SPECTROSCOPIC STUDIES OF INHIBITION OF CALMODULIN ACTIVITY BY SOME DRUGS

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Abstract — The effect of four inhibitors on calmodulin (CaM) were studied by a fluorescence and ultraviolet techniques. four compounds [N-(6-aminohexyl) 5-chloro - 1 - napthalenesulphonamide] (W-7), 1 - [bis - (4 - chlorophenyl) methyl] - 3 - [2, 4-dichloro - β - (2,4 - dichlorobenzyloxyl) phenethyl] imidazolium chloride (R24571), trifluoperazine (TFP), thiodiphenylamide chloride (TDPAC) showed inhibitory effect on bovine brain phosphodiesterase (PDE) induced by CaM. The Ca^{2+} / CaM activity (IC_{50}) and the Hill coefficient were correlating closely between the methods, K_i's and thermodynamic parameters for these interactions were estimated.
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INTRODUCTION

Ca^{2+} ion, as a second messenger in several different cell processes plays an important role in cell function (1). Evidences suggest that proteins such as calmodulin (CaM) can act as Ca^{2+} receptor in such processes (2-5). Some Ca^{2+} ion functions include, Ca^{2+} ability to regulate tropomyosin - troponin contractile system. Vascular smooth muscle contraction is also CaM dependent phosphorylation of myosin light chain kinase (6). CaM is a protein with a molecular wieght of 16500 dalton, and is ubiquitos in all eukaryotic cells (7). Many drugs of CaM inhibitory action were studied among which, are Ca^{2+} chelating agents, (8), smooth muscle relaxant (9), local anesthetics (10) neuropeptides and proteins (11-13). Many of these drugs contain hydrophobic region and a positive charge at physiological pH (12,13), which appear crucial in producing a direct complexation with CaM, although some other CaM antagonists, also inhibit protein kinase Competitively with its phospholipid cofactor (14). The modes of Ca^{2+} bindings to CaM and the sites of Ca^{2+} bindings are well established (15). It is suggested that upon such bindings conformational changes occur with large incrases in α - helical content of the protein (16,17). As a result of such bindings, hydrophobic binding sites that are capable of bindings to other proteins or inhibitors are exposed (18,19). A mechanism for the modes of activation and inhibition is described by Meltzer and coworkers (20).

CaM inhibitors have important pharmacological significance in that they provide both an understanding towards the mechanism by which drugs alter CaM action and also to explore further the physiological role of the calcium binding protein, i.e. the antipsychotic action associated with phenotiazine drugs, or the compound R24571 (Calmidazolium) is known to have antimycotic action. Other characteristics such as hypotensive effect is also found with some CaM inhibitors. To investigate further the modes of action and the relative effect of some CaM inhibitory drugs, we studied the inhibitory effect of compounds such as TFP, W-7, TDPAC, R24571 by fluorescence and UV, using direct method and also by using CaM activation of bovine brain phosphodiesterase inhibition and aquired kinetic and thermodynamic data which could reveal some mechanistic aspect of drug action as well as insight into the nature of bindings.

MATERIALS AND METHODS

Calmodulin purified from bovine brain with a 95-97% purity according to the method described previously (15,21). The degree of its purity was measured on the basis of its phosphodiesterase activation properties and electrophoretic migration in polyacrylamide gels, in the presence of sodium dodecyl sulfate (SDS) (22). Bovine brain cyclic 3', 5' nucleotide phosphodiesterase was prepared according to the method of Wallace and coworkers (23). W-7, phenothiazines, snake venom and buffer reagents such as mops, and tris, were obtained from Sigma. Calmidazolium (R24571) was obtained from Seikagaku Kogyo Co. (Tokyo, Japan). All other reagents were of analytical grade or the highest purity available. CaM concentrations were determined by measuring its phosphodiesterase activation as described previously (24). Extinction coefficient for the purified CaM ($\varepsilon^{1\%}_{220}$ = 3.3) was also used for such assays. Protein concentration was determined by Lowry method using serum albumin as standard (25). SDS gel electrophoresis

was performed as described by Laemmeli (22). The fluorescence emission intensities were determined at 310 nm with the excitation wavelength of 277 nm and 230 nm with RF-5000 Shimadzu fluorescence spectrophotometer. The IC_{50} was determined, using seven to nine concentrations for each inhibitor. Each point represents the mean value of 5 replicates.

RESULTS

The extent of activation of CaM by phosphodiesterase is estimated to be 7-13 folds, in the absence of inhibitory drugs. The effect of the drugs on the activation of bovine brain CaM - PDE induced by CaM is shown in (Table 1). The percent increase in fluoroscence intensity (F/Fo) of CaM in its reaction with each inhibitor were studied at excitation wavelength of 277 nm and the emission of 310 nm. It is noteworthy to say that the PDE - CaM activity as measured by UV, correlated closely with the results obtained by fluorescence of the direct interaction between CaM and the inhibitor drugs. The fluorescence intensity appeared to be enhanced by the inhibitors that had more affinity for CaM (Fig 1 and 2).

The affinity strength as measured by IC_{50} was determined in conditions where CaM concentration was kept constant with the increasing concentration of the inhibitor (Table 2). The inhibition constant K_i were determined from the intercept of Dixon plot (Fig 3). The apparent binding sites were determined from Hill plot for each inhibitor (Tabe 2).

Table 1. Effect of various drugs on activity and fluorescence intensity of calmodulin. FI: Fluorescence intensity.

No.	Drugs	FI	PDE-activity
1	W-7	43.27	0.097
2	Compound R24571	264.19	0.011
3	Trifluoperazine (TFP)	184.23	0.067
4	2-Chloro-phenothiazine	204.29	0.025
5	Thiodiphenylamide (TDPAC)	198.66	0.028
6	Cyclosporin A (CsA)	114.6	0.077

Table 2. Inhibition of calmodulin by direct interaction and by PDE activity by various drugs.

No.	Drugs	IC_{50} (μM)				K_i(M)	pK_i	n_H
		F.I[a]	F.I[b]	UV[a]	UV[b]			
1	W-7	42.0	44.66	54.0	53.7	3.1×10^{-5}	4.51	1.23
2	TFP	7.80	7.40	12.4	11.76	7.2×10^{-6}	5.14	1.06
3	TDPAC	4.70	5.25	5	5.20	3.0×10^{-6}	5.54	1.07
4	R24571	0.36	0.34	0.45	0.43	1.2×10^{-7}	6.92	1.40

a: obtained from Figure 4. b: obtained from Figure 3 The Ic_{50} value is defined as the concentration of the drug required to produce 50% inhibition of the enzyme activity. n_H derived from the slope of Hill plots representing the affinity of binding.

Table 3. Thermodynamic parameters characterizing drugs inhibition of calmodulin.

Drugs	ΔG (H2O) (kJ mol^{-1})	(D)1/2 (M)	m (kJ mol^{-1} M^{-1})
W-7	14.80	43×10^{-6}	3.44×10^{5}
TFP	17.0	8.60×10^{-6}	1.98×10^{6}
TDPAC	19.5	4.6×10^{-6}	4.24×10^{6}
R24571	22.5	0.41×10^{-6}	5.49×10^{7}

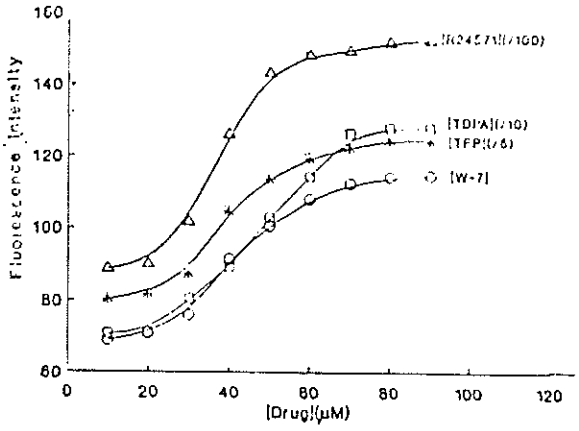

Fig. 1. The effect of various concentration of durgs as calmodulin antagonists on clamodulin fluorescence. The increase in fluorescence intensity for clamodulin was calculated at each concentration of drugs, the curves obtained with 40 μ M of CaM. pH = 7.4, T = 37°C, buffer tris 40 mM. Inhibitor concentration ranged from 10-95 μM. Each point represents the mean value of five determinations.

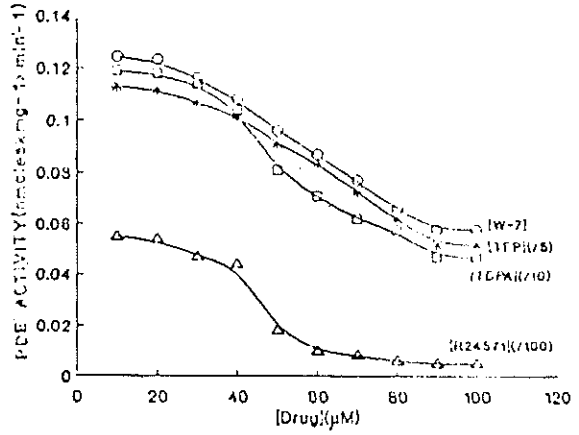

Fig. 2. Inhibition of calmodulin - induced activation of phosphodiesterase by calmodulin antagonists. Phosphodiesterase activity was determined in the presence and absence of clamodulin (40 μM) and various concentration of clamodulin antagonists (10-100 μM). Each point represents the mean value of five dterminations.

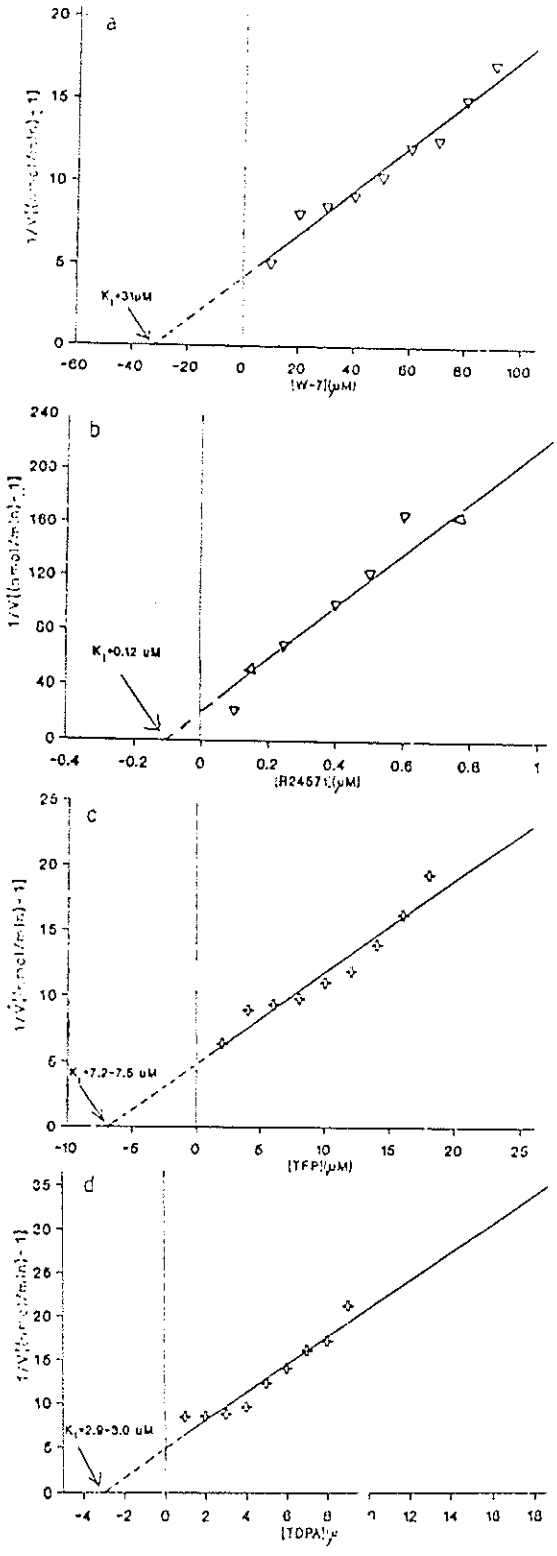

Fig. 3. Dixon plots for kinetic analysis of the inhibition (K_i) by calmodulin antagonists of calmodulin - stimulated phosphodiesterase activity. The calmodulin - induced increase in phosphodiesterase activity was determined in the presence CaM 100 μM, PDE = 1 mg/ml, other reaction condition were: pH = 7.4, T = 37°C, buffer tris 40 mM. Each point represents the mean value of five experiments.

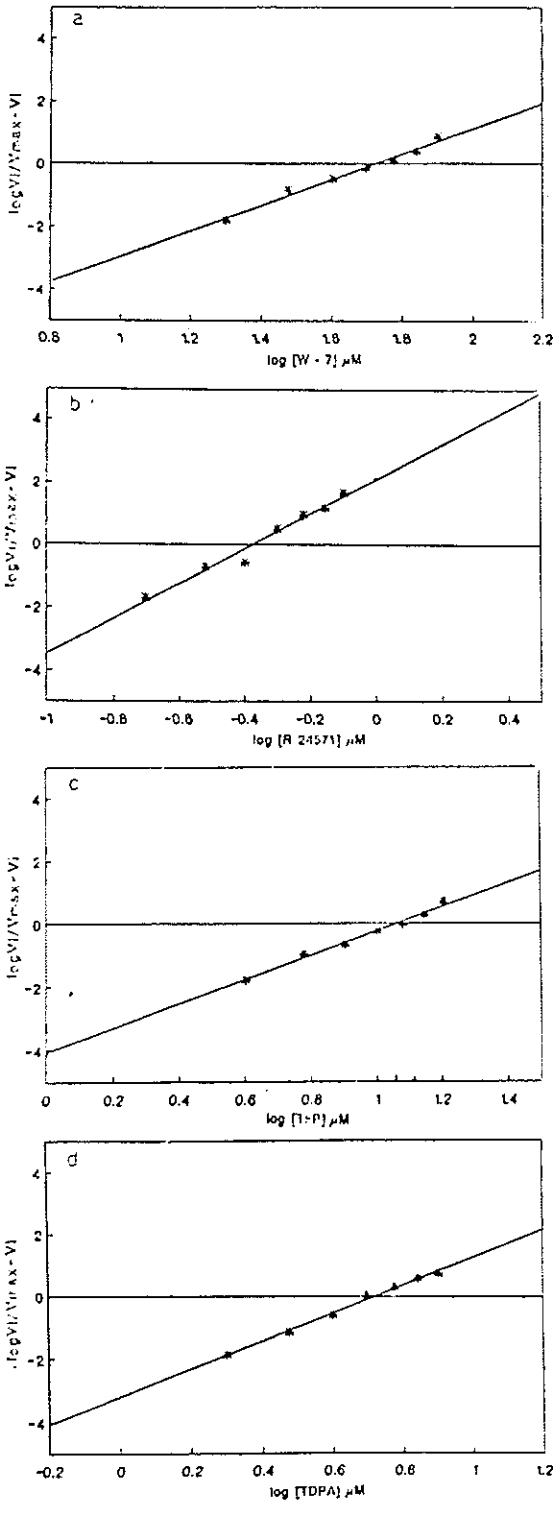

Fig. 4. Hill plots for the inhibition of CaM - activated phosphodiesterase by calmodulin antagonists. Bovine brain CaM-PDE activity was measured in the presence of 0.1 mM $CaCl_2$, CaM 100 μM, PDE = 1 mg/ml, other reaction condition were: pH = 7.4 , T = 37°C, buffer tris 40 mM and various concentrations of drugs. Each point represents the mean value of five determinations. a) W-7, b) R 24571, c) TFP, d) TDPAC

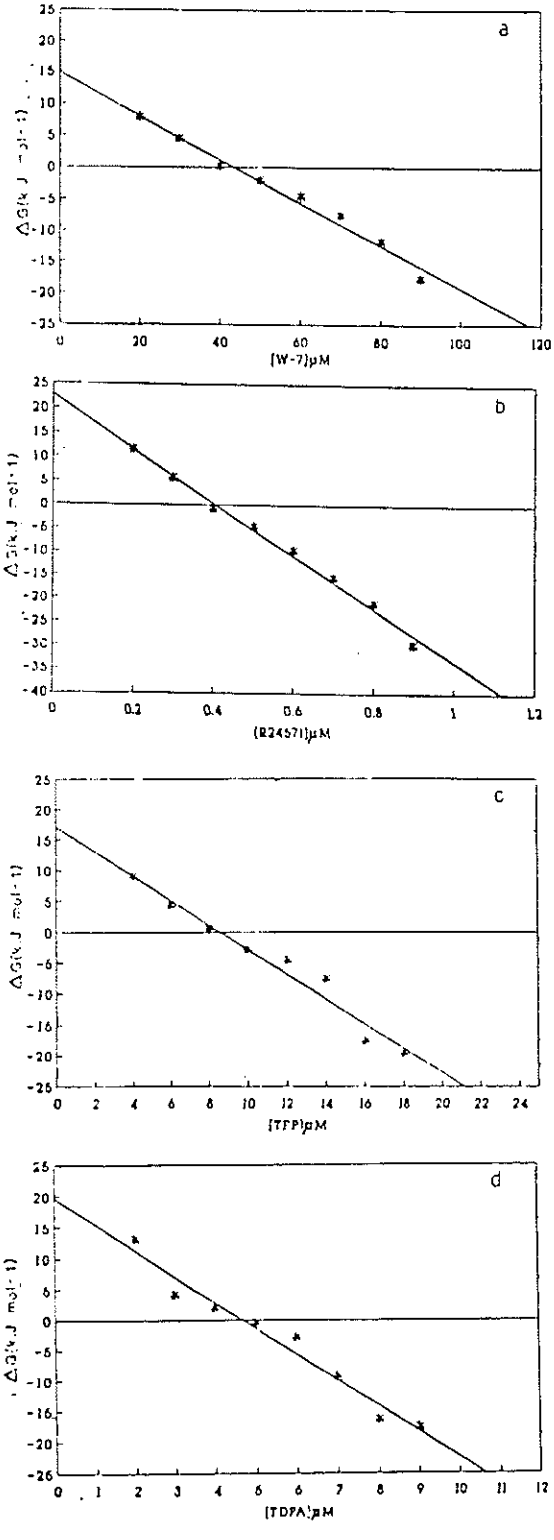

Fig. 5. Free energy, ΔG (calculated from the measurement in the transition region using Eq. (1). The data used were from sigmodial curves (Fig. 1) versus drugs concentration. a) W-7, b) R24571, c) TFP, d) TDPA.

DISCUSSION

The IC_{50} and K_i values obtained with inhibitors W-7 and TDPAC, which are compounds of phenothiazine structure and R24571, TFP indicate that the inhibitory effect of compounds are at least two orders of magnitude different (Table 2). The compounds under study could be arranged in decreasing order of inhibitory potential as follows:

R24571 > TDPAC > TFP > W-7

A generalization could be made that the more hydrophobic components present in molecule, is associated with stronger inhibitory capacity. Previous reports indicate that both hydrophobicity and the positive charge on the molecules are essential for inhibitory effect (18). This was proved by measuring the effects of acetylation of lysine residues of some inhibitory peptides such as melittin and mastoparan (26). The positive charges are supposed to interact with the negatively charged aspartate and glutamate residues on CaM (27). The use of a large number of drugs from different classes of compounds also point to hydrophobic effect in inhibition (18,19). Other factors such as size and nature of substituents are also considered important (19).

Our data indicates that the compounds under study the inhibition parameters obtained by PDE - CaM induced activation and inhibitory studies correlates closely with the studies on the direct interaction of CaM and inhibitors by fluorescence. This suggest a single mechanism by which the inhibitors are specifically bind to CaM and therefore factors arising from the complexity of two reaction appear minimal. Similar results have been obtained by others using similar CaM antagonists (31).

To study further, the nature of interactions it is worthwhile to link the kinetics parameters to that of thermodynamic obtained from sigmoidal curves (22). Because CaM are small globular proteins, they can assume conformational alterations in a two state mechanism (28), the treatment of the data is previously reported (29) assuming such mechanism an equation of state could be employed as follows:

$$F_d = (y_n - Y_{obs}) / (y_n - Y_d)$$

Where F_d is the fraction of protein altered in the presence of ligand, Y_{obs} is the observed variable parameter, Y_n and Y_d are the values of Y (e.g. fluorescence intensity, etc.) characteristics of altered and native conformation. The difference in free energy (ΔG) obtained between the native and altered conformation may be estimated using the, equation:

$$\Delta G = -RT\ln [Fd/1-Fd] = -RT\ln [(Y_n-Y_{obs}) / (Y_{obs}-Y_d)]$$

Where R is the gas constant and T is the absolute temperature, ΔG is plotted aginst concentration of inhibitors (Fig.5). A linearty was found between the ΔG and the inhibitors concentration obeying the following equation:

$$\Delta G = \Delta G (H_2O - m[D])$$

Where $\Delta G (H_2O)$ is the value of ΔG in the absence of inhibitors and m is the measure of dependence of ΔG on inhibitors concentration [D]. $\Delta G (H_2O)$ is the simplest estimate of the stability of the protein (30), m depicts the degree of the variation in the system. Table 3 shows the thermodynamic parameters obtained from inhibition of CaM by the drugs under study. These data correspond to kinetic data obtained in which the $\Delta G (H_2O)$ for all the inhibitors correlated to their degree of inhibition potentials, i.e. the compound R24571 possessing more inhibitory effects, produced more stable complex with CaM, as revealed by the value of free energy. The highest value of "m" and the lowest D1/2 were associated with R24571 compound, indicating a stronger inhibitory effect produced by the drug. The data obtained from Fig.4 is indicative of a competitve inhibition by the drugs. It is important to note that the value of the Hill coefficients tabulated in Table 2 for all the inhibitors are around unity except for R24571 which is 1.405 pointing to the fact that a small conformational change may also occur in the process of its binding to the protein. With a small change in the apparent value of coefficient.

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REFERENCES

1. Cheung WY, Calmodulin plays a pivotal role in cellular regluation. Science 207:19-27, 1980.

2. Klee CB and Vanaman TC, Calmodulin. In: Adv. Protein Chem vol.35, pp. 213-321, Academic Press New York, 1982.

3. Means AR and Dedman JR, Calmodulin - an intracellular calcium receptor. Nature (London) 285:73-77, 1980.

4. Vanaman TC, Structure, function and evolution of calmodulin. In: Calcium and cell function vol. I. (Ed. Cheung WY) pp. 41-58, Academic Press New York, 1980.

5. Wang JH and Waisman DM, Calmodulin and its role in the second messenger system. Curr. Top. Cell. Regul 15:47-107, 1979.

6. Dabrowoska R, Sherry JMF, Aramatorio DK and Hartsbome DJ, Modulator protein as a component of myosin light chain kinase from chicken gizard. Biochemistry 17:253-258, 1978.

7. Forsen S, Volgel HG and Drakenberg T, Biophysical studies of Calmodulin. In: Calcium and cell function vol. VI pp.113-157, Academic Press New York, 1986.

8. Lin YM, Liu VP and Cheung WY, Cyclic 3,5 - nucleotide phosphodiesterase activator. Fedn. Eur. Biochem. Soc. Lett 94:356-364, 1975.

9. Hidka H, Yamaki T, Naka M, Tanaka T, Hayashi H and Kobayashi R, Calcium - regulated modulator protein interacting agents inhibit smooth muscle calcium - stimulated protein kinase and ATP_{ase}. Mol. pharmacol. 17:66-72, 1980.

10. Volpi M, Shaafi R, Epstein PM, Andrenyak DM, and Feinstein MB. local anesthetics mepacrine and propranolol are antagonists of calmodulin. Proce. Natl. Acad. Sci. U.S.A 78:795-799, 1981.

11. Weiss B. Prozialeek WC, Barnete MS and Wallace TL. Pharmacological regulation of calmodulin. Ann. N.Y., Acad Sci. 365: 319-345, 1980.

12. Weiss B, Lehne R and Strada SJ. A rapid microassy of adenosine 3.5 monophosphate phsophodiesterase activity. Anal. Biochem. 45:222-235. 1972.

13. Barnette MS and Weiss B. Interaction of B - endorphin and other opiate peptides with calmodulin. Mol. Pharmacol 21:86-91. 1982.

14. Wolff DJ and Brostrom CO. Calcium - dependent cyclic nucleotide phosphodiesterase from brain: identification of phospholipids as calcium - independent activators. Arch Biochem Biophys. 173: 720-731, 1976.

15. Farzami B. Naderi GA and Moosavi - Movahedi AA. Elucidation of pKa values for Ca^{2+} binding sites incalmodulin by spectrofluormetry. Int. J. Biol. Macromol. In Press. 1994.

16. Dedman JR, Potter JD, Jackson RL, Johnson JD and Means AR. Physicochemical properties of rat testis Ca^{2+} dependent regulator protein of cyclic nucleotide phosphodiesterase. J. Biol. Chem 252:8415-8422. 1977.

17. Brostrom CO and Wolff DJ. Properties and functions of calmodulin. Biochem Pharmacol 30:1395-1405. 1981.

18. Tanaka T and Hidaka H. Hydrophobic regions function in calmodulin - enzymes interaction. J. Biol. Chem. 255: 11078-11080, 1980.

19. Laport DC, Wierman BM and Storm DR, Calcium induced exposure of a hydrophobic surface on calmodulin. Biochemistry 19: 3814-3819, 1980.

20. Mettzer, HI and Kassir S, Inhibition of calmodulin activated Ca^{2+} - ATP_{ase} by propranolol and nadolol. Biochim Biophys Acta 755:452-456, 1983.

21. Netwon DL, Krinks MH, Kaufman Jb, Shiloach J and Klee CB, Large scale preparation of clamodulin. Preparative Biochemistry. 18: 247-259, 1988.

22. Laemmli UK, SDS gel electrophoresis. Nature (Londong) 227: 680-685, 1970.

23. Klee CB, Crouch TH and Krinks MH, Subunit structure and catalytic properties of bovine brain Ca^{2+} - dependent cyclic nucleotide phsophodiesterase. Biochemistry. 18:722-729. 1979.

24. Klee CB, Conformational transition accompanying the binding of Ca^{2+} to the protein activator of 3.5 cyclic adenosine monophosphate phosphodiesterase. Biochemistry. 16: 1017-1024, 11977.

25. Lowry OH, Rosebrough NJ, Farr AL and RAndall RJ. Determination of total protein. J. Biol. Chem 193: 265-275. 1951.

26. Barnette MS, Daly R and Weiss B, Inhibition of calmodulin activity by insect venom peptides. Biochem Pharmocol 32: 2929-2933, 1988.

27. Klumpp S, Frey M, Kleefled G, Sauer A and Eger K. Pyrool [2,3-d] pyrimidines as inhibitors of CAMP - phosphodiesterase. Biochem Pharmacol. 38:949-953, 1989.

28. Privalov PL. Stability of proteins small globular protein. In: Adv. protein chem vol.33, pp. 167-241, Academic Press New York. 1979.

29. Mossavi - Movahedi AA, Naderi GA and Farzami B, The denaturation behaviour of calmodulin insodium n - dodecyl sulphate, dodecyl trimethy ammonium bromide, guanidine hydrochloride and urea. Thermochimica Acta In Press. 1994.

30. Pace NC. Measuring and increasing protein stability. Tibtech vol. 6. pp. 93-98. 1990.

31. Johnson JD and Wittenauer LA. A fluorescent calmodulin that reports the binding of hydrophobic inhibitory ligands. Biochem. J. 211:473-479, 1983.