Calcium Ion Dependent Covalent Modification of Calmodulin with Norchlorpromazine Isothiocyanate†

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ABSTRACT: Calmodulin forms a covalent, one to one, complex with ^{3}H-labeled norchlorpromazine isothiocyanate. Complex formation was monitored by high-performance liquid chromatography using a CN reverse-phase column which resolves calmodulin, the calmodulin–norchlorpromazine adduct, and norchlorpromazine isothiocyanate. Formation of the adduct requires Ca^{2+} and is not observed with norchlorpromazine. The one to one calmodulin–norchlorpromazine complex does not activate phosphodiesterase but can interact with the enzyme and competitively inhibit its stimulation by calmodulin. High concentrations of trifluoperazine inhibit whereas low concentrations stimulate complex formation. This apparent potentiation of the interaction of calmodulin with norchlorpromazine by another phenothiazine suggests that calmodulin contains at least two phenothiazine binding sites and that the binding of phenothiazine to calmodulin is cooperative.

Phenothiazines interact with calmodulin in a Ca^{2+}-dependent manner and prevent the interaction of calmodulin with its target proteins (Levin & Weiss, 1977; Weiss et al., 1982). They are widely used as calmodulin antagonists even though little is known about the calmodulin–phenothiazine interaction. Originally, this class of drugs was believed to have a high specificity for calmodulin, which was directly related to their antipsychotic activity (Levin & Weiss, 1977). Recent evidence, however, indicates that the affinity of phenothiazines for calmodulin is related to their hydrophobicity and does not correlate with their antipsychotic activity (Norman & Drummond, 1979; Roufogalis, 1981). Evidence for the lack of specificity has been reported by Marshak et al. (1981) and Moore & Dedman (1982). In spite of the lack of specificity, they remain potential tools to study the interaction of calmodulin with many of its target proteins. It was proposed by LaPorte et al. (1980) that the binding of Ca^{2+} to calmodulin exposes hydrophobic surfaces on calmodulin, which are recognized by phenothiazines and may also be recognized by target proteins. Thus, identification of the phenothiazine interaction site(s) on calmodulin may lead to the identification of the interaction sites of target proteins.

In this paper we describe a new approach to the study of the interaction of phenothiazines with calmodulin. Using a radiolabeled, acylating phenothiazine, norchlorpromazine isothiocyanate, we demonstrate the Ca^{2+}-dependent formation of a covalent, one to one, complex of calmodulin with the drug.

Materials and Methods

Trifluoperazine was a gift of Smith Kline & French Laboratories; 2-chloro-10-(3-aminopropyl)phenothiazine hydrochloride (norchlorpromazine) was generously provided by Dr. Albert Manian, Psychopharmacology Research Branch, National Institute of Mental Health; [^{3}H]H_2O (5 Ci/g) was purchased from New England Nuclear; thiophosgene was from Aldrich Chemical Co.; all HPLC[1] columns and solvents were from Waters Associates. Ram testis calmodulin and calmodulin-stimulated cyclic nucleotide phosphodiesterase were prepared as previously described (Klee, 1977; Klee et al., 1979).

Synthesis of 2-Chloro-10-(3-isothiocyanatopropyl)phenothiazine (**2**) (*CAPP-NCS*). A suspension of norchlorpromazine hydrochloride (**1**) (Figure 1) (200 mg, 0.61 mmol) in H_2O (20 mL) was treated with $NaHCO_3$ (206 mg, 2.45 mmol) and stirred with $CHCl_3$ (20 mL), yielding a clear biphasic mixture. Redistilled thiophosgene (70 μL, 0.93 mmol) was added with stirring at 20 °C, giving a transient red color. Analysis of the $CHCl_3$ layer (2 μL) at 20 min by thin-layer chromatography on 250-μm silica gel (10 cm × 2.5 cm, Analtech, Inc.) using $CHCl_3$–MeOH–NH_4OH, 9:1:0.1, as the developing solvent showed a single product spot (R_f 0.96) with no starting material (R_f 0.26). The $CHCl_3$ layer was removed and combined with a $CHCl_3$ extract (5 mL) of the residual aqueous layer. After filtration through $MgSO_4$, the solvent was evaporated under reduced pressure to yield **2** (Figure 1) as a syrup (208 mg, 100% yield). An infrared spectrum was recorded on a Beckman IR 4230. An electron impact ionization mass spectrum (EIMS) was obtained on a Hitachi Perkin-Elmer RMU-6E spectrometer (70 eV): EIMS 332, 334 (M^+); IR 2095 cm^{-1} (NCS).

Synthesis of [^{3}*H*]*-2-Chloro-10-(3-isothiocyanatopropyl)-phenothiazine* (**4**) ([^{3}*H*]*CAPP-NCS*). Norchlorpromazine hydrochloride (5 mg, **1**) was sealed in a Wheaton reactivial with tritiated H_2O (1 g) and CF_3CO_2H (50 μL). The vial was wrapped in foil and stirred in an oil bath at 130 °C. After 19 h the reddish solution was cooled to 20 °C and the CF_3CO_2H neutralized by the addition of solid $NaHCO_3$. The mixture was shaken with $CHCl_3$ (1 mL) and the aqueous phase removed and discarded. The purple $CHCl_3$ phase was exhaustively washed with saturated aqueous $NaHCO_3$ (15 × 1 mL washes) to remove exchangeable tritium. The resulting $CHCl_3$ layer was evaporated and purified by thin-layer chromatography using an aluminum-backed silica gel plate (200 μm, 2 cm × 7 cm) with $CHCl_3$–MeOH–NH_4OH,

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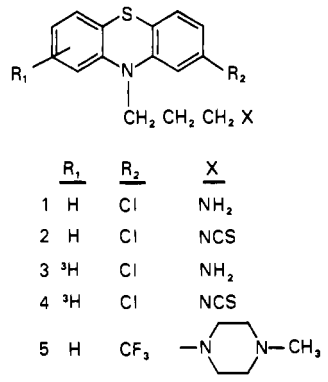
[1] Abbreviations: HPLC, high-performance liquid chromatography; CAPP, 2-chloro-10-(3-aminopropyl)phenothiazine; CAPP-NCS, 2-chloro-10-(3-aminopropyl)phenothiazine isothiocyanate (norchlorpromazine isothiocyanate); CAPP–calmodulin, calmodulin–norchlorpromazine isothiocyanate covalent adduct; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; RT, retention time; cAMP, adenosine cyclic 3',5'-monophosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.



	R_1	R_2	X
1	H	Cl	NH_2
2	H	Cl	NCS
3	^3H	Cl	NH_2
4	^3H	Cl	NCS
5	H	CF_3	

FIGURE 1: Chemical structures of phenothiazines used in this study.

9:1:0.1, as solvent. The product band **3** (Figure 1) was eluted from the plate with MeOH and evaporated under N_2 (87 μCi). This was dissolved in $CHCl_3$ (100 μL) and stirred at 20 °C for 25 min with saturated aqueous $NaHCO_3$ (100 μL) and thiophosgene (1 μL). The $CHCl_3$ phase was removed, combined with a $CHCl_3$ extract (100 μL) of the residual aqueous layer, reduced in volume under N_2, and purified by thin-layer chromatography (1 cm × 7 cm plate, $CHCl_3$–MeOH, 9:1). Autoradiography (10 min using Kodak X-Omat AR film) showed a single product band (R_f 0.96). Elution of the product band with MeOH yielded **4** (Figure 1, 41 μCi). Examination of an aliquot of **4** by thin-layer chromatography showed that 90% of the total radioactivity cochromatographed with authentic unlabeled standard **2**. Specific activity was calculated as 39.8 mCi/mmol on the basis of a 50% counting efficiency for tritium and a extinction coefficient for norchlorpromazine, ϵ_{258nm} = 45 000.

Preparation of CAPP–Calmodulin. All operations were carried out in the dark. Calmodulin (10 μM in 20 mM $NaHCO_3$–0.1 M NaCl, pH 8.2) was incubated at 37 °C in the presence of either 2 mM Ca^{2+} or 2 mM EGTA. Every 20 min, aliquots of [^{3}H]CAPP-NCS (stock solution, 0.54 mM in ethanol, 4500 cpm/nmol) were added until a final concentration of 30 μM was reached (15 additions). Incubation volumes varied between 0.5 (analytical procedures) and 70 mL (preparative procedures). At the end of the incubation period and prior to HPLC, all solutions were centrifuged for 5–10 min in a table-top Eppendorf centrifuge.

HPLC Purification of CAPP–Calmodulin [Modification of Wallace et al. (1981)]. Calmodulin, CAPP-NCS, and CAPP–calmodulin were resolved from each other on a Waters HPLC system equipped with two Model 6000A solvent delivery systems, a Waters Model 730 data module, a Model 440 absorbance detector, and a Model 450 variable wavelength detector to monitor absorbances at 280 and 215 nm, respectively. A Bondapak C18/Porasil B column (7.8 mm × 61 cm) was mounted between the two pumps to remove trace impurities in the aqueous solvents. The column used for the purification of CAPP–calmodulin was a Waters μBondapak CN reverse-phase column (10-μm resin diameter, 3.9 mm × 30 cm). For analytical procedures, aliquots (150 μL) were chromatographed with a linear gradient from 95% buffer A (0.01 M potassium phosphate, pH 6.25, 2 mM EGTA, 5% CH_3CN)–5% solvent B (100% CH_3CN) to 35% buffer A–65% solvent B over 20 min.

For preparative procedures, aliquots (8–12 mL) were applied as 2-mL fractions, with 2-min intervals, at 100% buffer A until the desired volume was loaded onto the column. The linear elution gradients in buffer A were 0–15% solvent B over 10 min, 15–25% solvent B over 20 min (to resolve CAPP–calmodulin from calmodulin), and 25–70% buffer A over 10 min

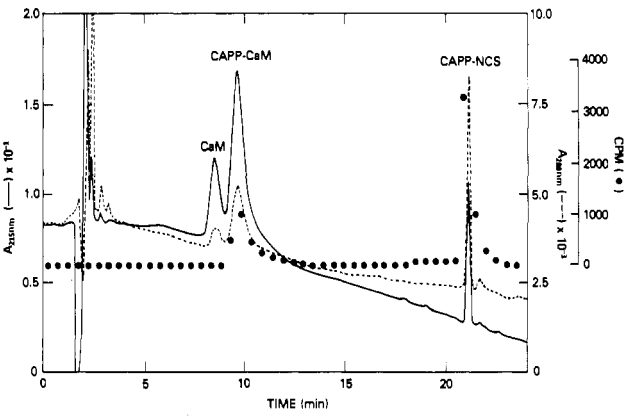


FIGURE 2: HPLC fractionation of calmodulin, CAPP-NCS, and [^{3}H]CAPP–calmodulin. Calmodulin and [^{3}H]CAPP-NCS were incubated in the presence of 2 mM Ca^{2+} for 6 h and analyzed by HPLC as described under Materials and Methods. Fractions (0.75 mL) were collected every 30 s and monitored for radioactivity. Components are identified on the figure. Recovery of protein was 59%; recovery of applied radioactivity was 83%.

(to elute CAPP-NCS). The UV-absorbing peaks were collected, pooled, and flash evaporated. The samples were then dissolved in 1 mL of 0.05 M NH_4HCO_3 and passed through a Pharmacia PD-10 column (Pharmacia Fine Chemicals), equilibrated and eluted with 0.05 M NH_4HCO_3, to remove EGTA and phosphate. Complete removal of EGTA required two passages through the PD-10 columns. [^{3}H]CAPP–calmodulin monitored by absorbance at 258 nm and radioactivity elutes from the PD-10 column between 3.0 and 4.5 mL. CAPP–calmodulin was stored as a solution in 0.05 M NH_4HCO_3. The concentration of CAPP–calmodulin was determined by amino acid analysis since the method of Lowry et al. (1951), using calmodulin as a standard, gave erroneous values. UV absorption spectra were measured in ethanol for CAPP-NCS and in 0.05 M NH_4HCO_3 for CAPP–calmodulin on a Cary Model 118 spectrophotometer.

Results

Ca^{2+}-Dependent Covalent Formation of a Complex between Calmodulin and Norchlorpromazine Isothiocyanate. Calmodulin and phenothiazines can be resolved by HPLC on a CN reverse-phase column (Figure 2). Calmodulin (monitored at 215 and 280 nm) and [^{3}H]norchlorpromazine isothiocyanate (CAPP-NCS) (monitored by radioactivity) are eluted with retention times of 8.5 and 21 min, respectively. When calmodulin is incubated with CAPP-NCS in the presence of Ca^{2+}, a second radioactive peak, eluting at 9.5 min, is detected. The increase in lipophilicity (increased retention time), association with radioactivity, and increase in relative absorbance at 280 nm over 215 nm compared to that of calmodulin suggest that a complex has been formed between calmodulin and CAPP-NCS. This complex will be referred to as CAPP–calmodulin (RT, 9.5 min). No complex was detected by HPLC when calmodulin was incubated with CAPP instead of CAPP-NCS (data not shown).

As shown in Figure 3 (left panel), the appearance of CAPP–calmodulin is accompanied by the disappearance of calmodulin. In an independent experiment, when the reaction was followed at two different concentrations of CAPP-NCS (10 or 20 μM) with 10 μM calmodulin, the rates of the reaction (0.4 nmol mL^{-1} h^{-1})[2] were linear and identical up to

[2] The very low solubility of CAPP-NCS prevented an accurate determination of the actual concentration of CAPP-NCS in the incubation mixture. It is probably responsible for the apparent independence of the reaction rate on added CAPP-NCS.

Table I: Separation of CAPP–Calmodulin, CAPP-NCS, and Calmodulin by HPLC[a]

	volume (mL)	protein (mg × 10^{-3})	radioactivity (cpm × 10^4)	yield		stoichiometry (mol of CAPP/ mol of CaM)
				protein	cpm	
(1) incubation mixture	0.5	82.5	5.25[b]	100	100	3:1
(2) HPLC						
(a) calmodulin		15.7[c]	0	19		0
(b) CAPP-calmodulin		33.3[d]	0.8	40	15	1.1–0.84[e]
(c) CAPP-NCS			1.6		30	
total recovery				59	45	

[a] Calmodulin (10 μM) was incubated with CAPP-NCS (30 μM) in the presence of Ca^{2+}, and the soluble fraction was analyzed by HPLC as described under Materials and Methods. [b] The specific activity of [^{3}H]CAPP-NCS was determined in the presence of bovine serum albumin and was 3500 cpm/nmol as opposed to 4500 cpm/nmol in the absence of protein (addition of protein always results in quenching of radioactivity). [c] Protein content was determined by absorbance at 215 nm using a calmodulin standard run under identical conditions. [d] Protein content was determined as in footnote *c* but was corrected for the different extinction coefficient of CAPP–calmodulin determined as described in the text. [e] The value of 1.1 mol of CAPP/mol of calmodulin was determined by using a specific activity, 3500 cpm/nmol, for [^{3}H]CAPP-NCS. The value of 0.84 mol of CAPP/mol of calmodulin was determined spectrophotometrically at 258 nm.

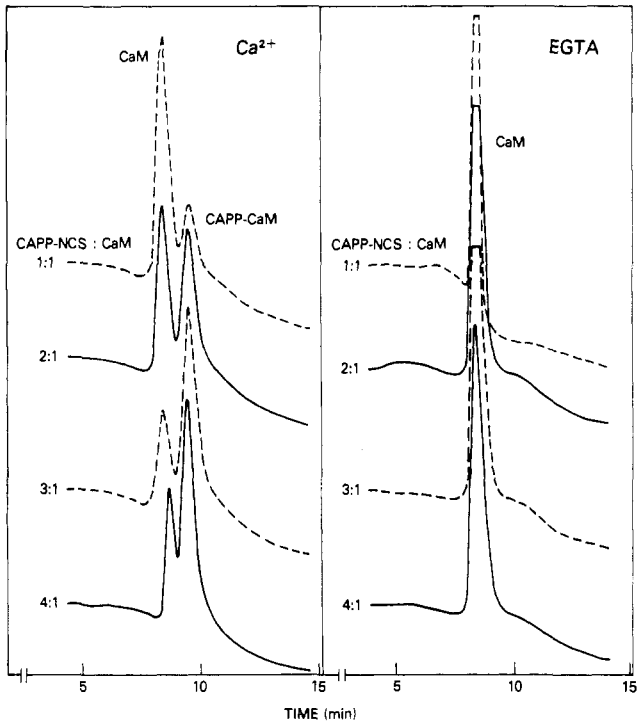


FIGURE 3: Ca^{2+}-dependent formation of CAPP–calmodulin. CAPP-NCS was added to a solution of calmodulin (10 μM) in 2 mM Ca^{2+} (left) or 2 mM EGTA (right) in successive aliquots up to 40 μM final concentration of CAPP-NCS. After each addition, the protein–CAPP-NCS mixture was incubated at 37 °C for 20 min, and an aliquot was immediately analyzed by HPLC.

4 h of incubation (data not shown). After 4 h, the rate of reaction was slightly larger in the presence of the high concentration of CAPP-NCS. Complex formation leveled off after 24 h, leaving 32% and 4% of calmodulin unmodified at the low and high concentrations of CAPP-NCS, respectively. In the presence of EGTA, no complex was detected (Figure 3, right panel), indicating that the reaction was Ca^{2+} dependent.

Characterization of the CAPP–Calmodulin Complex. A preparation of [^{3}H]CAPP–calmodulin is summarized in Table I. Forty percent of calmodulin was recovered as CAPP–calmodulin and 19% as calmodulin with an overall protein recovery of 59%. Whereas the recovery of radioactivity at the HPLC step was usually >80%, total recovery of radioactivity after incubation in the presence of Ca^{2+} was only 45% (15% as CAPP–calmodulin and 30% as CAPP-NCS). The combined loss of protein (40%) and radioactivity (55%) may reflect the formation of insoluble, multivalent complexes between CAPP-NCS and calmodulin. When the incubation was conducted in the presence of Ca^{2+}, precipitated material was always detected and removed by centrifugation before HPLC. In contrast, recovery of protein and radioactivity from the incubation mixture performed in the presence of EGTA (where no complex is formed) was always >90%.

Rechromatography of the CAPP–calmodulin peak with a very shallow gradient or stepwise isocratic conditions resulted in a symmetrical peak, suggesting that the modified protein is homogeneous. The amino acid composition of CAPP–calmodulin was identical with that of calmodulin. The molar extinction coefficients of CAPP–calmodulin, based on the protein concentration determined by amino acid analysis (see Materials and Methods), were $\epsilon_{215nm} = 318 \times 10^3$, $\epsilon_{258nm} = 41 \times 10^3$, and $\epsilon_{280nm} = 6.8 \times 10^3$ as opposed to $\epsilon_{215nm} = 190 \times 10^3$, $\epsilon_{258nm} = 2.55 \times 10^3$, and $\epsilon_{280nm} = 2.7 \times 10^3$ for calmodulin. The higher 280/215 ratio of CAPP–calmodulin (0.021) than that of calmodulin (0.014) reflects the relatively high absorbance of CAPP-NCS at 280 nm ($\epsilon_{280nm} = 2.18 \times 10^3$) and its low absorbance at 215 nm ($\epsilon_{215nm} = 37.6 \times 10^3$) compared to calmodulin. The stoichiometry of covalent drug binding to calmodulin determined spectrophotometrically at 258 nm (assuming additivity of the absorbances of calmodulin and CAPP-NCS) was 0.84 mol/mol of calmodulin. This value may be erroneously low since the absorbance of CAPP-NCS in ethanol may be different from that of CAPP-NCS bound to calmodulin measured in aqueous solvents. With radiolabeled CAPP-NCS, a stoichiometry of 1.1 mol/mol of calmodulin was determined (Table I).

Effect of Trifluoperazine on the Formation of CAPP–Calmodulin. In the presence of a 10-fold excess of trifluoperazine (Figure 1, **5**) over CAPP-NCS, a pronounced inhibition of complex formation was observed (Table II). After 4 h, 7% of calmodulin was modified in the presence of trifluoperazine as opposed to 33% in the absence of trifluoperazine. The pronounced inhibition observed at early times was less marked after prolonged incubations (>4 h). Since the reaction of calmodulin with CAPP-NCS is irreversible, it is expected, first, that the amount of modified calmodulin will eventually plateau at the same level in either the presence or absence of trifluoperazine and, second, that high concentrations of trifluoperazine should be required to achieve a significant inhibition of the irreversible interaction of CAPP-NCS with calmodulin. The competition between trifluoperazine and CAPP-NCS for phenothiazine binding sites on calmodulin suggests that phenothiazines and CAPP-NCS may bind at the same site.

In contrast, when low concentrations of trifluoperazine were used, an apparent potentiation of the interaction of calmodulin

Table II: Inhibition by Trifluoperazine of the Modification of Calmodulin by CAPP-NCS[a]

incubation time (min)	trifluoperazine (μM)	CAPP-NCS (μM)	modification[b] (%)
40	none	20	1.0
40	200	20	0
90	none	20	7.0
90	200	20	<1.0
240	none	20	32.7
240	200	20	6.6

[a] Calmodulin (10 μM) was incubated at 37 °C as described under Materials and Methods in the presence of Ca^{2+} and 20 μM CAPP-NCS (one addition) in the presence or absence of 200 μM trifluoperazine. Trifluoperazine (1.85 mM in H_2O) was added to the incubation mixture just before CAPP-NCS. Aliquots (150 μL) were analyzed by HPLC as described under Materials and Methods. [b] Percent of total calmodulin (sum of calmodulin and CAPP-calmodulin) recovered as CAPP-calmodulin. The integrated area of the UV-absorbing peaks measured at 215 nm was corrected for the different molar extinction coefficients of the two proteins.

with CAPP-NCS was observed. At equimolar concentrations of calmodulin, CAPP-NCS, and trifluoperazine (10 μM each), 82% of calmodulin was converted to CAPP–calmodulin after 24 h as opposed to 68% in the absence of trifluoperazine (data not shown). When calmodulin (10 μM) was incubated with CAPP-NCS (20 μM) and trifluoperazine (20 μM), the same plateau of incorporation was reached after 24 h of incubation whether or not trifluoperazine was present (96–98% of calmodulin was converted to CAPP–calmodulin). During the first 4 h, however, in both cases the rates of complex formation in the presence of trifluoperazine were faster than in its absence (0.4 nmol mL^{-1} h^{-1} in the absence and 0.8 nmol mL^{-1} h^{-1} in the presence of trifluoperazine). Protein recovery was not significantly affected by the presence of trifluoperazine; thus trifluoperazine does not appear to prevent the formation of multivalent, insoluble complexes.

Interaction of CAPP–Calmodulin with Phosphodiesterase. When CAPP–calmodulin was tested for its ability to activate calmodulin-dependent phosphodiesterase (Figure 4), no stimulation of the enzyme was observed up to 10^{-7} M (top panel). When the sensitivity of the assay was increased by increasing the level of enzyme (10^{-9} M) with 10^{-5} M cAMP, activation of the enzyme by CAPP–calmodulin was detected at concentrations greater than 10^{-7} M. This activation could be explained by a 0.05% contamination with calmodulin. CAPP–calmodulin, on the other hand, had a marked inhibitory effect on the ability of calmodulin to stimulate the enzyme (Figure 4, lower panel). This inhibition was relieved at high concentrations of calmodulin. Assuming competitive inhibition by CAPP–calmodulin, the following kinetic constants were obtained: $K_i = 3.0 \pm 1.0 \times 10^{-8}$ M, $K_m = 1.8 \pm 0.4 \times 10^{-9}$ M, and $V_{max} = 0.7$ μmol min^{-1} (mg of enzyme)$^{-1}$. It seems, therefore, that CAPP–calmodulin can still interact with phosphodiesterase at a calmodulin-binding site.

Discussion

The formation of a covalent bond between calmodulin and CAPP-NCS was suggested by the following: (1) No complex was observed by HPLC when CAPP rather than CAPP-NCS was incubated with calmodulin under identical conditions. (2) The addition of EGTA after completion of the incubation had no effect on the chromatographic behavior of the calmodulin–CAPP-NCS complex. Since CAPP–calmodulin was not formed in the presence of EGTA (Figure 3, right panel), the

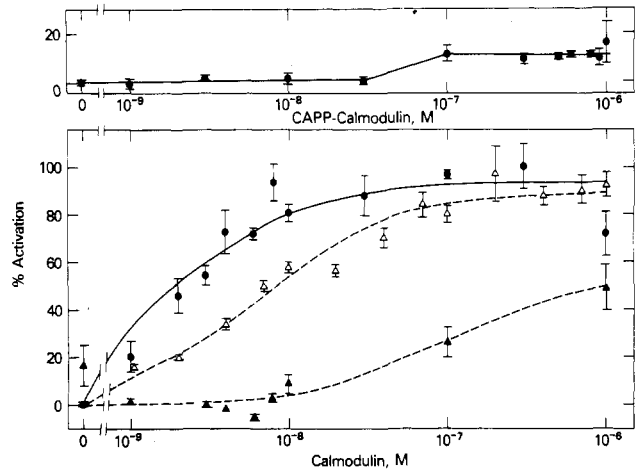


FIGURE 4: Effect of CAPP–calmodulin on phosphodiesterase activity. cAMP phosphodiesterase was assayed at 30 °C for 40 min as described by Klee (1977). The incubation mixture (0.1 mL) contained 40 mM Tris-HCl, pH 8.0, 3 mM $MgCl_2$, 1.0 mM $CaCl_2$, 0.2 M NH_4Cl, 1.0 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, 1 μM [^{3}H]-cAMP [(7.5–8.0) × 10^5 cpm/nmol], and 1.5 × 10^{-7} M [^{14}C]AMP (105 000–120 000 cpm/nmol) in the presence or absence of calmodulin. The reaction was initiated by addition of enzyme (5 × 10^{-11} M). (Top panel) Activation of phosphodiesterase by CAPP–calmodulin (●); (bottom panel) inhibition of the calmodulin stimulation of phosphodiesterase by CAPP–calmodulin [(●) calmodulin; (▲) calmodulin and 10^{-6} M CAPP–calmodulin; (△) calmodulin and 10^{-7} M CAPP–calmodulin]. Each point is representative of triplicate determinations. The data in the bottom panel were fitted to the following equation assuming competitive inhibition: $v = VA/[K(1 + I/K_i) + A]$. A curve-fitting program, MLAB (Knott & Reece, 1971), was used to analyze the data.

addition of excess EGTA to a complexed calmodulin–CAPP-NCS should dissociate the complex if it were noncovalent. (3) Under the conditions chosen to elute CAPP–calmodulin from PD-10 columns, free drug was retained on the column, whereas [^{3}H]CAPP–calmodulin was eluted in the void volume of the column with a >90% recovery of radioactivity and protein. (4) When CAPP–calmodulin was tested for its ability to inhibit the calmodulin stimulation of phosphodiesterase, the K_i value for CAPP–calmodulin (3.0×10^{-8} M) was approximately 3 orders of magnitude lower than that determined for CAPP-NCS (10^{-5} M). If the complex between calmodulin and drug were not covalent, the low level of drug present in CAPP–calmodulin used in the assay (10^{-7} M, Figure 4) would not be sufficient to inhibit the activation of the enzyme by calmodulin.

HPLC fractionation of calmodulin and aromatic ligands has been used in the development of an assay to detect the formation of Ca^{2+}-dependent, covalent adducts between calmodulin and a phenothiazine isothiocyanate derivative. A one to one adduct of norchlorpromazine isothiocyanate and calmodulin was isolated. Formation of this complex is potentiated by low concentrations of another anti-calmodulin drug, trifluoperazine, suggesting that calmodulin contains at least two phenothiazine binding sites. Trifluoperazine binding at one phenothiazine site could facilitate binding of CAPP-NCS at a second site. Such cooperative binding has been recently proposed by Johnson (1983) to explain the enhancement of felodipine binding to calmodulin by calmodulin antagonists and Ca^{2+} antagonists. The monosubstituted derivative described in this paper can still interact with phosphodiesterase and prevent calmodulin stimulation. Thus CAPP–calmodulin still contains a phosphodiesterase binding site. These results are in agreement with the recent demonstration of a (β-endorphin)$_2$–calmodulin complex (Giedroc et al., 1983). The

identity of the phenothiazine and phosphodiesterase binding sites on calmodulin remain to be identified.

Registry No. 1·HCl, 3763-80-2; 2, 87508-98-3; 3, 87508-99-4; 4, 87509-00-0; 5, 117-89-5; thiophosgene, 463-71-8; cyclic nucleotide phosphodiesterase, 9040-59-9; Ca, 7440-70-2; cAMP, 60-92-4.

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Articles

Rat Kidney Renin and Cathepsin D: Purification and Comparison of Properties†

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ABSTRACT: Renin and cathepsin D were purified by seven-step procedures involving five steps common to both enzymes. These common five steps were extraction of freeze-dried kidney powder in 30% methoxyethanol–water, diethylaminoethyl-cellulose (DEAE-cellulose) batch adsorption and elution, pepstatin–aminohexyl-Sepharose chromatography, Sephadex G-100 chromatography, and DEAE-cellulose chromatography. The renin component was purified further by passage through an anti-rat spleen cathepsin D immunoglobulin G–Sepharose (IgG–Sepharose) column followed by carboxymethyl-Sepharose (CM-Sepharose) chromatography which separated two renin components. Cathepsin D activity obtained by the fifth step was purified by passage through an anti-rat kidney renin IgG–Sepharose column followed by DEAE-Sephacel chromatography which separated three cathepsin D components. The homogeneity of renin and cathepsin D preparations was demonstrated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. The two components of renins showed molecular weights of 42000 and 36000 by gel filtration and 38000 and 36000 by SDS gel electrophoresis, respectively. They showed isoelectric points of 5.35 and 5.65 by electrofocusing in 5% polyacrylamide gels. Their optimum pHs of enzyme activity were 6.5 as determined by using nephrectomized rat plasma as a substrate. Their specific angiotensin I (Ang I) generation activities were 158 and 146 μg of Ang I (μg of protein)$^{-1}$ h^{-1}, respectively, which correspond to 1100 and 1020 Goldblatt units (mg of protein)$^{-1}$ h^{-1}. The three cathepsins showed molecular weights of 41 000, 43 000, and 41 000 by gel filtration and 46 000, 45 000, and 46 000 by SDS gel electrophoresis. They showed isoelectric points of 6.20, 6.05, and 6.00 by electrofocusing in 5% polyacrylamide gels. Each of the cathepsins showed two pH optima of 3.0 and 4.5 as examined by using bovine hemoglobin labeled with [^{14}C]-glycine methyl ester as substrate. The cathepsins showed an optimal angiotensin I generating activity of 0.98, 1.27, and 1.22 μg of Ang I (μg of protein)$^{-1}$ h^{-1}, respectively, at pH 4.5. However, at pH 6.5, the cathepsin D showed a much diminished renin-like angiotensin I generation activity.

Renin (EC 3.4.99.19) is a peptidase whose function is dedicated to the formation of decapeptide angiotensin I from its prohormone angiotensinogen by the cleavage of the singular Leu–Leu (Skeggs et al., 1957) or Leu–Val (Tewksbury et al., 1981) peptide bond located in the amino-terminal region of this substrate molecule. The discovery that the active site of renin shares many structural features commonly found in aspartic (acid) proteinases such as pepsin (Inagami et al., 1974; McKown & Gregerman, 1975; Misono & Inagami, 1980) and yet has little or practically no general protease activity has aroused interest concerning the mechanism underlying its exceedingly stringent substrate specificity.

The lysosomal aspartic proteinase cathepsin D (EC 3.4.23.5) of the brain and spleen, in a partially purified form, has been reported to possess a renin-like activity in catalyzing the formation of angiotensin I from angiotensinogen (Day & Reid,

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