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IN VITRO INTERACTION BETWEEN CALCITONIN AND CALMODULIN

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The effects of salmon calcitonin and human calcitonin on the fluorescence spectra of a fluorescent conjugate of calmodulin with dansyl-chloride and on the calmodulin mediated activation of phosphodiesterase have been studied. We showed that salmon calcitonin provoked calcium-dependent modifications of the spectra of dansyl-calmodulin and completely inhibited the calmodulin mediated activation of phosphodiesterase with a maximum effect at the dose of 5.8×10^{-7} M and 10^{-7} M respectively. No appreciable effect of human calcitonin was observed in either system. This interaction of salmon calcitonin with calmodulin is similar to the interaction of certain neuropeptides and antipsychotic drugs to calmodulin and may explain certain of the peptide's pharmacological effects.

CT is a 32-aminoacid single chain peptide, secreted from the parafollicular C cells of the mammalian thyroid, that is a major calcium-regulating factor (1). However the recent findings of immunoreactive CT-like material in brain tissue (2) and cerebrospinal fluid (3), and of CT binding sites in brain regions both in animals (4) and in man (2) may suggest CT as a possible neurotransmitter or modulator. The possible neurological actions of CT to date include inhibition of feeding behavior (5), suppression of water consumption (6) and gastric acid secretion (7), modulation of anterior pituitary hormone secretion (8,9), analgesia (10,11) and other behavioral effects (12). The mechanism

Abbreviations: CT=calcitonin, hCT=human calcitonin, sCT=salmon calcitonin, CaM=calmodulin, dansyl-CaM=dansylchloride labeled calmodulin, Mops=morpholinopropane sulfonic acid, SDS=sodium-duodecyl-sulphate, EDTA=ethylenediaminetetraacetic acid.

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by which CT alters central nervous system functions is unknown. However it has been suggested that CT may produce its central effects by altering neuronal calcium fluxes (7). The aim of the present paper was to evaluate the possible interaction of sCT and hCT with CaM, that is the major calcium receptor protein in brain that modulates the action of numerous calcium-dependent processes (13). To detect the interaction, a fluorescent conjugate of bovine CaM with 5-(dimethylamino)-1-naphthalenesulfonyl-cloride was prepared. This preparation has been shown to be exceptionally sensitive to both calcium and protein binding (14). Moreover we studied the effect of sCT and hCT on the in vitro CaM-induced activation of cyclic nucleotide phosphodiesterase.

MATERIALS AND METHODS

Reagents

sCT and hCT were purchased from Bachem (Torrance Ca.); CaM, CaM free phosphodiesterase, AMP and cAMP from Sigma Chemical Co.(St. Louis Mo.). All other chemicals were of reagent grade and obtained from common commercial sources.

Fluorescence spectra of dansyl-CaM

The dansyl-CaM was prepared as described by Anderson and Weber (15). The fluorescence spectra were taken in a Perkin-Elmer 650-10S fluorescence spectrophotometer and recorded with a Perkin-Elmer 56 recorder. The fluorescence studies were carried out at fixed excitation at 340 nm and with emission wavelenght variable between 400 and 600 nm with a maximum of fluorescence at 510 nm. All experiments were carried out in the presence of 200 mM KCl, 50 mM Mops, 1.8×10^{-6} M dansyl-CaM and 10^{-5} M $CaCl_2$ or 2 mM EDTA, pH 7.3 at 25 °C. We investigated the effects of the addition of sCT and hCT (from 10^{-8} to 2.5×10^{-5} M) on the fluorescence spectra of dansyl-CaM. Likewise the fluorescence of dansyl-CaM in the presence of fixed concentrations of sCT (5.8×10^{-7} M) or hCT (2.5×10^{-5} M) obtained at various concentrations of $CaCl_2$ (from 10^{-8} to 10^{-2} M) was evaluated.

Determination of phosphodiesterase activity

To determine the effects of hCT and sCT on CaM-induced activation of phosphodiesterase various concentrations of the two peptides (from 10^{-10} to 10^{-5} M) were added to a solution containing 2.5 mg/ml of a CaM-sensitive form of phosphodiesterase (Sigma cat.P05 20), 0.5 mg/ml of cAMP, 10^{-5} M $CaCl_2$, 0.01 M phosphate buffer, pH 7.0 in the absence or in the presence of 5 ug/ml of CaM. 20 ul of the solution was incubated at 37 °C. After 20 minutes the reaction was stopped with an equal volume of SDS 1%.

HPLC analysis of AMP and cAMP

The enzymatic activity of phosphodiesterase was determined injecting 25 ul of the SDS stopped solution in an HPLC apparatus to

detect the AMP formed and the cAMP residual. The liquid chromatograph used was equipped with a constant flow pump (Waters Ass. Chromatography pump 6000 A), a universal liquid chromatograph injector (Waters Ass. Model U 6K) and a 24x0.45 cm Rp8 column (Violet-Rome). Eluate adsorbance at 280 nm was monitored in a Model 450 variable wavelength UV detector (Waters Ass.). Adsorbance was recorded on a chart recorder Waters Data Module set at 0.5 cm/min. The HPLC analysis was performed using 6% CH_3CN, 12% MetOH, 82% NaH_2PO_4 10^{-2} M, tetrabutylammonium-hydroxide 1.26 mM to pH 4.9 as eluant, under isocratic conditions and at a flow rate of 1.5 ml/min.

RESULTS

Fluorescence spectra of dansyl-CaM

Increasing amounts of sCT produced a progressive rise in the fluorescence spectrum of dansyl-CaM. This variation was already shown at concentration of $5x10^{-8}$M with a maximum at $5.8x10^{-7}$M. hCT exerted negligible effects on the fluorescence spectrum of the conjugate also at the higher concentration used ($2.5x10^{-5}$M) (fig.1). When 2mM EDTA were present no detectable interaction between CT and dansyl-CaM occurred (data not shown).

Figure 2 shows the calcium related variations of the fluorescence spectrum of dansyl-CaM in the presence of $5.8x10^{-7}$M of sCT or

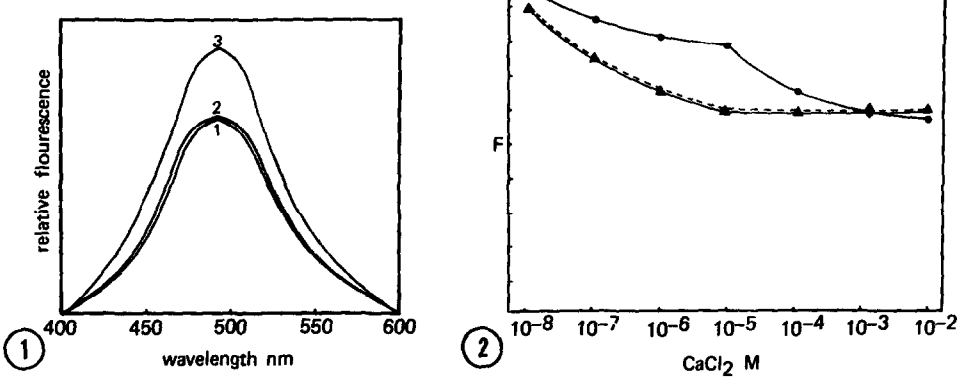


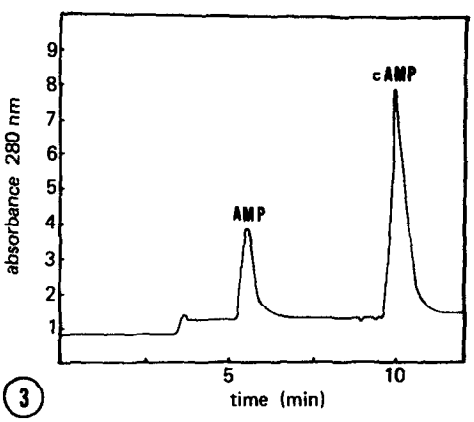
Fig.1 - Fluorescence emission spectra of dansyl-CaM. The spectra of $1.8x10^{-6}$M solutions of dansyl-CaM were recorded in the presence of 10^{-5}M $CaCl_2$ (1) and $2.5x10^{-5}$M hCT (2) or $5.8x10^{-7}$M sCT (3). The solutions also contained 200 mM KCl and 50 mM Mops, pH 7.3 at 25 °C; excitation 340 nm.

Fig.2 - Fluorescence of dansyl-CaM alone (▲--▲) and in the presence of sCT ($5.8x10^{-7}$M) (●—●) or hCT ($2.5x10^{-5}$M) (▲—▲) obtained at various concentrations of $CaCl_2$. For the experimental conditions see "Materials and Methods".

$2.5x10^{-5}$M hCT. In the presence of sCT the addition of $CaCl_2$ from 10^{-8} to 10^{-2}M determined a dual effect with a progressive increase in the fluorescence of dansyl-CaM from 10^{-8} to 10^{-5}M and a subsequent decrease from 10^{-4} to 10^{-2}M suggesting that subtle final conformational changes in CaM could occur by saturation of the weak calcium binding sites (16). By contrast,in the presence of hCT, there were no variations in the course of the fluorescence spectra of dansyl-CaM.

HPLC analysis

The HPLC provided complete separation of the reference standards of cAMP and AMP with 5.30 and 9.50 minutes as retention time respectively (fig.3). In our experimental conditions, the AMP, formed as a product of the phosphodiesterase activity, was well separated from the residual cAMP, the positions of the two peaks corresponding to those of the standards, and the time course of the enzymatic reaction products was linear with a near complete recovery of the compounds of interest (data not shown).



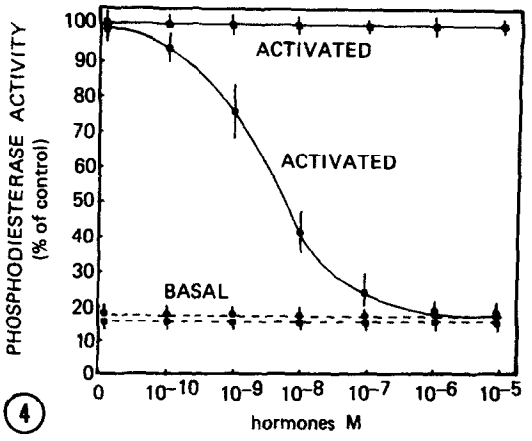


Fig.3 - Chromatographic profile showing separation of a mixture of 5 ul AMP (25 ug/ml) and 20 ul cAMP (25 ug/ml) on a Rp8 10u column (Violet-Rome). UV absorbance was measured at 280 nm. For conditions of separation see "Materials and Methods".

Fig.4 - Effect of various concentrations of sCT (●) and hCT (■) on phosphodiesterase activity in the presence (——) and absence (---) of CaM (5 ug/ml). The phosphodiesterase activity is expressed as per cent of the activated form of phosphodiesterase. Each point represents the mean of four determinations. Verticalbrackets indicate the standard error.

Effects of sCT and hCT on CaM-induced activation of phosphodiesterase

Figure 4 shows the effects of various concentrations of sCT and hCT on the basal and CaM-induced phosphodiesterase activity expressed as per cent of the maximum activity of phosphodiesterase. CaM by itself activated the CaM deficient phosphodiesterase by more than five-fold; this effect was inhibited in a dose dependet way by sCT. Moreover sCT concentrations that totally inhibited the activation of phosphodiesterase induced by CaM had no effect on the basal phosphodiesterase activity. The concentration of sCT that reduced the activation of phosphodiesterase by 50% (IC 50 value) was approximately 5×10^{-9} M. hCT had no effect on the basal and CaM induced phosphodiesterase activity within the concentration range studied.

DISCUSSION

In the last few years it has been shown that, among the very different classes of drugs that can modify the activity of CaM, numerous neuropeptides bind this protein and influence its action (17). Recently reports from a number of laboratories have kindled the idea that CT or CT-related endogenous peptides in the central nervous system may function as neuropeptides to modify or regulate important biological events.

Our results indicate that CT undergoes absolutely calcium-dependent interaction with CaM and inhibits the CaM-mediated activation of phosphodiesterase without altering the basal phosphodiesterase activity.

It is of interest that while nanomolar concentrations of sCT are very active in modifing CaM activity even micromolar amounts of hCT do not induce any alterations. The failure of hCT to interact with CaM was not unexpected. sCT has an aminoacid sequence that differs 50% from that of hCT and shows a higher potential for helical structure and a higher net positive charge than hCT (18,19). These observations, coupled with the notion that these characteristics have been shown to be crucial in the interaction between CaM and the known binder peptides, might possibly account for the

failure of hCT to interact with CaM. On the other hand, it has been known that sCT is much more active than hCT in eliciting biological effects. sCT is 50 to 70 times more potent than hCT in exerting analgesia (20), food and water consumption inhibition (6), hypocalcemia (21) and has 1000 times more binding affinity than hCT to brain membrane preparations (2). The reported observation that in brain homogenates, in contrast with the bone and kidney (22), CT produces a dose-dependent inhibition of basal adenylate cyclase activity (4) could be consistent with the fact that sCT inhibits the CaM dependent enzyme activation and suggests a possible physiological meaning of this interaction at the brain level

There are, furthermore, evidences that suggest an involvement of the calcium ions in the central effects of CT. In fact the reduction of gastric acid secretion induced by intraventricular sCT in rats is inhibited by concomitant administration of calcium chloride (7), the analgesia induced by intracisternal injection of porcine CT in mice is reversed when calcium and CT are injected in combination (23) and using a hypothalamic explant culture system CT depresses $^{45}Ca^{++}$ uptake (24). The interaction of sCT with the calcium-binding protein CaM could support the hypothesis that alterations in the levels of intracellular calcium may be closely related to the central actions of CT. Since CaM and CT are normally in different cellular compartments our observation regarding the in vitro interaction between CT and CaM may have physiological significance considering that the possibility of CT internalization in target cells has been suggested (25) and that this process is generally important for cellular function (26).

In conclusion the observation of the interaction of CT with CaM is novel and could be of value for comprehension of the cellular action of the peptide.

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REFERENCES

1. Foster,G.V. (1981) New Engl.J.Med. 279,349.
2. Fischer,J.A.,Tobler,P.H.,Kaufmann,M.,Born,W.,Henke,H.,Cooper,

P.E.,Sagar,S.M. and Martin,J.B. (1981) Proc.Natl.Acad.Sci.USA 78,7801.
3. Pavlinac,D.M.,Lenhard,L.W.,Parthermore,J.G. and Deftos,L.J. (1980) J.Clin.Endocrinol.Metab. 50,717.
4. Rizzo,A.J. and Goltzman,D. (1981) Endocrinology 108, 1672.
5. Freed,W.J.,Perlow,M.J and Wyatt,R.J. (1979) Science 206, 850.
6. Twery,M.J.,Obie,J.F. and Cooper,C.W. (1982) Peptides 3, 749.
7. Morley,J.E.,Levine,A.S. and Silvas,S.E. (1981) Science 214, 671.
8. Fujita,T.,Chihara,K.,Iwasaki,J.,Iwasaki,Y.,Abe,H. and Minamitani,N. (1981) Hormonal control of calcium metabolism pp. 287-292, Excerpta Medica, Amsterdam.
9. Olgiati,V.R.,Guidobono,F.,Netti,C. and Pecile,A. (1982) Endocrinology 111, 641.
10. Pecile,A.,Ferri,S.,Braga,P.C. and Olgiati,V.R. (1975) Experientia 31, 332.
11. Fraioli,F.,Fabbri,A.,Gnessi,L.,Moretti,C.,Santoro,C. and Felici,M. (1982) Eur.J.Phamacol. 78, 381.
12. Nicoletti,F.,Clementi,G.,Patti,F.,Canonico,P.L.,DiGiorgio,R. M., Matera,M.,Pennisi,G.,Angelucci,L. and Scapagnini,U. (1982) Brain Res. 250, 381.
13. Cheung,W.Y. (1980) Science 207, 19.
14. Malencik,D.A. and Anderson,S.R. (1982) Biochemistry 21, 3480.
15. Anderson,S. and Weber,G. (1966) Arch.Biochem.Biophys. 116,207.
16. Crouch,T.H. and Klee,C.B. (1980) Biochemistry 19, 3692.
17. Malencik,D.A. and Anderson,S.R. (1983) Biochemistry 22, 1955.
18. Potts.J.T.,Jr. and Aurbach,G.D. (1977) Chemistry of the calcitonins, in Handbook of Physiology; Section 7:Endocrinology; Vol. 7 Parathyroid. Greep,R.O. and Astwood,E.B.(eds.),Baltimore, Williams & Wilkins, p.423.
19. Merle,M.,Lefevre,G. and Milhaud,G. (1979) Biochem.Biophys. Res.Comm. 87, 455.
20. Fabbri,A.,Fraioli,F.,Pert,C.B. and Pert,A. Brain Res. (submitted for publication).
21. Munson,P.L. (1976) Handbook of Physiology pp.443-464, American Physiological Society, Washington D.C.
22. Heersche,J.N.M.,Marcus,R.,Aurbach,G.D. (1974) Endocrinology 94, 241.
23. Satoh,M.,Amano,H.,Nakazawa,T. and Takagi,H. (1979) Res.Comm. Chem.Path.Pharm. 26, 213.
24. Koida,M.,Yamamoto,Y.,Nakamuta,H.,Matsuo,J.,Okamoto,M. and Morimoto,T. (1982) Japan J.Pharmacol. 32, 981.
25. Fisher,J.A.,Sagar,S.M. and Martin,J.B. (1981) Life Sci. 29, 663.
26. King,A.C. and Cuatrecasas,P. (1981) New Engl.J.Med. 305, 77.