# CAPP-calmodulin: a potent competitive inhibitor of calmodulin actions

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A covalent adduct of norchlorpromazine (CAPP) and calmodulin is a very potent antagonist of calmodulin activation of several enzymes. The phenothiazine–calmodulin complex (CAPP-calmodulin) acts as a pure antagonist with phosphodiesterase and myosin kinase or a partial agonist with the phosphoprotein phosphatase, calcineurin. Because of its potency and the selectivity inherent to its calmodulin moiety, CAPP-calmodulin should be a uniquely useful probe of calmodulin actions.

*Phosphodiesterase* *Myosin kinase* *Phenothiazine*
*CAPP-calmodulin* *Calcineurin* *Protein phosphatase 2B*

## 1. INTRODUCTION

In the presence of $Ca^{2+}$, phenothiazines bind to calmodulin and thereby prevent the activation of calmodulin-regulated enzymes (review [1]). Because phenothiazines lack specificity [2–4], have a low affinity for calmodulin [1] and interact also with calmodulin target proteins [5–7], their usefulness as probes of calmodulin function is limited. Recently, we described the $Ca^{2+}$-dependent formation of a covalent, one to one, complex of calmodulin with norchlorpromazine isothiocyanate (CAPP-NCS) [8]. This drug-calmodulin adduct (CAPP-calmodulin) lacks the ability to stimulate the calmodulin-dependent cyclic nucleotide phosphodiesterase, but can still interact with the enzyme and prevent the stimulation of the enzyme by calmodulin. Thus, CAPP-calmodulin contains a phosphodiesterase binding site. We demonstrate here that by covalently attaching a phenothiazine to calmodulin, we have created a calmodulin antagonist which not only is specific for calmodulin-dependent reactions, but is 50–300 times more potent than unconjugated phenothiazines as a calmodulin antagonist.

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## 2. MATERIALS AND METHODS

Trifluoperazine (TFP) was a gift from Smith Kline and French Laboratories; all other reagents were as in [8,9]. Ram testis calmodulin [9], CAPP-calmodulin [8], calmodulin-stimulated cyclic nucleotide phosphodiesterase [10] and calcineurin [11] were prepared according to published methods. Dephosphorylated chicken gizzard smooth muscle myosin light chains [12], and myosin kinase [13] were gifts of Dr David R. Hathaway, Indiana University School of Medicine.

cAMP phosphodiesterase was assayed after a 40 min incubation at 30°C as in [8]. The reaction was initiated by addition of enzyme $5 \times 10^{-11}$ M). Myosin kinase incubations [13] were at 37°C for 15 min. The reaction mixture (0.06 ml) contained 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, $10^{-5}$ M myosin light chains, 0.4 mM $Ca^{2+}$ or 2 mM EGTA, 0.1 mM [$\gamma$-$^{32}$P]ATP ($4.0 \times 10^5$ cpm/nmol) in the presence or absence of calmodulin ($10^{-5}$–$10^{-10}$ M). The reaction was initiated by the addition of en-

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zyme ($10^{-10}$ M) and was terminated by applying 50-$\mu$l aliquots to Whatman 3 MM filter discs which were immediately immersed in chilled 10% trichloroacetic acid containing 40 mM sodium pyrophosphate. The filter discs were washed 3 times with trichloroacetic acid and once with 95% EtOH (15 min each time) and were counted in a Beckman LS 335 scintillation spectrometer after addition of 7.5 ml aquasol. Calcineurin was assayed using *p*-nitrophenylphosphate as substrate [9]. Calcineurin concentration was $10^{-8}$ M. The reaction was initiated by the addition of substrate to a final concentration of 2.5 mM. The linear rate of appearance of *p*-nitrophenolate was monitored at 400 nm at 23°C over 15 min. Appropriate aliquots of TFP (stock solution, 5.6 mM in $H_2O$) or CAPP-calmodulin (stock solution, $2.3 \times 10^{-5}$ M in 0.05 M $NH_4HCO_3$) were added as indicated. Serial dilutions of TFP were made in $H_2O$ and those of CAPP-calmodulin in 0.5 mg/ml bovine serum albumin.

## 3. RESULTS

In the experiments reported here, the activity of TFP is compared with that of CAPP-calmodulin. TFP rather than CAPP was used as the phenothiazine antagonist because TFP is more soluble than CAPP and has a $K_i$ value similar to that of CAPP as an antagonist of calmodulin. CAPP-calmodulin and TFP inhibit the stimulation of phosphodiesterase by calmodulin (fig.1). The inhibition is competitive in both cases. TFP inhibition was completely overcome by increasing the concentration of calmodulin to $10^{-5}$ M. In the case of CAPP-calmodulin, complete reversal was observed only when the inhibitor was present at less than $10^{-6}$ M (not shown). CAPP-calmodulin was about 50 times more potent than TFP in antagonizing the calmodulin stimulation of the enzyme. Assuming simple competitive inhibition, the following kinetic constants were obtained: $K_a$ for calmodulin, $3 \pm 0.5 \times 10^{-9}$ M; $K_i$ for CAPP-calmodulin, $3.0 \pm 1.0 \times 10^{-8}$ M; $K_i$ for TFP, $1.5 \pm 0.3 \times 10^{-6}$ M (the $K_i$ value for CAPP is $0.9 \pm 0.2 \times 10^{-6}$ M).

Similar results were obtained with myosin kinase (fig.2). Again CAPP-calmodulin was more effective (about 300-fold) than TFP in preventing activation by calmodulin, and the inhibition was

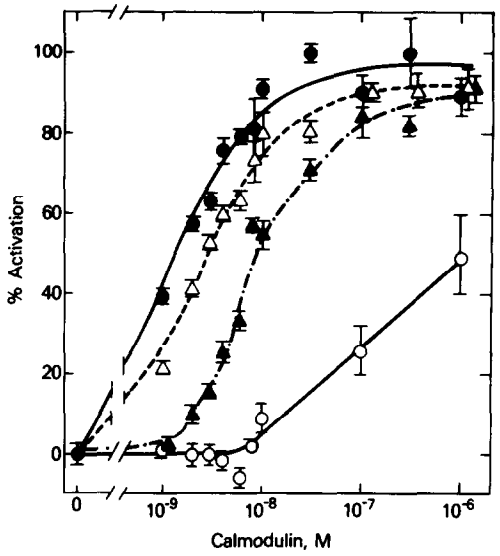


Fig. 1. Inhibition of the calmodulin stimulation of phosphodiesterase by TFP and CAPP-calmodulin. cAMP phosphodiesterase was assayed as described in section 2. Activation of phosphodiesterase by calmodulin (●); inhibition of calmodulin stimulation of phosphodiesterase by $10^{-5}$ M TFP (▲), and $10^{-6}$ M TFP (△) or $10^{-6}$ M CAPP-calmodulin (○). Each point is representative of triplicate determinations.

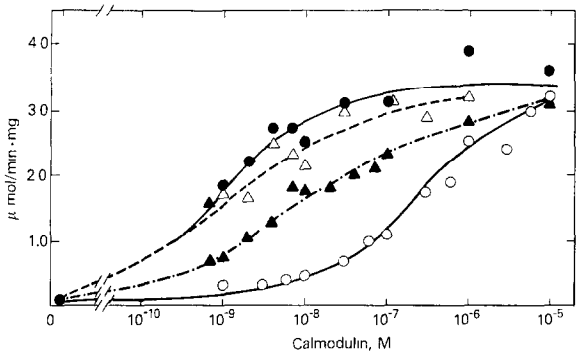


Fig.2. Inhibition of calmodulin stimulation of myosin kinase by TFP and CAPP-calmodulin. Myosin kinase was assayed as described in section 2. Activation by calmodulin (●); inhibition of calmodulin activation by $10^{-5}$ M TFP (▲), and $10^{-6}$ M TFP (△) or $10^{-6}$ M CAPP-calmodulin (○).

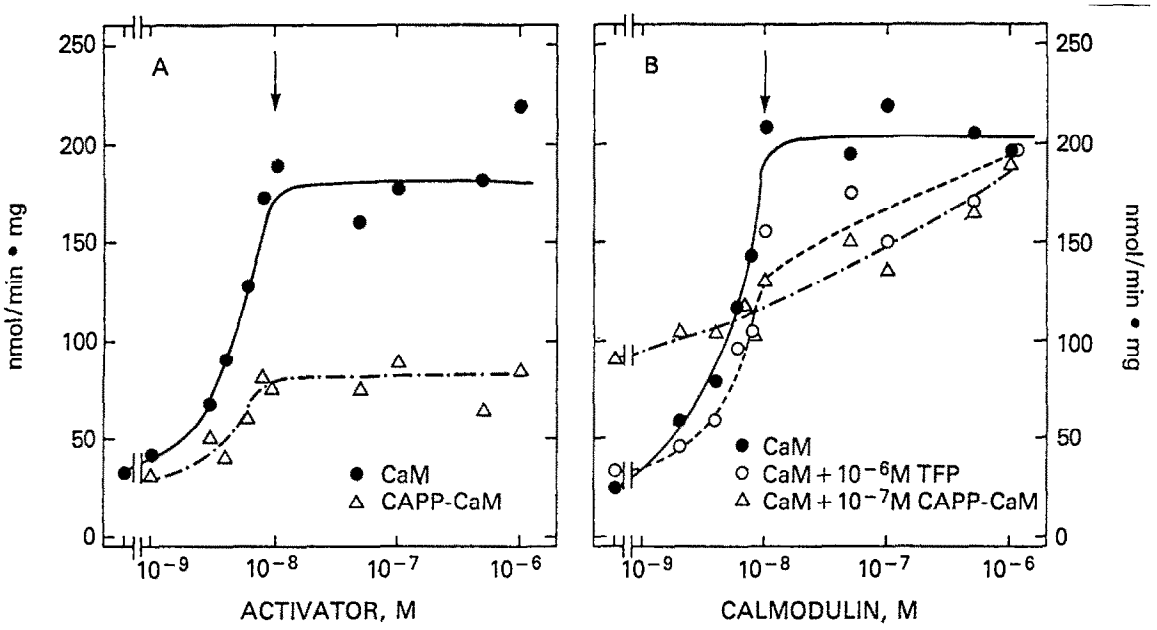


Fig.3. (A) Stimulation of calcineurin phosphatase activity by calmodulin and CAPP-calmodulin. Calcineurin was assayed as described in section 2. (B) Inhibition of calmodulin stimulation of calcineurin by TFP and CAPP-calmodulin. The arrows indicate the concentration of calcineurin in the assay mixture.

competitive. Based on the same assumption, a $K_a$ value for calmodulin of $7 \pm 2 \times 10^{-9}$ M, a $K_i$ value for CAPP-calmodulin of $3.4 \pm 1.0 \times 10^{-9}$ M and a $K_i$ value for TFP of $1.0 \pm 0.4 \times 10^{-6}$ M were obtained.

TFP and CAPP-calmodulin were also inhibitors of the calmodulin stimulation of calcineurin, a phosphoprotein phosphatase [14], but with more complex kinetics. Whereas CAPP-calmodulin does not activate phosphodiesterase or myosin kinase (fig.1,2), it does activate calcineurin to 40–50% of the maximal level achieved by calmodulin (fig.3A). The activation is $Ca^{2+}$-dependent. In contrast, TFP is unable to activate calcineurin in the presence or absence of $Ca^{2+}$. Because CAPP-calmodulin activation of calcineurin is only partial, it becomes inhibitory at calmodulin concentrations high enough to produce near maximal activation (fig.3B). As observed with phosphodiesterase and myosin kinase, the inhibition can be overcome by sufficiently high calmodulin concentrations (fig.3B). The kinetic constants were not calculated here because the activation curves were determined at $10^{-8}$ M calcineurin so that the concentration of free calmodulin was far from the amount added. Under these conditions, the affinities of CAPP-calmodulin and calmodulin for calcineurin were both $\leq 5 \times 10^{-9}$ M.

## 4. DISCUSSION

The covalent linking of 1 mol of the antipsychotic drug, norchlorpromazine, to 1 mol of calmodulin transforms this low affinity and nonspecific inhibitor of calmodulin into a high affinity and specific calmodulin antagonist. Whereas TFP (or CAPP) inhibits calmodulin-regulated reactions with a $K_i$ value near $10^{-6}$ M, CAPP-calmodulin inhibits the same reactions with a $K_i$ value 2–3 orders of magnitude lower ($3 \times 10^{-8}$ to $3 \times 10^{-9}$ M). Thus, the one to one phenothiazine–calmodulin complex interacts with the 3 enzymes studied with an affinity only slightly lower than that of calmodulin. Phenothiazines can therefore inhibit calmodulin actions in two ways. The first mechanism, as proposed by other investigators [15–17], involves the formation of a calmodulin–phenothiazine complex which cannot interact with the enzyme, thereby preventing enzyme activation. A second mechanism of inhibition, as shown here,

is by forming a phenothiazine-calmodulin complex which will interact with the enzyme with a relatively high affinity via its calmodulin moiety but either does not activate the enzyme (as with phosphodiesterase and myosin kinase) or activates the enzyme only partially (as with calcineurin). Whether one or both types of mechanisms of inhibition operate normally is not yet clear. It is clear, however, that CAPP-calmodulin is a much more specific probe of calmodulin function than are the phenothiazines themselves.

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