啓明醫大論文集 第11券 第4號
The Keimyung Univ Med J
Vol 11, No. 4, December, 1992

Experimental study of the calmodulin activity of the supernatant and particulate fraction in nephric organ of normal adult guinea pig

Department of Anesthesiolgy Keimyung University, School of Medicine Taegu, Korea

Jung Koo Lee, MD; Jin Mo Kim, MD; Jung Kil Chung, MD

INTRODUCTION

Recent studies have suggested that many of the effects of ionic calcium on enzymes and other calcium-dependent proteins are mediated through an intracellular calcium binding regulator protein, which has been called calmodulin[1,2,3]. Calmodulin was first discovered in extracts of brain tissues as an activator of cyclic nucleotide phospodiesteras e[4,5]. It is now well accepted as the major calcium-binding protein in nonmuscle cells. This uni que protein acts as an intracellular receptor for calcium ion, and its presence lends credance to the long-touted role of calcium ions as a "second messenger" analogous to cyclic adenosine monophosphate. Calmodulin is now generally believed that calmodulin is a major intracellular receptor of calcium, and that it mediates many of the calcium effects in eukaryotes[6,7,8]. Calmodulin, also called calcium-dependent regulator protein (CDR), mediates the activation by calcium ion of a variety of enzyme such as cyclic phosphodiesterase[2,9] brain adenyl cyclase[10,11]. erythrocyte calcium ion-magnesium ion dependent adenosine triphosphatase[12,13] myosin light chain kinase[14,15] and skeletal-muscle phosphorylase kinase[16,17]. The high affinity calcium ion binding properties of calmodulin in addition to its activating properties for many enzymes form the basis of the hypothesis that calmodulin is the link between intracellular calcium ions availability and the calcium dependent activation of these enzymes.

Calmodulin is a protein of 16,500 dalton. Its thermal stability in the presence of calcium ions and its acidic nature have been used in the purification procedures, which generally include successively ammonium sulfate fractionation, heat treatment, anion exchange chromatography and gel filtration[18, 19, 20, 21]. It is a highly coiled molecule that calmodulin has 148 amino acid residues and is characterised by the presence of trimethyllysine and the absence of cystein and tryptophan[22].

It is well established that calmodulin contains four calcium binding sites, with a dissociation constant within the range of 10^{-6} M. Upon calcium binding, calmodulin undergoes a conformational change which is necessary for its biological activity[23].

One of the potential functions of calmodulin in the regulation of intracellular calcium ions is to regulate calcium ion levels by altering the enzymatic system of cellular membranes.

In addition to binding a large proportion of the total calcium ion intracellularly released upon stimulation and to mediate the intracellular effects of calcium ion, calmodulin may act to decrease calcium ion level.

This action would constitute a self regulating mechanism for returning to a low steady state calcium concentration and terminiating the effects of calcium

* 이 논문은 1992년도 계명대학교 율종연구비 및 동산의료원 조사연구비로 이루어졌음.

ions.

Calmodulin has been reported to stimulate a plasma membrane calcium-adenosine triphosphatase which is coupled to an active calcium pumcytoplasmicp that extrudes calcium ion from the cytoplasmic compartment. Other mechanisms for restoring the low intracellular calcium ion level have been described, particularly a Na^+ : Ca^+ exchange. It would be important to know if calmodulin affects this exchange and what is the relative participation of the various mechanisms to calcium ions extrusion in various cell types. In the squid axon, the calcium pump seems predominant in the physiological int racellular calcium range[24,25,26,27].

In heart muscle cells, calmodulin stimulate calcium ion transport in sarcoplasmic reticulum as a consquence of the activation of a membrane bound protein kinase that phosphorylates phospholamban. It has been known for several years that phospolamban can also be phosporylated at a different site by a cyclic adenosine monophosphate-dependent protein kinase, and this phosporylation also increases calcium ion transport. Thus, in heart cells both calmodulin and cyclic adenosine monophosphate cooperate positively to decrease intracellular free calcium concentration. This mechanism may account for the reduction of cardiac systole which characterizes the effects of beta-adrenergic agents, together with a simultaneous increase in force of contraction. Cyclic adenosine monophosphate also seems to be implicated in the latter effect, since cyclic adenosine monophosphate-dependent phosphorylation of a plasma membrane protein may be responsible for the increase in calcium ion conductance that occurs under beta-adrenergic stimulation of heart cells. This would allow more calcium ions to penetrate the cells and to induce a larger release of calcium ions from sarcoplasmic reticulum, thereby increasing the force of contraction in the cardiac muscle cells[28,29,30,31].

The intracellular calcium ions may influence muscle contraction, relaxation and the enzymatic activities in cells and the mechanism controlling their levels may therefore be of considerable importance in the regulation of the cellular activity.

Author have therefore investigated enzymes involved with synthesis and degradation of cyclic adenosine monophosphate and cyclic guanosine monophosphate, i. e., nucleotide cyclase and nucleotide phospodiesterase.

Previous studies on the distribution and ontogenetic development of the above cyclic nucleotides and enzymatic activities have shown that enzymatic activities were evenly distributed and changed among the different part of subcellular fraction in ureters of normal adult guinea pig.

In this paper we have demonstrated the contents of calmodulin in the whole homogenate, 40,000 x g supernatant and particulate fraction in the cortical and medullary tissues of nephric organ of normal adult guinea pig in order to understand and the subcellular distribution of their properties in cellular levels.

MATERIALS AND METHODS

1. Materials:

The experimental animals were normal Hartley guinea pigs without sexual discrimination. Three years old animals of 995± 50 gram of live weight were used in this study. All animals were killed by decapitation and both kidney were removed immediately. Following removal of the surrounding capsules, connective tissues, fats, and blood vessels, the kidney were quickly frozen in liquid nitrogen.

The cortical and medullary tissues were separated from both kidney with the aid of ophthalmological scissors upon the placement of tissues on the vinyl surface of ice-cold vinyl bag and all of tissues were pooled into a cold ice-bag.

A adequate piece from the pooled tissues was weighted to aid the experimental samples.

A weighed amount of the medullary and cortical tissues were placed into the tissue extract solution containing 10 mM TES, 1 mM DTT and 1 mM EGTA solution (PH 7.4), homogenized with the aid of Polytron (Brinkman Inst.) into 10 percent (w/v)

homogenate using three pulses at speed 7 at 4°C and was subjected for a further subcellular fraction.

2. Separation and preparation of subcellular fraction:

The 10 percent whole homogenate as prepared as above was placed into centrifuge vinyl tube of 3.5 ml volume and centrifuged at 40,000 x g for 30 minutes at 0°C with the aid of Model L5-50 ultracentrifuge (Beckman Inst.) equipped with aluminium angle rotor and the subsequent supernatant fraction (soluble fraction) was collected into a vinyl tube of 5.0 ml capacity whereas the sediments was washed twice with the tissue extract solution (pH 7.4) and brought into a fraction of same concentration to the whole homogenate fraction with the aid of same solution to serve as particulate fraction (pellet fraction) throughout the trial.

All of the subcellular fractions are boiled at 100°C for 5 minutes. The heat-denatured protein was removed by centrifugation at 5,000 x g for 5 minutes after cooling the preparation to 4°C in ice-water. Prior to the determination of calmodulin contents in the subcellular fraction was properly diluted.

3. Assay for the calmodulin content:

The assay for calmodulin content in the tissue homogenate or subcellular fractions was based on the ability to activate the activity of calmodulin-deficent brain phosphodiesterase.

An appropriate dilutions were made from each of the heat-treated samples solutions and they were assay for their abilities to activate the calmodulin-deficient phosphodiesterase activity prepared from bovine brain.

The procedure was described by Wallace, Robert W. et al.[5]. and Kakiuchi, Shiro, et al[4].

The 10 μl of smple containing known or unknown quantities of calmodulin were added to a mixture containing 400 μM $CaCl_2$, 200 μM EGTA, 7 μCi[^{3}H]−cGMP, 100 μl reaction mixture (125 mM TES, 6 μM $MgCl_2$, BSA 16 mg/ml and 4 μM cGMP, pH 7.5) and 20 μl calmodulin-deficient phosphodiesterase containing 0.2 μg protein in a final volume 200 μl.

The concentration of calmodulin-deficient phosphodiesterase added was such that no more than 5-30% of the substrate cyclic [^{3}H]-GMP was hydrolyzed during the course of reaction. The reaction was initiated by the addition of the calmodulin-deficient phosphodiesterase solution.

The mixture was vortexed and the tubes were incubated at 30°C for 30 minutes. The conversion cyclic GMP to 5'-GMP by the calmodulin-deficient phosphodiesterase was stopped by the addition of 25 μl of a termination medium containing 50 mM EDTA, 5 mM cyclic GMP and 25 μl of snake venom (crotalus atrox, 10 mg/ml) is then added and the solution is further incubate for 10 minutes at 30°C to convert 5'-GMP to guanosine.

At end of incubation, 0.75 ml of 0.1 mM guanosine added. The each sample is applied to small column of QAE Sephadex A-25 (0.7 x 2.0 cm) which has been equilibrated with 20 mM ammonium formate (pH 7.4).

The samples are eluted with 3.5 ml of ammonium formate solution. The elute was dissolved in Aqueous Counting Scintillant Solution (ACS, Amersham Co.) and radioactivity of [^{3}H]-guanosine was determined in a liquid scintillation spectrometer (Beckman Co.). All assay were performed in triplate and a typical calmodulin assay protocol consists of standard curve of calmodulin in ranging 1.0 to 10.0 ng per 10 μl of calmodulin, tubes of blank containing everything except the calmodulin-deficient phosphodiesterase.

The results are expressed ng of calmodulin per mg of protein per 30 minutes of time.

The protein was determined by the method of Lowry et al.[45]. using bovine serum albumin as the standard.

4. Chemicals and reagents:

Ethyleneglycol-bis(beta-aminoethyl ether) N, N'-tetraacetic acid (EGTA), N-tris (hydroxylmethyl) methyl 1-2-aminethanesulfonate (TES); cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), DL-dithiothreitol (DTT), bovine serum albumin, theophylline, adenosine,

guanosine, sodium chloride, urea, sodium dodecylsulfate (SDS), potassium chloride, and crotalus atrox venom were purchased from Sigma chemical Co.. Cyclic [^{3}H]-adenosine-3', 5'-monophosphate (specific activity 33.5 Ci/mmol), and cyclic [^{3}H] guanosine-3', 5'-monophosphate (3.45 Ci/mmol) were puchased from New England Nuclear Co.. Isobutylmethylxanthine (MIX), unlabeled AMP, unlabeled GMP, ethylendiaminotetraacetate (EDTA), trifluoperazine (TEP), calmodulin-deficient phosphodiesterase enzyme were obtained from Sigam Chemical Co..

QAE Sephadex A-25 (Pharmacia fine Chemicals) was equilibrated with the column buffer prio to use. All other reagents were of the best available grade from commercial sources.

RESULTS

1. Subcellular localization of the calmodulin content in the nephric cortical and medullary tissues of normal adult guinea pig.

The concentration of calmodulin contained sub cellular fractions was determined by differentiation of the whole homogenate into the supernatant fraction (soluble fraction) and particulate fraction (pellets fraction) with the aid of ultracentrifugation by the Model L5-50 ultracentrifuge and results were compiled as in Table I. The concentration of calmodulin in subcellular fraction was expressed as a specific concentration, ng of calmodulin per mg of protein per 30 minutes.

The content of calmodulin of the whole nephric tissues is divided into 6.1 percent for medullary tissues and about 93.9 percent for cortical tissues in the present trial.

The comparison of the amount of calmodulin between the medullary and cortical tissues reveals a ratio of about 1 : 15.

As can be seen in Table I. the specific concentration of both medullary and cortical tissues were localized predominantly in the supernatant fraction. The small amount of remaining calmodulin was found in the particulate fraction.

However, in the present experiment the fractional calmodulin was compared on the base of 12.6 ng of calmodulin per mg of protein found in the whole homogenate of the medullary tissues as 100 percent; the supernatant fraction was found to be the 11.5 ng of calmodulin as 91.6 percent within the given time, 1.06 ng of calmodulin per mg of protein for the particulate fraction as 8.40 percent.

In the case of cortical tissues the amount of calmodulin was compared on the base of 194.17 ng of calmodulin per mg of protein found in the whole homogenate preparation as 100 percent; the supernatant fraction was found to contain the amount in 190.0 ng of calmodulin per mg of protein 97.8 percent, 4.17 ng of calmodulin per mg of protein for the particulate fraction as 2.2 percent. The subcellular concentration of calmodulin in the both medullary and cortical tissues were mainly localized to be the supernatant fraction.

Expressed otherwise, the intracellular distribution of calmodulin in the supernatant fraction was almost 90 percent more than that of particulate fraction in the both medullary cortical tissues.

2. The effect of heat on the calmodulin level in supernatant fraction of the renal medullary tissues and cortical tissues.

As can be seen in Figure I and Figure 2, in the present trial, the calmodulin levels of supernatant fraction in the medullary tissues was raised by the heat-treatment versus the dilution factors of samples.

Therefore, the effect of heat-treatment on the calmodulin levels in supernatant fraction of the renal cortical tissues as observed in the present trial was compiled in Figure 3 and Figure 4.

When the effect of heat-treatment versus the dilution factor of samples on the concentration of calmodulin is compared in the cortical and medullary tissues, they are similar; 3, 4 folds at the dilution factors of 50 times for the medullary tissues. Similar was the case with the dilution factors of 25 times or 100 times; no change in the increa-

Table 1. Subcellular Distribution of Calmodulin in the Supernatant and and Particulate Fraction of the Nephric Medullary and Cortical Tissues of Normal Adult Guinea Pig

The reaction mixture contained 400micromoles of $CaCl_2$, 200 micro moles of EGTA, 7 microCi of [^{3}H]-cGMP, 100 micromoles of reaction mixture buffer, and 20 micromoles of calmodulin-deficient phosphodiesterase solution in a final volume of 200 microliter at 30°C for 30 minutes in water bath

The reaction was stopped by the addition of 25μl of a termination medium containing 50 mM EDTA, 5 mM cGMP, and 25μl of snake venom (crotalus atrox, 10mg/ml) and the solution is further incubated for 10 minutes at 30°C. At end of incubation, 075 ml of 0.1 mM guanosine added. All of samples were applied on OAE Sephadex (A-25, 0.7 x 20 cm) column. The elutes were dissolved in Aqueous Counting Scintillant Solution and radioactivity of [^{3}H]-guanosine were determined in a liquid scintillation spectrometer. The result were expressed by the ng of clamodulin per mg of protein per 30 minutes of times. Each value was expressed from the pooled samples of 7 renal medullary and cortical tissues in normal adult guinea pig

Fraction	MEDULLA		CORTEX	
Sample number	soluble fraction	particulate fraction	soluble fraction	particulate fraction
	ng of calmodulin x 100/mg of protein			
1.	11.21	1.42	192.14	4.26
2.	10.98	0.92	182.12	3.96
3.	12.86	1.66	188.27	4.45
4.	11.45	1.68	190.96	4.26
5.	10.78	0.72	189.42	3.87
6.	11.24	0.86	190.98	4.11
7.	12.11	0.77	192.76	4.40
8.	11.91	0.98	188.41	3.98
9.	10.99	1.01	189.92	4.09
10.	11.49	0.99	190.46	4.11
11.	12.01	0.97	193.04	4.29
12.	11.76	0.96	191.77	4.22
Ranges	10.78-12.86	0.72-1.68	182.12-193.04	3.96-4.45
Means	11.557	1.063	190.001	4.167
Standard errors	± 0.599	± 0.334	± 2.941	± 0,178
t-test	score: 19,29 p<0.001	3.183 0.001<p<0.005	64.600 p<0.001	23.41 p<0.001

sement of ratio of the calmodulin levels was registered with the dilution factors of supernatant fraction.

The results of stastical analysis are presented in Figure 2 and 4 even though its statistical anlysis demonstrated a very high significance.

3. The effect of heat exposure time on the supernatant-calmodulin of different organs

There are a number of ways to the exposure time of heat-treatment on the supernatant fluid in different organs of normal adult guinea pig.

The major objective is to bring the supernatant fluid up to the exposure time of heat-treatment(30 minutes) and down (30 seconds). One approach is to 0.1 ml of supernatant fluid in different organs and immerse it in a boiling water-bath for the exposure time of heat-treatment as indicated. At specific time during the heat exposure time at 100°C. The agglutinated suspension can then be directly clarified by centrifugation at 5,000 x g for 5 minutes.

The lower represents the case for cardiac calmodulin, medullary-calmodulin, and cortical-calmodulin together with individual range whereas the

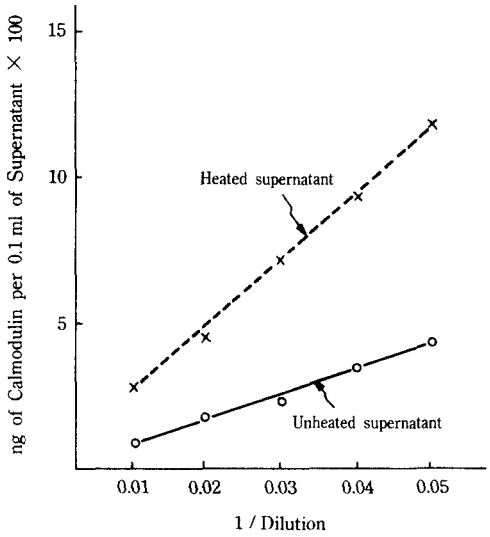

Fig. 1. The Effect of Heat on Calmodulin Level of Supernatant in the Renal Medullary Tissues of Normal Adult Guinea Pig.
The incubation condition and reaction mixture were as described in Table I except that varying dilution factor of calmodulin contents were added to the reaction mixture as indicated. Each point represents the mean of 15 experiments.

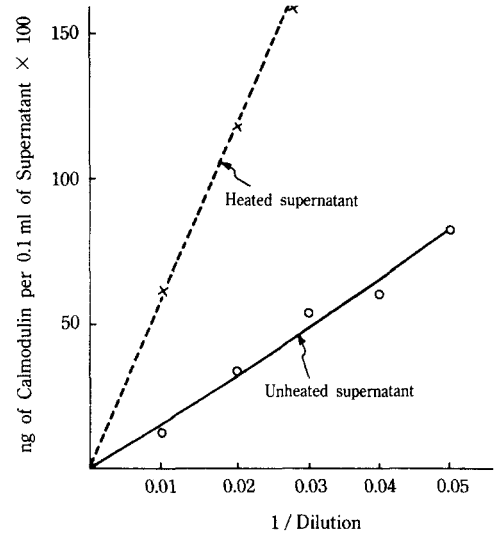

Fig. 3. The Activatory Effect of Heat on Concentration of Calmodulin in the Supernatant Fraction of the Nephric Cortical Tissues of Normal Adult guinea Pig.
The reaction mixture were the same as described in Table I. Each point represents the awerage value of 15 experiments.

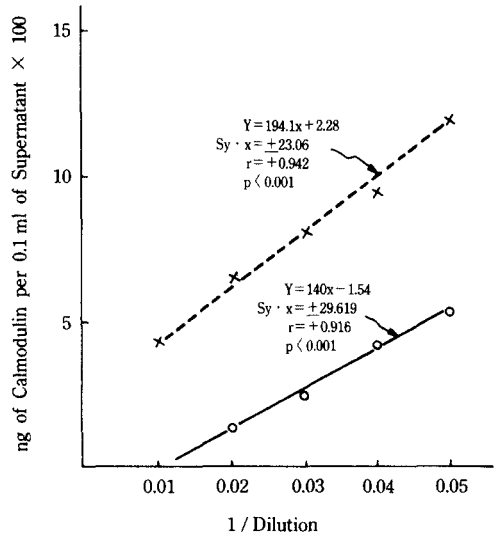

Fig. 2. Regression Equation of the Nephric Calmodulin Content of Supernatant Between Heated Treatment and Unheated Treatment in Medullary Tissues.
×---× ; heated supernatnat
o—o ; unheated supernatant

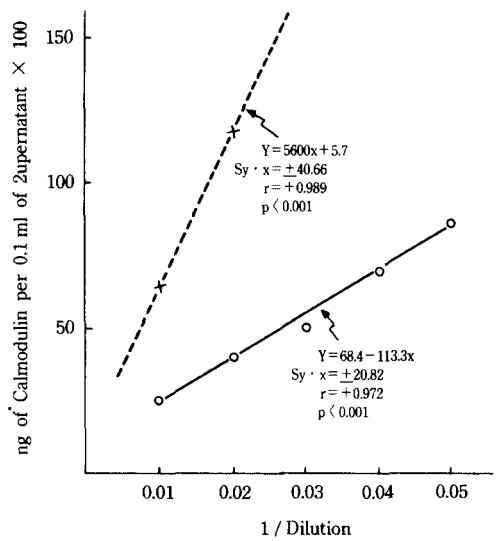

Fig. 4. Regression Equation of the Nephric Calmodulin Content of Supernatant in Cortical Tissues Between Dilution Factors and concentration of Calmodulin.
The reaction mixture were as described in Table I. Each point represents the mean of 15 experiments.
×---× ; heated supernatant
o—o ; unheated supernatant

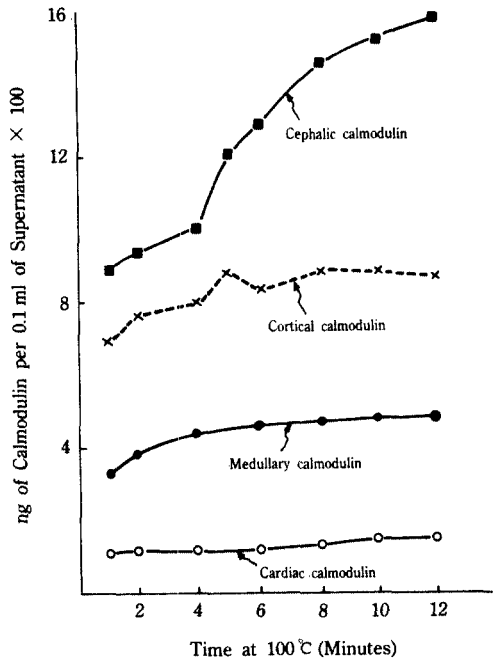

Fig. 5. The Effect of Heat Exposure Time on the supernatant Calmodulin of Different Tissues of Normal Adult Guinea Pig.
The experimental details were as described in Tabl I. Each value is the average value of 15 experiments.

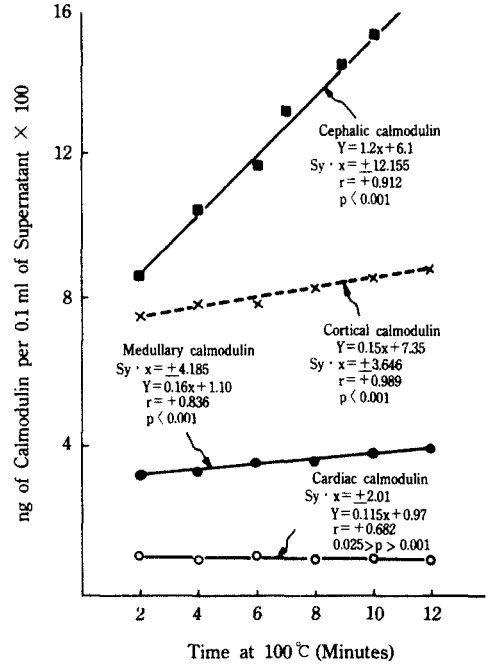

Fig. 6. Regression Equation of Different Calmodulin Content of Supernatant in Different Tissues Between Heating ?Time and Concentration of Calmodulin.
The reaction mixture were as described in Table I. Each point represents the mean of 15 experiments.

upper line represents the case of cephalic calmodulin in normal adult guinea pig as is seen in Figure 5 and Figure 6.

As can be seen in Figure 5, the levels of calmodulin in the cortical and medullary tissues were increased in function of the heat exposure time until 5 minutes whereas that of cardiac calmodulin was not affected by the increase in the heat exposure times. By contrast the levels of cephalic calmodulin was increased by the increase of the heat exposure time to the supernatant fluid as can be seen in the upper line of Figure 5 and Figure 6.

4. The effect of salt on the calmodulin assay in cortical medullary tissues

The authentic calmodulin was assayed in the presence of the concentration of NaCl indicated in the Figure 7 and 8.

Since NaCl or KCl was routinely included in tissues preparation, the effect of carryover of salt into the calmodulin assay was examined. Figure 7 and shows a results with NaCl, and an identical result was obtained with KCl. The results shows that, under the conditions of the present study, carryover of salt into the assay mixture of the lesser than 150 mM did not interfere with the assay, although higher concentration of salt were inhibitory in the medullary-calmodulin.

However, when the addition of NaCl became 300 mM at final concentration, the calmodulin assay was not inhibited by more than 99.9 percent in the case of cortical-calmodulin.

By contrast calmodulin assay in the case of cortical-calmodulin was affected by the increase of concentration of the addition of salt solution to the mixture as can be seen in Figure 7.

An inhibition of 65 percent was noted in the case of medullary-calmodulin for the calmodulin assay with a 50 percent (I_{50}) inhibition at 300 mM of NaCl, and the corresponding 50 percent (I_{50}) inhi-

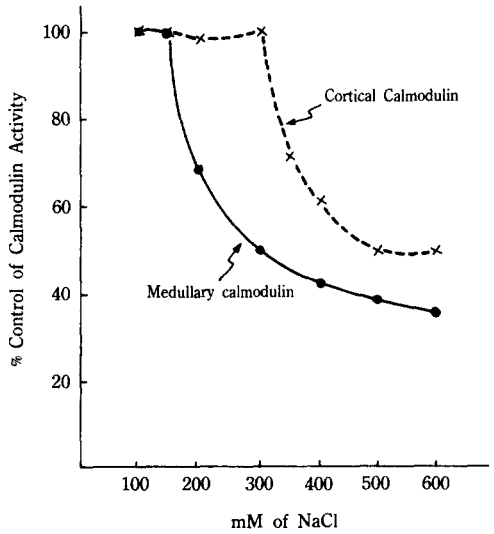

Fig. 7. The Effect of Salt on Calmodulin Assay in Supernatant Fraction of the Nephric Medullary and Cortical Tissues of Normal Adult Guinea Pig.
The authentic calmodulin was assayed in the presence of the concentration of NaCl indicated in the figure. Each point presents the means of 15 experiments.

bition was found to be 500 mM of NaCl for the calmodulin assay of the cortical tissues.

5. The effect of additives on the calmodulin assay in supernatant fraction of cortical and medullary tissues

Since it was found that salt solution does inhibit the calmodulin assay the present experiment was directed to find out the inhibitory effect on this calmodulin assay by other organic compounds and the experimental results were compiled in Table II in the expression of its relative activity.

The inhibitory effect of NaCl or KCl at a concentration of 350 mM ON the calmodulin levels was found to be about 55 percent in the case of medullary tissues, but the addition at the same concentration of NaCl or KCl resulted in 93 percent inhibition of the case of cortical tissues.

The addition of 20 mM EGTA, known to be a chelating agents, resulted in an inhibition of about 60 percent in the case of medullary tissues and about 95 percent in the case of cortical tissues whereas the addition of 200 mM EDTA, an inhibition of about 65 percent for the case of medullary tissues and about 97 percent for the case of cortical tissues.

The addition of 6 M urea, a substance knows to disrupt the hydrogen bonding of enzyme apoprotein and inhibit the enzymatic activity, to the react did not interfere to the calmodulin levels of both medullary and cortical tissues.

In particular, the addition of 100 mM SDS, the compound of the decreasement of surface tension, did not affect the calmodulin levels in both medullary and cortical tissues in the present trial.

DISCUSSIONS

Originally described as a cyclic nucleotide phosphodiesterase activator the calcium binding protein, calmodulin, has since been shown to be an important intracellular regulatory factor. In the past few years, a number of excellent review articles have appeared in which the structure, function, mechansim of action and distribution of calmodulin have been described[2,4,5].

In order to determine the intracellular distribution of calmodulin levels in the medullary and cortical tissues of normal adult guinea pig, 10 percent whole homogenate was centrifuged at 40,000 x g for 30 minutes into the supernatant fraction and particulate fraction and the levels of calmodulin in each fraction were compiled as in Table I.

The subcellular localization of tissue calmodulin of the both medullary and cortical tissues in normal adult guinea pig were almostly 91.6 percent for the case of medullary tissues and about 97.8 percent for the case of cortical tissues in the present experiment. There are a small amount of bounded calmodulin in the presence of particulate fraction within about 8.4 percent for the medullary tissues and about 2.1 percent for the cortical tissues.

According to the report of Giebert, C. White II. et al[32] in 1981, the calmodulin levels of platelets to indicate that platelets function may be regulated by alteration in cytoplasmic concentration of calcium ions, calmodulin levels was localized and compared between the calmodulin levels of intracellular cy-

toplasm and aggregation of blood coagulation. The distribution of calmodulin in resting platelets was examined by subcellular fractionation. Gel filtered platelets were frozen and thawed three times, sonicated, and separated into soluble and particulate fractions by centrifugation. The result show that 84 percent of calmodulin activity in the homogenate was present in the soluble, and 14 percent in the particulate fraction of platelets cells. The specific activity of calmodulin in the soluble fraction was 1520 units per mg of protein while that in the particulate fraction was 320 units per mg of protein.

Studies with bovine brain have suggested that portion of soluble calmodulin may be translocated to a particulate distribution in the presence of calcium ions[33,34,35].

To examine this possibility in platelets, platelets were recalcified to a final concentration of 2 mM and homogenized and fractionated. There are significant shift in protein from the particulate (decreased from 4.19 mg/ml to 2.53 mg/ml) to the soluble (increased from 0.94 mg/ml to 1.31 mg/ml) fraction so that the specific activity of the calmodulin in the particulate fraction increased (320 μ/mg of protein to 380 μ/mg) and that in the soluble fraction decreased (1520 μ/mg to 1130 μ/mg). Nevertheless, the distribution of calmodulin between soluble and particulate fraction was essentially unchanged under the condition used.

The levels of calmodulin in the supernatant fraction of the cortical and medullary tissues in the

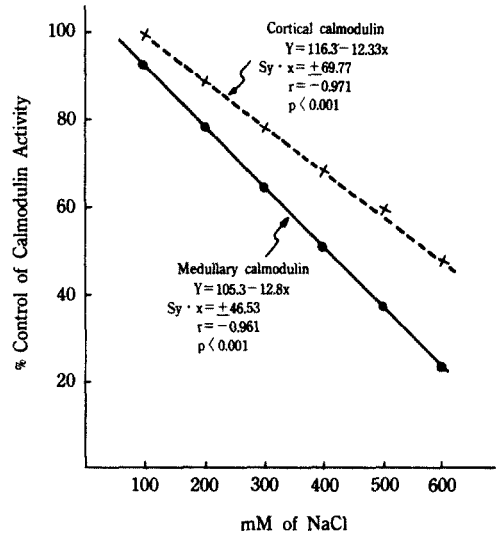

Fig. 8. Regression Equation of Calmodulin Assay in Supernatant Fraction of the Nephric Medullary and Cortical Tissue of NOrmal Adult Guinea Pig Between Concentration of Sodium Chloride and Calmodulin Assay. The experimental details were as described in Table I. Each point represents the mean of 15 experiments.

Table 2. Effect of Various Additives on the Calmodulin Assay in Supernatant Fraction of Renal Medullary and Cortical Tissues of Normal Adult Guinea Pig
The reaction mixture were as described in Table 1.
Each value represents the mean± S. E. of 17 experiments

Additives	MEDULLA		CORTEX	
	ng of calmodulin /mg of protein	Relative activity(%)	ng of calmodulin /mg of protein	Relative activity(%)
None	1,121.0± 59	100.0	19,214.0± 294	100.0
NaCl, 350 mM	516.8± 45	45.6	1,391± 191	7.2
KCl, 350 mM	532.8± 40	47.5	1,289.4± 176	6.7
EGTA, 20mM *	472.9± 31	42.2	972.6± 91	5.1
EDTA, 200mM *	396.4± 26	35.4	601.9± 82	3.1
TFP, 10 mM *	227.5± 25	20.3	279.3± 19	1.5
Urea, 6 M	1,123.0± 59	100.0	19,212.0±	99.9
SDS, 100mM *	1,118± 57	99.7	19,216± 298	100.0

* EGTA: Ethyleneglycol-bis(beta-aminoethyl ether) N,N'-tetracetate
EDTA: Ethylenediaminotetraacetic acid
TFP : Trifluoperazine
SDS : Sodium dodecylsulfate

present experiement are 21.4-folds higher than those reported by Giebert, C. White II. Altthough this difference is unexplained and have to study in greater detail with respect to their calmodulin content and function.

A large portion of the calmodulin activity in the medullary and cortical tissues of nephric organ of normal adult guinea pig was found to be the supernatant fraction.

Others have reported that the binding of metal ions to calmodulin depends on the salt concentration[36,37]. Thus altering the salt concentration results in some magnesium binding.

Since NaCl or KCl was routinely included in tissue preparation, the effect of the concentration of salt on the calmodulin assay was examined. The effect of the differential concentration of salt solution on the calmodulin levels in supernatant fraction of both medullary and cortical tissues were compiled as in Table II, Figure 7 and Figure 8.

As can be seen in Table II, Figure 7, and Figure 8, the activity of calmodulin was inhibited in function of NaCl concentration whereas that of supernatant-calmodulin of cortical tissues was almostly resistered by the same concentration of salt solution more than the supernatant-calmodulin in medullary tissues.

For each of the tissue fractionated sample, a plot of sample concentration and the degree of inhibition of calmodulin levels by the differential concentration of salt solution were prepared. From this curve, the I_{50} value which means the concentration of the salt solution for 50 percent inhibition was calculated. Fifty percent inhibitory concentration of salt solution on calmodulin levels of the supernatant fraction in medullary tissues was found to be 300 mM and 500 mM for the calmodulin levels in cortical tissues.

Calmodulin is a highly coiled molecules. In the absence of metal, 40 percent of the protein exists in an alpha-helical configuration. Calcium binding to calmodulin increases the helicity to greater than 50 percent. This change in conformation is mandatory for the regulation of all calmodulin-modulated enzyme molecules described so far. Also the regulation of an enzyme by calmodulin can be affected in two ways.

One way is to increase the calcium concentration in the presence of a fixed amount of calmodulin. The second method is to increase the calmodulin concentration in the presence of a fixed amount of calcium. As the calmodulin concentration is increase, the amount of calcium required to stimulate a reaction is markedly reduced[38,39,40,41].

The t-test for the probability in the effect of salt solution on the calmodulin levels in the supernatant fraction of both cortical and medullary tissues was found to be $p<0.001$ or very highly significant in statistical terminolgy.

The effect of the increase in the dilution factors of supernatant fluid of both medullary and cortical tissues upon the calmodulin levels after the heat-treatment at 100°C for 5 minutes could be increased about 3.51-folds for the calmodulin levels of cortical tissues at 25 diluted supernatant-calmodulin and that of the medullary-calmodulin by 2.65-folds at same dilution factors.

Upon the heat-treatment on supernatant-calmodulin was highly noted in the case of cortical-calmodulin that that of medullary-calmodulin.

It was generally noted that calmodulin levels in supernatant fraction of nephric organ was stablized on the heat-treatment.

In order to better present the relationship of the heat-treatment between calmodulin levels of supernatant fraction and the dilution factors of samples were plotted and summarized in Figure 2, and Figure 4.

To be noted in passing is that the regression coefficient for the case of medullary-calmodulin was found to be $r=+0.942$ and that of cortical-calmodulin by $r=0.989$.

It was observed in this experiment that the degree of calmodulin levels in supernatant fraction of cortical tissues due to the heat-treatment was highly activated than in the medullary tissues, suggesting that the structure of calmodulin protein and its properties of the configuration of calcium binding sites found in both tissues were slightly differed

in the mode of membrane-bounded form in the protein layer.

Means, AR, and Dedman, JR[42]. in 1980 reported that multiple role for calmodulin are suggested by its multiple localization in all tissues, best illustrated in the rat cerebellum. Calmodulin was concentrated in Purkinje and glial cell bodies where it was predominatly associated with free and bound ribosome, the nuclear membranes, coated membranes of smooth and rough endoplasmic reticulum, mitochondrial and plasma membranes. Calmodulin was also found in the neuronal dendrites of the Purkinje celles, in which it coated the surface of smooth endoplasmic reticulum, small vesicle, and inner and outer mitochondrial membrane[43].

The present experiment was conceived and carried out to determine the calmodulin levels within differential organ i. e., heart, kidney, and brain tissues in the one hand and the effect of the heat exposure time on this calmodulin activity in supernatant fraction and the results were figurized as in Figure 5, and Figure 6.

As can be seen in Figure 5, all of the supernatant-calmodulin in kidney and brain tissues did affected by the heat-treatment until 5 minutes whilst that of cardiac calmodulin did not influenced by the increase of heat exposure time.

The activatory effect of the heat exposure time at a time of 4 minutes on the supernatant fraction of brain tissues was found to be about 721 ng of calmodulin per mg of protein but the increase at 10 minutes of heat exposure time was resulted in 1920 ng of calmodulin per mg of protein. The increase of heat exposure time on cephalic calmodulin was predominantly raised by 2.660folds.

It was found in the present experiment in which the heat exposure time for the activation of supernatant calmodulin was needed by about 5 minutes, but the case of cardiac calmodulin in supernatant fraction did not affected by the degree of the heat exposure time as can be seen in Figure 5.

The fact was further confirmed by the findings that the changes in added the differential chemical compounds did affected by the additives. Compiled in Table II are the result of the determination of supernatant-calmodulin levels due to the effect of the various concentration of additives.

Green, S, and Dobrijansky, A[44]. in 1971 reported that 6 M urea reversibly or irreversibly inactivated nicotinamide adenine dinucleotide glycohydrolase from mammalian liver with the dissociation of NAD-glycohydrolase into inactive subunits. Many enzymes are known to be inacivated by the rupture of the hydrogen bondings of the enzymes proteins in the high concentration of urea, which some enzyme are reported to be activated since urea removed inhibitors, if any, from the enzyme-inhibitor complex[45,46,47,48,49,50].

Accordingly, the effect of urea on the concentration of calmodulin in supernatant fraction of both medullary and cortical tissue was investigated that addition of 6 M urea to the reaction mixture of calmodulin assay was not changed.

Also, the effect of 100 mM sodium dodecylsulfate on the above calmodulin levels in both medullary and cortical tissues was not affected by the substance of the related surface tension.

The comparison of the structural configuration of calmodulin protein in supernatant fraction between the medullary and cortical tissues was differed due to differing only in the hydrogen bondings of the each protein conformation.

Therefore, in order to further detail the inhibitory effect of 20 mM EGTA, 200 mM EDTA, and 10 mM trifluoperazine on the calmodulin levels in supernatant fraction of both medullary and cortical tissues were schematically summerized in Table II.

Certain authors[51,52] reported the inhibition of brain cyclic nucleotide phosphodiesterase by phenothiazine neuroleptics, found that trifluoperazine and related phenothiazine derivatives are potent inhibitor of the calcium sensitive phosphodiesterase, at a concentration which has no influence on the basal activity of the enzyme in the absence of calcium. they subsequently demonstrated that these drugs bind to calmodulin in its calcium-activated form and inhibit its biological properties.

The antipsychotic effect of neuroleptics is unrelated

to the inhibition of calmodulin activity, but trifluoperazine and other inhibitors of calmodulin activity, have been used to inhibit and identify the role of calmodulin-regulated reaction in various cells. Calmodulin binds 2 moles of trifluoperazine with a dissociation constant of 10^{-6} M; upon binding trifluo-perazine, calmodulin become biologically inactive.

The inhibitory effect is to be interpreted as can increased formation of calmodulin-inhibitor(TEP) complex which the calmodulin can not bind which would an increasing disruption the calmodulin-calcium complex by the combination of inhibitor at site of calcium bindings in calmodulin molecules.

These experimental findings, therefore, indicate that the calmodulin of both medullary and cortical tissues are located on the same nephric organ, but its properties and characteristics are remarkedly differed from those for the degree of its concentration in subcellular space and author can be concluded that the calmodulin of both medullary and cortical tissues have to discussed with regard to the nephric functions.

It will be intersting to see in future studies how and why what mechansim calmodulin is bound to the inhibitor and what is the biochemical significance of biomodal distribution of calmodulin in both the supernatant and particulate fraction of the cell.
The present results for the determination of calmodulin in the supernatant and particulate fraction represents a step toward such work of the medical aspects in future.

Summary

In order to determine the subcellular localization of the calmodulin activity (ng of calmodulin per mg of protein) of the supernatant fraction and particulate fraction in nephric organ of normal adult guinea pig by a differential centrifugal method was carried out and the effect of various inhibitors together with certain other organic compounds on this calmodulin levels was determined and the following conclusions were obtained:

The calmodulin levels of supernatant fraction in medullary tissues of normal adult guinea pig was found to be 1,155.7± 59.9 in comparison to that of 106.3± 33.4 for particulate fraction.

The concentration of calmodulin of supernatant fraction in cortical tissues of nephric organ of normal adult guinea pig was determined to be 19,000± 294.1 whilest that of particulate fraction to be about 416.7± 17.8.

The effect of heat-treatment versus the dilution factors between the medullary tissues and cortical tissues was proportionally activated due to the increase of dilution factors, and the activatory effect of heat-treatment on the calmodulin levels of supernatant fraction in cortical tissues was 1.44-folds higher than that of medullary tissues.

The activatory effect of heat-treatment on the calmodulin levels in supernatant fraction was very highly significant in terms of statistics ($p<0.001$).

The duration of the heat exposure time on the calmodulin levels in supernatant fraction of both medullary and cortical tissues was found to be 5 minutes.

Fifty percent inhibitory concentration (I_{50}) of salt solution on the calmodulin assay in supernatant fraction of medullary tissues was found to be about 300 mM in comparison to that of 500 mM for cortical tissues.

The inhibitory effect of salt solution on calmodulin assay in supernatant fraction of medullary tissues was essentially inhibited 1.67-fold higher than that of cortical tissues at the same concentration of salt.

A stastically significant correlation coefficient of $r=-0.971$ was found between the concentration of salt solution added and the calmodulin assay with a probability of less than 0.001 and a regression equation (y) of 116.3-12.32x whilest a case of medullary tissues by that of $r=-0.961$, $P<0.001$, and $y=105.3-12.8x$.

No effect was exerted on the supernatant-calmodulin in both medullary and cortical tissues by the addition of urea and sodium dodecylsulfate.

The addition of trifluoperazine to the reactant at a concentration of 10 mM inhibited on the calmo-

dulin levels in supernatant fraction of cortical tissues by 98.5 percent whilst that of medullary tissues, by 79.7 percent.

These experimental findings, therefore, indicate that the calmodulin of both medullary and cortical tissues are located on the same nephric organ, but its properties and characteristics are remarkedly different from those for the degree of its concentration in subcellular space and author can be concluded that the calmodulin of both medullary and cortical tissues have to discuss with regard to the nephric functions.

REFERENCES

1. Blum, JJ, Hayes A, Jamieson GA, et al: Calmodulin confers calcium sensitivity on ciliary dunein ATPase. *J Cell Biol* 1980; 87: 386-388.
2. Cheung WY: Cyclic 3', 5'-nucleotide phosphodiesterase; Determination of an activator. *Biochem Biophys Res Commun* 1970; 38: 533-536.
3. Kakiuchi S, Yamazaki H: Properties of a heatstable phosphodiesterase activating factor isolated from brain extract. *Proc Jpn Acad Sci* 1970; 46: 587-588.
4. Kakiuchi S, Yasuda S, Yamazaki R, et al: Quantitative determination of caimodulin in the supernatant and particulate fraction of mammalian tissue. *J Biochem* 1982; 92: 1041.
5. Wallace RW, Tallant EA, cheung WY: Assay of calmodulin by Ca^{2+}-dependent phosphodiesterase. *Method Enzymol* 1983; 102: 39.
6. Autric FC, Ferraz MO, Kihoffer C, et al: Large-scale purification and characterization of calmodulin from ram testis; its metal-ion-dependent conformer. *Biochem Biophys Acta* 1980; 631: 139-141.
7. Jamieson GA Jr, Vanaman TC, Blun JJ: Presence of calmodulin in tetrahymena. *Proc Natl Sci USA* 1979; 76: 6471.
8. Wang JH, Waisman DM: Calmodulin and its role in the the second-messenger system. *Curr Top Cell Reg* 1979; 15: 47.
9. Kakiuchi S, Yamazaki R: Calcium dependent phosphodiesterase activity and its activating factor (PAF) from brain. *Biochem Biophys Res Commun* 1970; 41: 1104.
10. Brostrom CO, Huang YC, Breckenridge B Mcl, et al: Identification of calcium-binding protein as a calcium-dependent regulator of brain adenyl cyclase. *Proc Natl Acad Sci USA* 1975; 72: 64-67.
11. Cheung WY, Bradham LS, Lynch TJ, et al: Protein activation of cyclic 3', 5'-nucleotide phosphodiesterase of bovine or rat brain also activates its adenyl cyclase. *Biochem Biophys Res Commun* 1975; 66: 1055-1059.
12. Giopinath RM, Vincenzi FF: Phosphodiesterase protein activator mimics red blood cell on cytoplasmic activator of Ca^{2+}-ATPase. *Biochem Biophys Res Commun* 1977; 77: 1203-1206.
13. Jarrett HW, Penniston JT: Partial purification of the Ca^{2+}-Mg^{2+}-ATPase activator from human erythrocytes: its similarity to the activator of 3', 5'-cyclic mucleotide phosphodiesterase. *Biochem Biophys Res Commun* 1977; 77: 1210.
14. Beale EG, Declman JR, Means AR: Isolation and Regulation of the protein kinase inhibitor and the cyclic nucleotide phosphodiesterase regulator in the sertoli cell-enriched testis. *Endorinology* 1977; 101: 1621-1623.
15. Dabrowska R, Sherry JMF, Aromatorio DK, et al: Modulator protein as a component of the myosin light chain kinase from chicken gizzard. *Biochemistry* 1978 17: 253-254.
16. Cohen P, Buohell A, Foulkes JG, et al: Idendification of the Ca^{2+}-oependent modulation protein as the four the subunit of rabbit skeletal muscle phosphorylase kinase. *FEBS Letter* 1975; 92: 287.
17. Cohen PP, Cohen T, Shenolikar S, et al: The role of calmodulin in the structure and regulation of phosphorylase kinase from rabbit skeletal muuscle. *Eur J Biochem* 1979; 100: 329-331.
18. Childers SR, Siegel FL: Isolation and purification of calcium-binding protein from eletroplax of lelectro-phorus eletricus. *Biocheim Biophys Acta* 1975 405: 99-101.
19. Cook WJ, Deman Jr, Means AR, et al: Crystal-lization and preliminary x-ray investigation of calmodulin, *J Biol Chem* 1980 255: 8152-8153.
20. Forrester IT, Bradley MD: Identification of calmodulin-like activity in human seminal plasma. *Biochem Biophy Res Commun* 1980; 92: 994-995.
21. Garbers PL, Hansbrough JR, Radany EW, et al: Purification and characterization of calmodulin from sea urchin spermatozoa. *J Reprod Fertil*

1980; 59: 377.
22. Grand RJ, Shenolikar AS, Cohen P: The amino acid sequence of the delta-subunit (calmodulin) of rabbit skeletal muscle phosphorylase kinase. *Eur J Biochem* 1981; 113: 359-360.
23. Kretsinger RH: Structure and evolution of calmodulin-modulated protein. *Crit Rev Biochem* 1980; 8: 119.
24. Bond GH, Clough DL: A soluble protein activator of mg-Ca^{2+}-dependent ATPase in human red cell membranes. *Biochim Biophys Acta* 1973; 323: 592-594.
25. Brown EM: Calcium-regulated phosphodiesterase in bovine parathyroid cell. *Endocrinology* 1979; 107: 1988.
26. Hinds TR, Larsen FL, Vincenzi FF: Plasma membrane Ca^{2+}-transport: Stimulation by soluble protein. *Biochem Biophys Res Commun* 1978; 81: 455-456.
27. Pershadsingh HA, Medaniel ML, Landt M, et al: Ca^{2+} activated ATPase and ATP-dependent calmodulin-stimulated Ca^{2+} transport in islet cell plasma membrane. *Nature* 1980; 288: 492.
28. Brostrom CO, Brostrom MA, Wolff DJ: Calcium-dependent adenyl cyclase from rat cerebral cortex. *J Biol Chem* 1977; 252: 5677-5679.
29. Dieter P, Marme D: Calmodulin activation of plant microsomal Ca^{2+} uptake. *Proc Natl Acad Sci USA* 1980; 12: 7311.
30. Egrie JC, Nagle SW: Ave the sea urchin mitotic Ca^{2+}-ATPase and Ca^{2+}-sequenstering activities affected by calmodulin. *Ann NY Acad Sci* 1980; 356: 376-377.
31. Lopaschuk G, Richter B, Katz S: Characterization of calmodulin: effect on calcium transport in cardiac microsomes enriched in sarcoplasmic reticulum. *Biochemistry* 1980; 19: 5603.
32. Giebert CW II, Steven NL, Alton NS: Platelet calmodulin-dependent protein; Identification and localization of the calcium-dependent regulator, calmodulin, in platelets. *Am J Hemat* 1981; 10: 358-359.
33. Conn PM, Marian J, McMillian M: Evidence for calcium mediation of gonadotoropin releasing hormone action in the pituitary. *Cell Calcium* 1980 1: 7-8.
34. Conn PM, Chafouleas JG, Roger D, et al: Gonadotropin releasing hormone stimulates calmodulin redistribution in the rat pituitary. *Nature* 1981 292: 264.
35. Teshima Y, Kakiuchi S: Membrane-boun forms of Ca^{2+}-dependent protein modulation: Ca^{2+}-dependent and independent binding of modulator protein to the particulate fraction from brain. *J Cyclic Nucl Res* 1978; 4: 219.
36. Klee CB: Conformational transition accompanying the binding of Ca^{2+} to the protein activator of 3', 5'-cyclic adenine monophosphate phosphodiesterase. *Biochemistry* 1977; 16: 1017.
37. Klee CB, Crouch TH, Richman PG: Calmodulin. *Ann Rev Biochem* 1980; 49: 489.
38. Deman JR, Potter JD, Jackson RL, et al: Physiochemical properties of rat testis Ca^{2+}-dependent regulator protein of cyclic nucleotide phosphodiesterase; relationship of Ca^{2+}-binding, conformational changes and phosphodiesterase activity. *J Biol Chem* 1977 252: 8415-8416.
39. Deman JR, Jackson RL, Schreiber WE: Sequence homolgy of the Ca^{2+}-dependent regulator of cyclic nucleotide phosphodiesterase from rat testis with other Ca^{2+} binding protein. *J Biol Chem* 1978; 253: 343-344.
40. Epel DE, Wallace RW, Cheung Y: Calmodulin activates NAD kinase of sera urchin eggs: an early event of fertilization. *Cell* 1981 23: 543.
41. Gitelman SE, Witman GR: Purification of calmodulin from chlamydomonas. *J Biol Chem* 1980; 97: 764.
42. Means AR, Dedman JR: Calmodulin: an intracellular calcium receptor. *Nature* 1980; 285: 73.
43. Dedman JR, Lin T, Marcum JM, et al: CalmodulinL its role in the mitotic apparatus, In Siegel FL calcium binding proteins, Structure and function, 1980 181.
44. Green S, Dobrijansky A: Inactivation of NAD glycohydrolase from livers of different mammalian species by NAD. *Biochemistry* 1971; 10: 4533-4536.
45. Christensen, HN, Polmer GA: *In enzyme kinetics,* ed 1. WB saunder Co, Philadelphia, 1967, p. 68.
46. Kaplan NO, Everse J, Admiral J: Significance of substrate inhibition of dehydrogenase. *Ann N. Y. Acad Sci* 1968; 161: 400.
47. Kim YT: Studies on reticulocytes ribonuclease with special reference to inhibitor-free enzyme. *Chonnam Med J* 1967 6: 265.
48. Vales BL, Riordan JF: Chemical approaches to

the active site of eznyme. 1969; 38: 733-734.
49. Withycombe WA, Plummer DT, Wilkilson JH: Organ specificty and lactate dehydrogenase activity; Differential inhibition by urea and related compoun. *Biochemistry* 1965; 94: 384.
50. White A, Handler P, Smith EC: Kinetics of enzymic reaction, In *Principles of Biochemistry* ed 6. McGraw-Hill, 1973, p. 203.
51. Cheung WY: Calmosulin plays a pivotal role in cellular regulotion. *Science* 1980; 29-07: 19.
52. Claude Stoclet Jean: Calmodulin. *Biochem Pharm* 1981; 30: 1723-1724.

=국문초록=

정상 성숙 guinea pig 신장의 가용성 calmodulin에 대한 특징적 성상에 관한 실험적 연구

계명대학교 의과대학 마취과학교실

이정구 · 김진모 · 정정길

정상 성숙 guinea pig 신장으로부터 피질조직과 수질조직으로 분리하고 이로부터 분별원심침전법을 이용하여 가용성 분획과 침전분획을 분리하였다.

각 분획에 분포되어 있는 calmodulin농도에 대한 세포내 국소재를 정하고, 이 농도(ng of calmodulin per mg of protein)에 미치는 여러 가지 억제제와 유기물질의 영향을 관찰한 바 다음과 같은 결과를 얻었다.

정상 성숙 guinea pig 신장의 수질조직으로부터 분리한 가용성 분획에 존재하는 calmodulin 농도는 약 1,155.7± 59.9이었고, 침전 분획에서는 약 106.3± 33.4로 나타났다.

정상 성숙 guinea pig 신장의 피질조직으로부터 분리한 가용성 분획에 분포되어 있는 calmodulin 농도는 약 19,000± 294.1이었고, 침전 분획에는 약 416.7± 17.8로 나타났다.

수질조직과 피질조직내 존재하는 가용성 calmodulin은 회석배수에 대한 열 처리에 의해 비례적으로 활성화되었으며, 피질조직의 가용성 calmodulin에 대한 열 처리의 효과가 수질조직의 가용성 calmodulin농도에 비하여 약 1.44 배가 더 높았다.

가용성 calmodulin에 대한 열 처리의 활성화 효과는 통계학적으로 매우 높은 의의를 가지고 있었다.

피질조직과 수질조직에서 분리한 가용성 calmodulin에 대한 열 처리 시간에 대한 기간은 약 5분으로 나타났다.

수질조직의 가용성 calmodulin량 측정에 대한 염 용액의 50% 억제 농도는 약 300mM로 나타났으며, 피질조직의 경우는 약 500mM로 나타났다.

수질조직의 가용성 calmodulin량 측정에 미치는 염 용액의 억제적 효과는 동일한 염 농도에서 피질조직의 경우보다 약 1.67배가 더 심하였다.

염 용액 첨가 농도의 증가에 대한 수질조직의 가용성 calmodulin량 측정에 대한 통계학적 의의 중 상관계수(r)는 −0.971로서 상관관계의 유의성으로 나타났다.

또 t−검정은 P−값이 $P<0.001$이었고 회기방정식(y)는 $116.3-12.33\chi$로 나타났다.

그리고 피질조직의 경우는 $r=-0.981$, $P<0.001$과 회기방정식 (y)는 $105.3-12.8\chi$로 나타났다.

피질조직과 수질조직의 가용성 calmodulin에 대한 urea와 sodium dodecylsulfate의 첨가는 아무런 영향을 주지 아니하였다.

피질조직의 가용성 calmodulin에 미치는 10mM trifluoperazine의 첨가는 약 98.5%를 억제하였고, 수질조직의 경우는 약 79.9%을 억제하였다.

이상과 같은 실험성적으로 미루어 보면 신장의 피질조직과 수질조직에 존재하는 calmodulin은 세포내 국소재와 그의 특징적인 성상이 서로 달리하고 있으므로 신장의 피질조직과 수질조직에 존재하는 calmodulin은 신장의 기능과 깊은 관련이 있으리라 사료된다.