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Evidence for Non-mediation of Calcitonin in the Action of Hypocalcemic Protein from Bovine Parotid Gland[1)]

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It was found by radioimmunoassay of serum calcitonin in rabbits that a hypocalcemic protein from bovine parotid gland lowers serum calcium without increasing the secretion of calcitonin. Binding of calcitonin and anti-calcitonin was not inhibited by the parotid protein. Furthermore, when calcitonin was administered to rabbits in addition to a maximally hypocalcemic dose of the parotid protein, it caused an additional decrease of blood calcium level. These results suggest that the target organs of the parotid protein and calcitonin are different, or that the parotid protein and calcitonin activate different receptors in the same target organ.

Leucocyte-increasing activity was demonstrated in both the parotid protein and calcitonin, but the effect of calcitonin developed more rapidly than that of the parotid protein, as was the case with hypocalcemic activity. It was also found that the parotid protein caused a significant increase of rosette-forming cell activity in the spleen of neonatal mice, whereas calcitonin did not. These observations suggest that the biological effects of the parotid protein are not mediated by calcitonin.

Keywords——hypocalcemic activity; parotid protein; calcitonin; serum calcium; radioimmunoassay; leukocyte-increasing activity; rosette-forming cell activity; chemical modification of parotid hypocalcemic protein

In 1955, Ogata *et al.*[3)] found a hypocalcemic protein from bovine parotid gland which affects the serum calcium level in rabbits, and named it parotin. Mizutani *et al.*[4)] purified this substance to homogeneity, as determined by polyacrylamide gel disc electrophoresis and sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. The molecular weight of this protein was determined to be 46500±1500 daltons by various methods, and its amino acid composition and secondary conformation were also reported.[4,5)] In addition to its hypocalcemic activity, it was recently confirmed that this protein increases the activity of antibody-producing cells in neonatal mice.[6)]

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3) *a*) T. Ogata, *Endocrinol. Japan*, **2**, 247 (1955); *b*) T. Ogata and Y. Ito, *Yakugaku Zasshi*, **64**, 146 (1944); *c*) Y. Ito and A. Mizutani, *Yakugaku Zasshi*, **72**, 244 (1952).
4) A. Mizutani, T. Kitamura, N. Yamada, H. Inaba, Y. Naito, and T. Mizutani, *Chem. Pharm. Bull.* (Tokyo), **22**, 1955 (1974).
5) A. Mizutani, T. Mizutani, and P-F. Kuo, *Chem. Pharm. Bull.* (Tokyo), **24**, 2733 (1976).
6) A. Mizutani, T. Mizutani, I. Suzuki, and P–F. Kuo, *Chem. Pharm. Bull.* (Tokyo), **25**, 1601 (1977).

Meanwhile, Copp *et al.*[7] in 1962 and Hirsch *et al.*[8] in 1963 discovered calcitonin, a thyroid gland hormone which is related to the regulation of calcium metabolism and which rapidly lowers blood calcium level. It should be noted that the molecular weight of calcitonin is small, 3600.[9] Because hypocalcemia develops more slowly in the case of the parotid protein than in the case of calcitonin, it has been suspected that the action of this parotid protein is mediated by the secretion of calcitonin.

In the present study, we examined the changes of serum calcitonin by radioimmunoassay after injection of the parotid protein into rabbits. Other biological activities of these two substances, such as leucocyte-increasing activity and rosette-forming cell (RFC) activity, were also studied to elucidate the difference in the actions of parotid protein and calcitonin.

Materials and Methods

Materials

The hypocalcemic protein was purified from bovine parotid gland according to the method of Mizutani *et al.*[4] It was extracted from the parotid glands with deionized water (pH 8.0) and purified by isoelectric precipitation at pH 5.4, fractional precipitation with $(NH_4)_2SO_4$, diethylaminoethyl-cellulose chromatography, gel electrophoresis, and finally by gel chromatography on Sepharose 6B. The hypocalcemic protein was homogeneous on analytical disc electrophoresis.

Porcine calcitonin containing gelatin was obtained from Armour Laboratory through Yamanouchi Pharmaceutical Co., Tokyo. Purified porcine calcitonin was generously supplied by the Research Laboratory of Yamanouchi Pharmaceutical Co.

Methods

Preparation of Antibody to Calcitonin[10,11]——Porcine calcitonin (160 MRCU) and porcine serum albumin (10 mg) were dissolved in 2.5 ml of 0.1 M phosphate buffer (pH 7.0). One ml of 0.021 M glutaraldehyde solution was added dropwise to this solution with constant stirring at 4°. Excess glutaraldehyde was eliminated by adding 50 μl of 1 M lysine solution, and the reaction mixture was dialyzed overnight at 4° against 0.9% NaCl solution. The solution was emulsified with an equal volume of Freund's complete adjuvant (Iatron Laboratories, Tokyo) and was injected subcutaneously into toe pads and back of 3 guinea total dose of about 50 MRCU of calcitonin per animal. This procedure was repeated pigs in a 6 times at 2-week intervals, and finally a booster injection of the same dose of the same antigen (without adjuvant) was given subcutaneously.

Radioiodination and Separation of Calcitonin——Pure porcine calcitonin was labeled with carrier-free $Na^{125}I$ (New England Nuclear, U.S.A.) to a specific radioactivity of about 50 μCi/μg of calcitonin according to the method of Greenwood *et al.*[12,13] with minor modification as follows: 0.02 ml of 0.01% purified porcine calcitonin and 0.01 ml of 0.2% chloramine-T in 0.5 M phosphate buffer were added simultaneously to 0.5 mCi of $Na^{125}I$ solution (pH 7.5); the solution was mixed for 10 sec, then the reaction was stopped by adding 0.1 ml of 0.1% $Na_2S_2O_5$ in 0.5 M phosphate buffer. Finally, 1.5 ml of 0.1 M Tris buffer (pH 7.5) containing 5% egg albumin and 0.05 M mercaptoethanol was added. The labeled calcitonin was isolated from the reaction mixture by the procedure of Tashjian;[13] it was absorbed on microfine granules of silica, Quso G-32 (Philadelphia Quartz Co.), then eluted with 1 ml of a solution containing 20% acetone, 1% AcOH, and 0.05 M mercaptoethanol after the gel had been washed several times with 0.05 M mercaptoethanol.

Radioimmunoassay of Calcitonin——Serum samples for the determination of calcitonin were collected from three groups of six normal mature male rabbits. The animals had been fasted for about 24 hr. The parotid protein or pure calcitonin was dissolved in physiological saline and intravenously injected into the rabbits of the test groups at a dose of 0.05 mg/0.5 ml/kg or 4 MRCU/0.5 ml/kg, respectively. The control group was injected with 0.5 ml/kg of physiological saline. Blood was drawn before, and 2, 3, 4, and 5 hr after the injection. Incubation for radioimmunoassay was carried out in flint glass test tubes (75 × 10 mm) in a non-equilibrium system. The dilution buffer was 0.1 M Tris (pH 7.5) containing 0.5% egg albumin, 5 mM mercaptoethanol, and 0.01% merthiolate. Antiserum was used at a dilution of 1: 1000, and a mixture of 0.1 ml of this diluted antiserum, 0.25 ml of dilution buffer, and 0.1 ml of the unlabeled calcitonin solution

7) D.H. Copp, E.C. Camerson, B.A. Cheney, A.G. Davidson, and K.G. Henze, *Endocrinology*, **70**, 638 (1962).
8) P.F. Hirsch, G.F. Gauthier, and P.L. Munson, *Endocrinology*, **73**, 244 (1963).
9) J.T. Potts, Jr., H.D. Niall, H.T. Keutmann, H.B. Brewer, Jr., and L.J. Deftos, *Proc. Nat. Acad. Sci. U.S.A.*, **59**, 1321 (1968).
10) A.H. Tashjian, Jr., P.H. Bell, and L. Levine, *Proc. Second Int. Symp. Calcitonin*, **1969**, 359.
11) M. Reichlin, J.J. Schnure, and V.K. Vance, *Proc. Soc. Exp. Biol. Med.*, **128**, 347 (1968).
12) F.C. Greenwood, W.M. Hunter, and J.S. Glover, *Biochem. J.*, **89**, 114 (1963).
13) A.H. Tashjian, Jr., *Endocrinology*, **84**, 140 (1969).

as a standard or of a serum sample was preincubated at 4° for 2 days. Labeled calcitonin solution (0.05 ml) was then added and incubation was continued for a further 2 days. Dextran-coated charcoal was prepared by the method of Herbert *et al.*[14] The buffer used was prepared by dissolving 14.714 g of sodium barbital, and 9.714 g of AcONa in 500 ml of distilled water. To 100 ml of this buffer, 1800 ml of 0.85% NaCl and 100 ml of 0.1 N HCl were added to yield a final solution of pH 7.4. Charcoal Norit A (Wako Pure Chem. Co., Tokyo) was suspended in the buffer (5 g/100 ml). Dextran 80 (Seikagaku Kogyo Co., Tokyo) was dissolved in the buffer to 0.5 g/100 ml. Dextran-coated charcoal was prepared by mixing equal volumes of the charcoal suspension and dextran solution. The mixture was shaken for 10 sec and stored at 4°. Since a small portion of the labeled calcitonin is inevitably decomposed during incubation, and ^{125}I released behaves together with antibody-calcitonin complex in the separation step, samples without the antiserum were treated in the same manner as a control. After incubation, free calcitonin was separated from bound calcitonin by absorption on dextran-coated charcoal. After addition of 0.5 ml of dextran-coated charcoal suspension, the mixture was stirred for several seconds, and centrifuged for about 15 min at 3000 rpm to separate the supernatant for the measurement of radioactivity with a scintillation counter (mark II, Searle Analytic Inc.). Meanwhile, in a similar manner, the parotid protein at various concentration instead of unlabeled calcitonin was preincubated with anti-calcitonin at 4° for 2 days and subsequently incubated with the labeled calcitonin for a further 2 days. The inhibitory effect of the parotid protein on the cross-reaction of calcitonin and anti-calcitonin was examined.

Bioassay of Hypocalcemic Activity——The hypocalcemic activity was assayed by the method described previously.[4] Each group consisted of six male rabbits of more than 2 kg body weight. The rabbits were fasted for 1 day before the experiments and the sample dissolved in physiological saline in a volume of 0.5 ml/kg body weight was injected into their aural vein. The parotid protein was injected intravenously into the rabbits at a dose of 0.05 mg/kg and calcitonin at 4 MRCU/kg. The control rabbits received physiological saline. Blood was drawn from the aural vein before the injection and 2, 4, 5, 6, 8, and 24 hr after injection, and the decrease in serum calcium was expressed as a percentage of the preinjection level. In the case of the parotid protein, the average of the decreases at 4,5 and 6 hr after injection was taken as the hypocalcemic rate. The difference between the test and control groups was statistically evaluated by means of the *t*-test at the 5% level of significance. Serum calcium was determined with a Shimadzu AA-610 atomic absorption spectrophotometer. In the experiment where calcitonin of 4MRCU/kg was injected intravenously 4 hr after the injection of the parotid protein at 0.15 mg/kg, blood was drawn before, and 4, 5, and 6 hr after the injection of the parotid protein, and serum calcium was determined as described above. The dose of the parotid protein used above causes a maximum hypocalcemic response.[15]

Measurement of the Number of Leucocytes——The sample solutions were prepared as described above and injected intravenously into rabbits. Blood was drawn before, and 2, 4, 6, 8, and 24 hr after the injection. The number of leucocytes stained with Gentian Violet was counted, using a melangeur and a Bürker–Türk hemocytometer. The difference between the test and control groups was examined by means of the *t*-test as above.

Measurement of RFC Activity——The method of Jerne and Nordin[16] as modified by Ceglowski *et al.*[17] was employed. Litter mates of neonatal mice of the ICR strain within 6—12 hr after birth were divided into two groups. One group of mice was injected with the sample intraperitoneally and the other group was given physiological saline as a control. After 14 days, the mice of both groups were injected intraperitoneally with 0.4 ml of 20% sheep red blood cells (SRBC) in physiological saline, and the spleen was excised 4 days later. Spleen cell suspension was prepared by loosening the spleen into small pieces in 2 ml of Dulbecco's phosphate buffer in the absence of Mg^{2+} and Ca^{2+}; it was strained with a stainless steel sieve of 200 mesh, then the cells were washed 3 times with the same buffer solution by centrifugation. Eagle's minimum essential medium (2 ml, 0.9%) was added to the sedimented cells to give a cell suspension. The number of cells in this suspension was counted by using a mélangeur and a Bürker–Türk hemocytometer. An aliquot of 0.4 ml of the cell suspension was mixed with 0.2 ml of 2% SRBC and centrifuged at 1000 rpm for 6 min at 4°, followed by gentle stirring. The resulting suspension was placed in a Bürker–Türk hemocytometer and the rosettes formed were counted with a dark-field, phase-contrast microscope. The number of rosette-forming cells/10^6 cells was calculated, and the sample was considered to be effective if the difference between the mean of the test group and that of the control group was significant at the 5% level (*t*-test).

Preparation of chemically Modified Parotid Protein——1) Modification of Lysyl Free Amino Groups: Lysyl free amino groups were modified with trinitrobenzenesulfonic acid (TNBS) according to the method of Habeeb.[15,18] TNBS (1 ml, 0.1%) was added to a mixture of 2 ml of the sample solution (0.6—0.7 mg/ml)

14) V. Herbert, K–S. Lau, C.W. Gottlieb, and S.J. Bleicher, *J. Clin. Endocrinol.*, **25**, 1375 (1965).
15) A. Mizutani, T. Mizutani, and P–F. Kuo, *Chem. Pharm. Bull.* (Tokyo), **25**, 2850 (1977).
16) N.K. Jerne and A.A. Nordin, *Science*, **140**, 405 (1963).
17) W.S. Ceglowski, T.L. Hand, and H. Friedman, "Thymic Hormones," ed. by T.D. Luckey, University Park Press, Baltimore, 1973, p. 185.
18) A.F.S.A. Habeeb, *Anal. Biochem.*, **14**, 328 (1966).

and 1 ml of phosphate buffer (pH 7.5) or 4% $NaHCO_3$ solution (pH 8.5). The resulting mixture was incubated at 40° for 2 hr, then subjected to dialysis and lyophilization after gel filtration on Sephadex G-25.

2) Modification of Disulfide Bonds: Mercaptoethanol (1%) was added to a solution of 5 mg of the sample in phosphate buffer (pH 8.0), and the mixture was allowed to react at room temperature for 30 min. It was then mixed with 0.2 ml of 3.96% 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in phosphate buffer (pH 7.0), and the mixture was incubated for 10 min.[15,19] The reaction mixture was lyophilized after gel filtration on Sephadex G-25.

Results

1. Time Course of the Hypocalcemic Action of the Parotid Protein and Calcitonin

As shown in Fig. 1, the parotid protein at a dose of 0.05 mg/kg caused maximum decrease (9.63±1.01%) of serum calcium 5 hr after the injection and the calcium level returned to normal in 24 hr, whereas calcitonin at a dose of 4 MRCU/kg caused a 12.75±0.75% decrease of serum calcium as early as 1 hr after injection, and the calcium level returned to normal in 6 hr. It can be concluded that the action of calcitonin is much more rapid than that of the parotid protein.

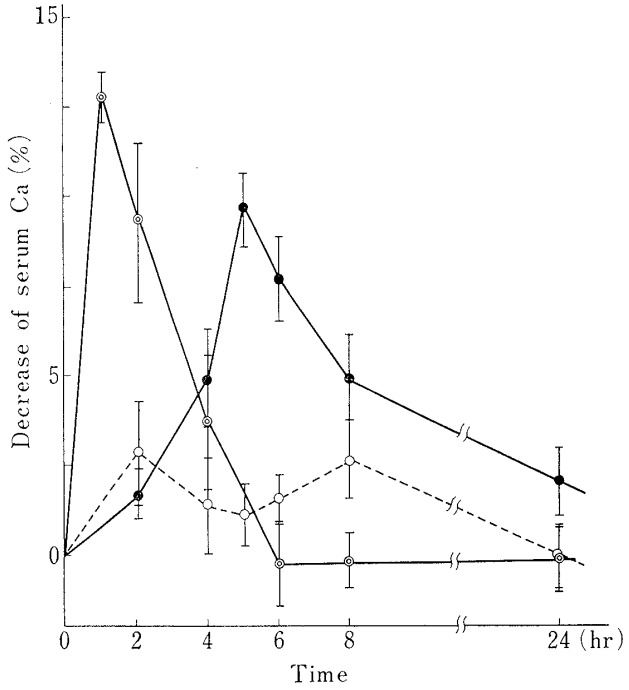

Fig. 1. Time Courses of Hypocalcemic Activity in Rabbits

●: the parotid protein (0.05 mg/kg),
◎: calcitonin (4 MRCU/kg),
○: control.

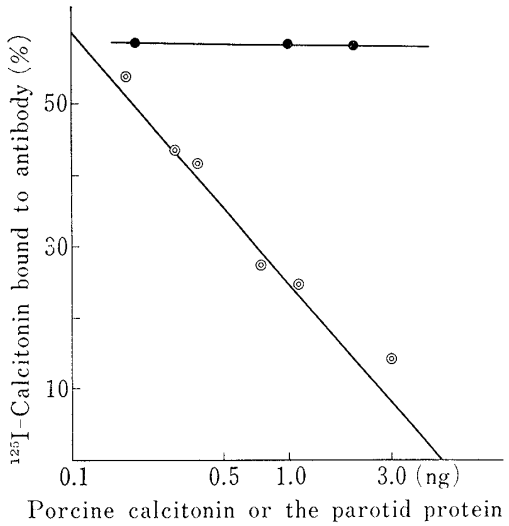

Fig. 2. Standard Curve for Porcine Calcitonin

◎: standard curve for porcine calcitonin,
●: after incubation of a solution containing the parotid protein and anti-calcitonin, the labeled calcitonin was added.

2. Change of Serum Calcitonin Concentration after Injection of the Parotid Protein in Rabbits

Figure 2 shows the standard radioimmunoassay curve of calcitonin. Using this radioimmunoassay, the effect of intravenous injection of 0.05 mg/kg of the parotid protein on the serum calcitonin level was examined. As shown in Fig. 3, only a slight, statistically insignificant difference was observed between the animals receiving the parotid protein and those receiving saline alone. To test the reliability of the assay method, the change of serum calcitonin concentration was checked after the injection of a hypocalcemic dose of calcitonin (4 MRCU/kg); the results are shown in Fig. 4. The concentration of calcitonin in serum was elevated about 6 times after 10 min compared with the concentration at 0 min, then decreased

19) G.L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).

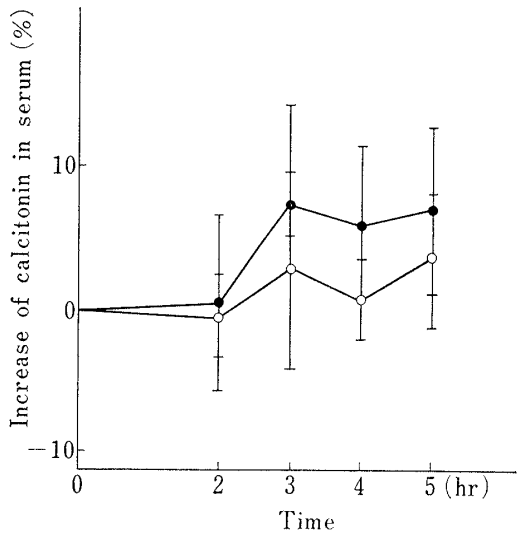

Fig. 3. Time Courses of the Concentration of Calcitonin in Rabbits after the Parotid Protein Injection

●: the parotid protein (0.05 mg/kg),
○: control.

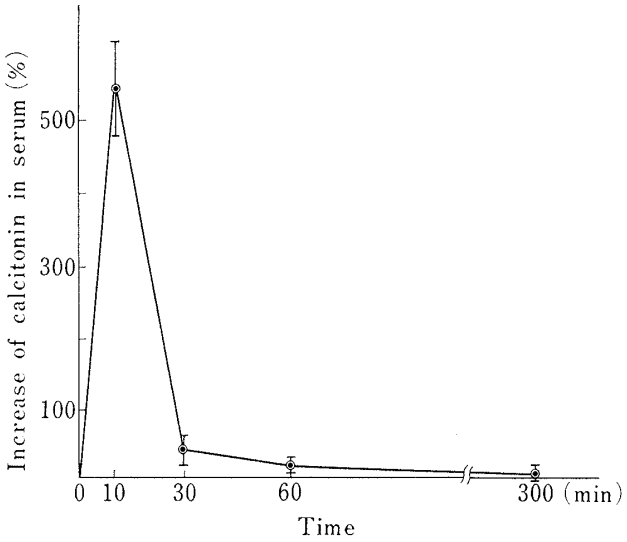

Fig. 4. Disappearance of Calcitonin from Serum

Calcitonin was injected intravenously into 6 rabbits at a dose of 4 MRCU/kg.

rapidly to 38.9% after 30 min and to 16.1% after 60 min, even though the maximum change of the serum calcitonin level after injection of the parotid protein was 7.5%.

The parotid protein at various concentrations did not inhibit the binding of calcitonin to anti-calcitonin (Fig. 2). This shows that the parotid protein and calcitonin do not have a common antigenic determinant. The unchanged serum concentration of calcitonin after administration of the parotid protein and the failure of the parotid protein to bind to anti-calcitonin suggest that the parotid protein is not a precursor of calcitonin.

3. Hypocalcemic Activities of the Parotid Protein and Calcitonin

When 4 MRCU/kg of calcitonin was injected 4 hr after the injection of 0.15 mg/kg of the parotid protein, the serum calcium level decreased by 18.63±1.15% at the maximum, while the injection of 0.15 mg/kg of the parotid protein alone caused only 10.20±1.70% decrease of the serum calcium (Fig. 5). The difference between the two groups is statistically significant ($p<0.05$). These results suggest that the target organ of the parotid protein differs from that of calcitonin, or that there are different receptor sites for the parotid protein and calcitonin in the same target organ.

4. Leucocyte-increasing Activity

Figure 6 shows the increase in the number of leucocytes in blood after injection of the parotid protein (0.05 mg/kg) or calcitonin (4 MRCU/kg). The parotid protein temporarily decreased the leucocyte number 2 hr after injection and then increased it gradually to a maximum (120.0±11.3%) 8 hr after the injection. On the other hand, calcitonin increased the leucocytes number more rapidly than the parotid protein and did not produce a temporary decrease. The effects of the parotid protein observed 6 and 8 hr after the injection are significant at the 5% and 1% levels, respectively. The effect of calcitonin is significant at the 1% level at 2, 4, 6, and 8 hr, and the average maximum value was 92.2±26.9% at the 8-hr point.

5. RFC Activity

The RFC and hypocalcemic activities of both the parotid protein and calcitonin are summarized in Table I. The parotid protein showed RFC activity at a dose of 10 μg/mouse, but calcitonin was ineffective at doses of 0.05, 0.25, 1.0, and 2.5 MRCU/mouse.

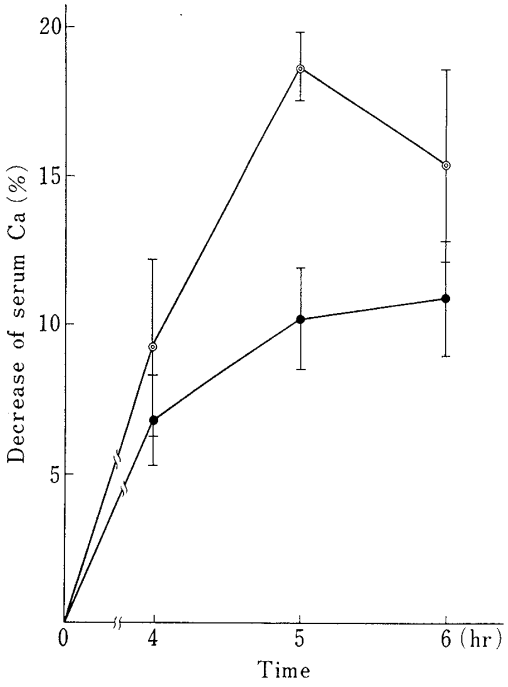

Fig. 5. Time Courses of Hypocalcemic Activity in Rabbits

●: the parotid protein (0.15 mg/kg), ◎: calcitonin (4 MRCU/kg) injection at various times after injecting the parotid protein (0.15 mg/kg).

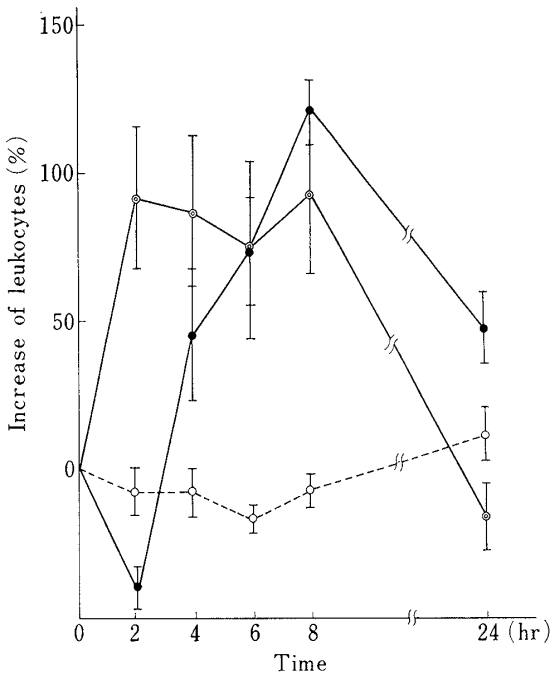

Fig. 6. Time Course of Leukocyte-increasing Activity in Rabbits

●; the parotid protein (0.05 mg/kg), ◎: calcitonin (4 MRCU/kg), ○: control.

TABLE I. Hypocalcemic and RFC Activities of the Parotid Protein and Calcitonin

Sample	Hypocalcemic activity (Rabbits)		RFC activity (Mice)		
	Dose (mg for the parotid protein or MRCU for calcitonin per kg)	Percent decrease in serum Ca Mean ± S.E. ($n=6$)	Dose (μg for the parotid protein of MRCU for calcitonin per mouse)	n	No. of RFC/10^6 cell Upper: control Lower: sample
Native parotid protein	0.05	10.40 ± 1.23[a]	10	4	2742.5 ± 208.4
				4	7014.0 ± 1683.1[b]
Modified parotid protein (TNBS at pH 7.5)	0.05	9.72 ± 0.83[a]	10	5	1231.6 ± 276.9
				7	1503.8 ± 261.6
Modified parotid protein (TNBS at pH 8.5)	0.05	7.09 ± 0.88[a]	10	4	3402.3 ± 304.0
				4	3920.4 ± 482.4
Modified parotid protein (DTNB after reduction)	0.05	4.12 ± 1.48	10	5	6998.2 ± 490.0
				5	6734.7 ± 428.6
Calcitonin	4.0	13.45 ± 1.10[a]	2.5	5	2454.4 ± 551.7
				5	2348.4 ± 465.1
			1.0	6	2409.1 ± 665.2
				5	4627.2 ± 1556.2
			0.25	6	4669.3 ± 1126.8
				5	5093.8 ± 722.1
			0.05	4	5872.7 ± 502.5
				5	4372.3 ± 467.9

n=number of animals.
a) Significantly different from control $p<0.01$.
b) Significantly different from control $p<0.05$.

6. Leucocyte-increasing, RFC, and Hypocalcemic Activities of Chemically Modified Parotid Protein

Figure 7 shows the leucocyte-increasing activity of the parotid protein after modification of 24.6% of the lysyl free amino groups and after modification of the disulfide bonds with DTNB. The effects of these chemical modification of the parotid protein on RFC and hypocalcemic activities are shown in Table I.

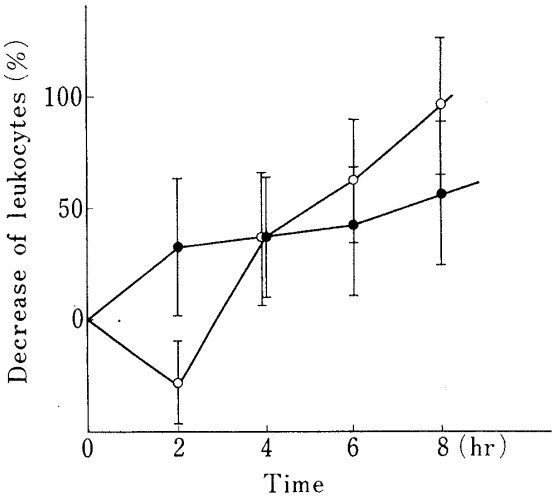

Fig. 7. Time Courses of Leukocyte-increasing Activity in Rabbits

○; the parotid protein modified with TNBS (pH 7.5), ●; the parotid protein modified with DTNB after reduction.

The leucocyte-increasing and hypocalcemic effects of the TNBS derivative of the parotid protein are significantly different from the control (physiological saline) at the 1% level, but the RFC activity is not statistically significant. After modification of the disulfide bonds, the parotid protein lost all three activities. After modification of 51.6% of the lysyl free amino groups with TNBS, the RFC activity was destroyed but the hypocalcemic activity was maintained (Table I).

Discussion

Based on the different time courses of hypocalcemia caused by the parotid protein and by calcitonin, it seemed possible that the hypocalcemic activity of the parotid protein is due to stimulation of the secretion of calcitonin. However, the determination of serum calcitonin level by radioimmunoassay showed that the parotid protein decreased serum calcium level without stimulation the secretion of calcitonin. Thus, it appears that the mechanism of hypocalcemic action of the parotid protein is different from that of calcitonin.

The finding that the molecular weight (M.W.) of the parotid protein (M.W. 46500) is much greater than that of calcitonin (M.W. 3600) raised the possibility that the parotid protein may produce a calcitonin-like substance upon decomposition in the animal body. However since the antigenicity of the parotid protein was different from that of calcitonin, this seems unlikely.

Intravenously injected calcitonin was rapidly lost from the serum and completely disappeared within 3 hr after the injection (Fig. 4). In general, it seems that disappearance of polypeptide hormones is very rapid. For instance, the biological half-life of parathormone (M.W. 8500) is as short as 20 or 30 min.[20] Injection of 4 MRCU/kg of calcitonin resulted in a hypocalcemic rate of 12.75% in 1 hr, whereas the serum calcitonin concentration was highest at 10 min. When the parotid protein was injected intravenously at a dose of 0.05 mg/kg, serum calcitonin was not increased significantly, and hypocalcemia of 9.63% occurred as much as 5 hr after the injection. This result suggests that the parotid protein exhibits hypocalcemic activity that does not involve stimulation of the secretion of calcitonin. Furthermore, when calcitonin was administered after injecting a maximally hypocalcemic dose of the parotid protein, a greater hypocalcemia occurred than was caused by the parotid protein alone (Fig. 5). This also supports the view that the action of the parotid protein is independent of that of calcitonin.

20) R.A. Melick, G.D. Aurbach, and J.T. Potts, Jr., *Endocrinology*, **77**, 198 (1965).

It was shown that both the parotid protein and calcitonin possess leucocyte-increasing activity, but the action of calcitonin is more rapid. This is consistent with the rapidity of hypocalcemic action of calcitonin. Chemical modification of free amino groups of the lysyl residues of the parotid protein did not abolish the hypocalcemic and leucocyte-increasing activity, but modification of the disulfide bonds resulted in the loss of both activities. This result suggests that the hypocalcemic and leucocyte-increasing activities may be related.

The parotid protein showed RFC activity, whereas calcitonin did not. Furthermore, neither of the derivatives of the parotid protein mentioned above exhibited RFC activity. It appears that the RFC activity of the parotid protein is not directly related either to the hypocalcemic activity or to the leucocyte-increasing activity.

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