57BL/6 mice was studied in vitro. Preliminary studies on the effect of 0.5 mg/ml of P–MSY indicated that an incubation time of 10 min gave the maximum response. This incubation time was used in dose-response studies. In thymic cells, the increase of cAMP content was dose-dependent (2—3.6 fold increase in response to 1 mg/ml P–MSY), but little change of cGMP was observed. The ratio of cAMP/cGMP increased about 1.7—2.5 times at a concentration of 0.2 mg/ml P-MSY. cAMP did not increase as much in the splenic cells as in the thymic cells. The difference between the thymus and spleen in response to P–MSY is probably due to the relatively greater influence of P–MSY on T-cells of the thymus.

Keywords——P–MSY; parotid hypocalcemic protein; antitumor protein; cyclic AMP; cyclic GMP; thymic and splenic cell

We have purified P–MSY, a protein with hypocalcemic action, from bovine parotid gland,[3)] and reported its immunestimulating effect.[4)] P–MSY also occasionally shows an inhibitory effect on the proliferation of sarcoma 180 and solid tumor of Ehrlich carcinoma cells.[5,6)] The effect of this substance on antibody producing cells and lymphocyte ratio (T-cell, B-cell) in the peripheral blood was recently reported.[7)]

In the present study, the effect of P–MSY on the cyclic adenosine-5′-monophosphate (cAMP)[8)] and cyclic guanosine-5′-monophosphate (cGMP)[9)] contents in mouse thymic and splenic cells was examined, and we investigated whether hypocalcemic protein, P–MSY, increases cAMP or cGMP in mouse thymic cells and how the changes after P–MSY addition correlate with the cellular cyclic nucleotides.

Materials and Methods

Materials——The acetone precipitate was obtained by the method of Mizutani *et al.*[3)] from the supernatant at pH 5.4 of aqueous extract of bovine parotid gland. This material was extracted with glacial acetic acid and the extract (PAIA) was then purified by diethyl aminoethyl (DEAE)-cellulose chromatography and gel filtration through Sephadex G-100, to obtain the finally purified material, P–MSY. The hypocalcemic action of this material in rabbit was assayed by the method already reported.[10)] At a dose of 10 μg/kg, P–MSY significantly decreased the serum calcium by $13.57 \pm 1.61\%$ ($p<0.01$). This effect was greater than that of saline. As the control drug, bovine serum albumin (Katayama Chemical Co.) was used.

Animals——Mice of C57BL/6 strain purchased from Shizuoka Agricultural Co-operative for Experimental Animals, Hamamatsu, Japan were used at 4 weeks of age.

Experimental Procedure——Cell Preparation: C57BL/6 mice (4—5 weeks of age, weighing 16—20 g) were decapitated and the thymus (about 64 mg) and spleen were quickly removed. These organs were placed in 10 volumes of Eagle's MEM medium (produced by Nissui Kagaku) containing 0.029% L-glutamine and gently homogenized once in a Potter type glass homogenizer. The cell suspension was filtered through a stainless steel screen of 200—400 mesh. The solution containing the cells was centrifuged at $250 \times g$ for 10 min, then the precipitated cells were resuspended in 0.017 M Tris-HCl buffer containing 0.75% ammonium chloride (pH 7.0) to lyse the red cells.[11)] After incubation for 10 min at room temperature and filtration

through the above screen, the cells were centrifuged at $250 \times g$ for 10 min, then resuspended in Eagle's MEM medium and washed twice by centrifugation. The cell concentration was adjusted to 2.5×10^7 cells/ml. The cells were incubated for 10 min at 37°C to allow them to stabilize.

Cyclic Nucleotide Assays: A solution (0.5 ml) containing cells at a concentration of 2.5×10^7/ml was mixed with 0.5 ml of Eagle's MEM medium containing P–MSY or PAIA and the mixture was incubated at 37°C. The reaction was stopped by immersion of the mixture for 1 min in a dry ice-acetone bath followed by 3 min in a boiling water bath according to the reported methods.[9b] Assays for cAMP or cGMP were performed on duplicate samples of the supernatants of a 10 min, $2000 \times g$ spin. Contents of cAMP and cGMP in the tissue cells were measured by the use of an RCC Assay Kit. The values were obtained from the standard curve based on the mean of duplicate experiments for each sample.

cAMP in 0.05 ml aliquots of the supernatants was measured as follows; 0.05 ml of [^{3}H] cAMP (5 μCi/10 ml) and 0.1 ml of the binding protein were added to each supernatant sample and the mixture was incubated for 2 h at 2°C. Then 0.1 ml of charcoal suspension was added, and the mixture was centrifuged at $8000 \times g$. The radioactivity in 0.2 ml of the supernatant was measured with a liquid scintillation counter (Mark II, Searle Analytic Inc.). The scintillant used consisted of one part of Triton X-100 and two parts of toluene containing PPO (3.2 g/l) and POPOP (0.24 g/l).

For the measurement of cGMP, 0.1 ml of the supernatant was mixed with 0.05 ml of [^{3}H] cGMP (1.6 μCi/10 ml) and 0.05 ml of antiserum. The solution was incubated at 2°C for 1.5 h, then 1 ml of ammonium sulfate solution was added and the mixture was centrifuged at $13000 \times g$ for 2 min. The precipitate was dissolved in 1.1 ml of distilled water and 1 ml of the solution was used for scintillation counting.

Other General Methods: Hypocalcemic action was assayed by the method described previously.[10,12] Protein concentration was determined by the method of Lowry *et al.*[13] Polyacrylamide disc gel electrophoresis was carried out according to Davis.[14]

Results and Discussion

Figure 1 shows the changes of contents of cAMP and cGMP on incubation of equal volumes of thymic cell suspension (2.5×10^7 cells/ml) and 0.5 mg/ml P–MSY solution for 0, 2, 5, 10 and 30 min at 37°C. The cAMP content increased 2—3 times, and peaked (3.2-fold increase) after 10 min of incubation. The peak of cGMP (about 2.3-fold increase) was seen after 5 min, somewhat earlier than in the case of cAMP. The cAMP/cGMP ratio in thymocytes (14.61) reached a maximum of about 1.8 times the zero-time level (8.08) at 10 min. The dose-response relationship was therefore studied with incubation for 10 min as the standard condition.

Figure 2 shows the dose-response relationship for P–MSY in thymic cells. A clear dose-

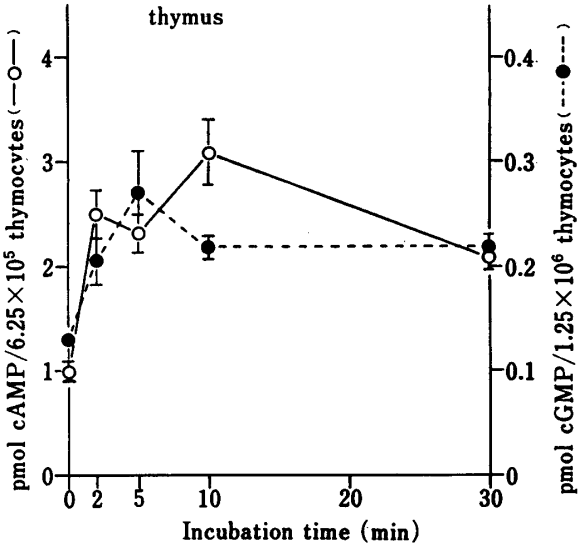

Fig. 1. Time Courses of the Effect of P–MSY (0.5 mg/ml) on the Levels of cAMP and cGMP in Thymus Cells

Each point represents the mean ± S.E. of 3—5 experiments.

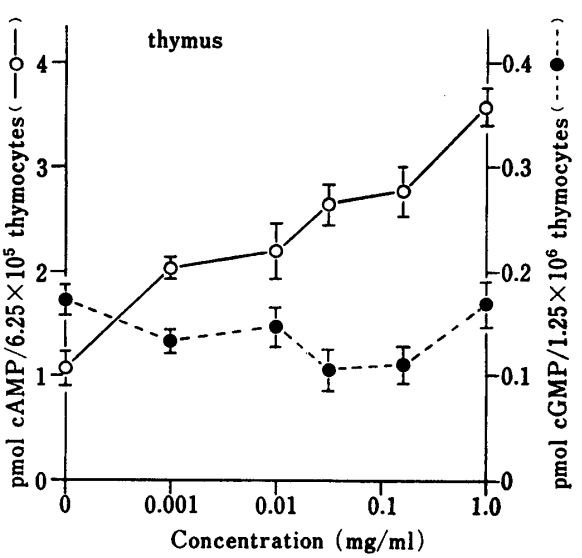

Fig. 2. Dose-response Relationships for P–MSY Action on the Levels of cAMP and cGMP in Thymus Cells

Each point represents the mean ± S.E. of 3—5 experiments.

dependent increase of cAMP was noted. At a concentration of 1 mg/ml P–MSY, cAMP increased about 3.6 times. In contrast, cGMP did not increase.

The time course of the effect of 0.5 mg/ml P–MSY on splenic cells was then studied (Fig. 3). The levels of both cAMP and cGMP peaked after 10 min of incubation. The dose-response relationship was therefore studied with incubation for 10 min as the standard condition, as in thymic cells. It was found that cAMP did not increase as markedly as in thymic cells, as shown in Fig. 4. cAMP increased about 2-fold in response to 1 mg/ml P–MSY. The content of cGMP in the spleen at 0.1 mg/ml P–MSY was only 0.7 times the zero-time level. The cAMP/cGMP ratio increased about 2.1 times at the concentration of 0.2 mg/ml P–MSY.

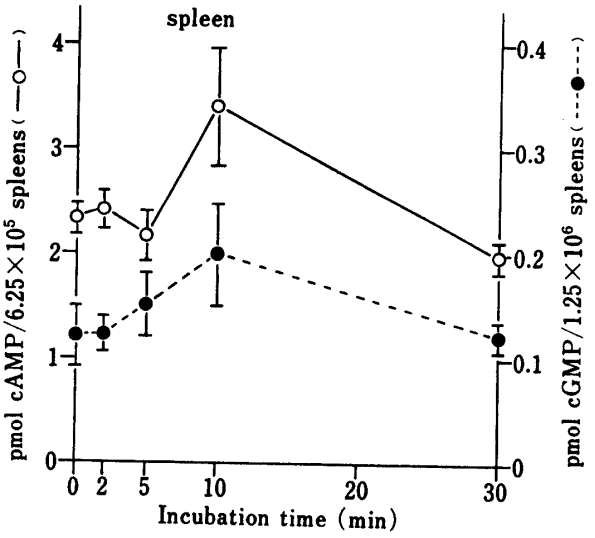

Fig. 3. Time Courses of the Effect of P–MSY (0.5 mg/ml) on the Levels of cAMP and cGMP in Spleen Cells

Each point represents the mean ± S.E. of 4—7 experiments.

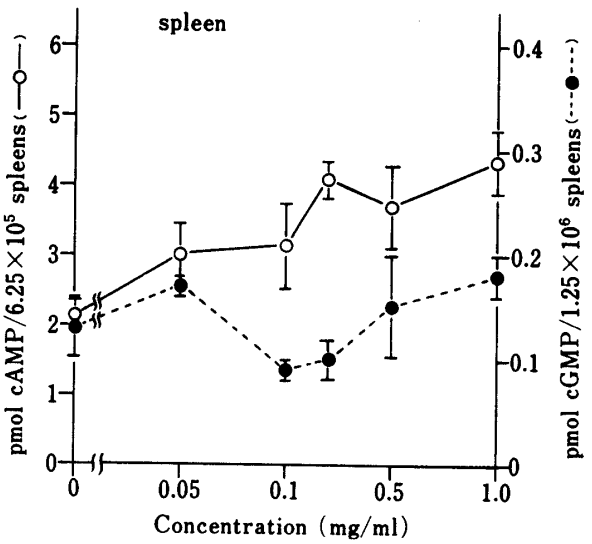

Fig. 4. Dose-response Relationship for P–MSY Action on the Levels of cAMP and cGMP in Spleen Cells

Each point represents the mean ± S.E. of 3—7 experiments.

Control experiments on the dose-response relationship of thymic cells to bovine serum albumin (BSA) produced almost no change of cAMP, while cGMP showed approximately a two-fold increase. The cAMP/cGMP ratio decreased about 0.7 time in response to 1 mg/ml albumin. The pattern of response was different from that to P–MSY.

The dose-response relationship of splenic cells to BSA was then studied. Scarcely any change of cAMP or cGMP was noted. The pattern of response was evidently different from that to P–MSY, which caused a definite increase of cAMP.

Partially purified material, PAIA,[3] tended to increase the cAMP/cGMP ratio in thymic and splenic cells at a relatively low concentration of 0.5 mg/ml, but tended to decrease it at high concentrations (0.5—1.0 mg/ml). This might represent the influence of various contaminants.

According to these results, P–MSY increased cAMP in the thymic and splenic cells but failed to cause much change of cGMP. This is in contrast to the general observations that thymopoietin,[9a] thymosin,[9b] and LPS[15] increase cGMP in lymphatic tissue. Humoral thymic factors such as SF[8b] and tetrahydrofuran (THF),[8c] on the other hand, probably have actions similar to those of P–MSY, in view of their reported cAMP-increasing effects.

P–MSY appears to exert a greater influence on the thymus or T-cells than on the spleen. Further studies with isolated T-cells and B-cells appear to be necessary. These results confirm the previously reported finding that guinea pigs dosed *i.p.* at the neonatal stage with 10 or 20 μg of P–MSY displayed a significant elevation of the T-cell percentage in peripheral blood with

a significant relative decrease in the B-cell subpopulation ($p<0.05$).[7] Such a difference is of profound interest in view of the previous studies on T- and B-cells in the peripheral blood, indicating that P–MSY increases T-cells.

According to the studies of Hirata *et al.*,[16] β-adrenergic agonists and β-adrenergic receptors bind to each other with subsequent coupling to adenylate cyclase. This reaction then prompts the methyl group transfer reaction of phospholipid. As a result, membrane fluidity increases, adenylate cyclase is activated and tissue cAMP increases. In view of the dose-dependent increase of P–MSY in the thymus and spleen, it is possible that P–MSY activates adenylate cyclase to increase cAMP.

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References and Notes

1) Presented at the 101st Annual Meeting of the Pharmaceutical Society of Japan, Kumamoto, April 1981.
2) Present address: *The 2nd Division, Daiyukai Institute of Medical Sciences, Matsufuri-dori, Ichinomiya 491, Japan.*
3) A. Mizutani, I. Suzuki, and H. Yamamoto, *Chem. Pharm. Bull.*, **26**, 1957 (1978).
4) A. Mizutani, I. Suzuki, Y. Ohba, T. Nakamura, and S. Adachi, *Chem. Pharm. Bull.*, **26**, 2927 (1978).
5) A. Mizutani, H. Yamamoto, and I. Suzuki, *Gann*, **69**, 291 (1978).
6) H. Yamamoto, I. Suzuki, and A. Mizutani, *Chem. Pharm. Bull.*, **30**, 2133 (1982).
7) H. Yamamoto and A. Mizutani, *Chem. Pharm. Bull.*, **29**, 3298 (1981).
8) *a*) R. Hiramatsu, T. Mizutani, and A. Mizutani, *Chem. Pharm. Bull.*, **28**, 413 (1980); *b*) A. Astaldi, G.C.B. Astaldi, P.TH.A. Schellekens, and V.P. Eijisvoogel, *Nature* (London), **260**, 713 (1976); *c*) A.I. Kook, N. Traini, *J. Exp. Med.*, **139**, 193 (1974); *d*) S. Ikehara, Y. Hamashima, and T. Masuda, *Nature* (London), **258**, 335 (1975).
9) *a*) G.H. Sunshine, R.S. Basch, R.G. Coffey, K.W. Cohen, G. Goldstein, and J.W. Hadden, *J. Immunol.*, **120**, 1594 (1978); *b*) P.H. Naylor, H. Sheppard, G.B. Thurman, and A.L. Goldstein, *Biochem. Biophys.* Res. Commun., **73**, 843 (1976).
10) A. Mizutani, K. Yamamoto, H. Sato, and Y. Saito, *Yakugaku* Zasshi, **91**, 297 (1971).
11) W. Boye, *Transplantation*, **6**, 761 (1968).
12) A. Mizutani, T. Mizutani, and P.-F. Kuo, *Chem. Pharm. Bull.*, **22**, 1955 (1974).
13) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
14) B.J. Davis, *Ann. N.Y. Acad. Sci.*, **121**, 409 (1964).
15) J. Watson, *J. Exp. Med.*, **141**, 97 (1975).
16) F. Hirata and J. Axelrod, *Science*, **209**, 1082 (1980).