# Calcium inhibition of a heat-stable cyclic nucleotide phosphodiesterase from *Neurospora crassa*

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*Neurospora crassa* had a heat-stable (up to 95°C), soluble cyclic nucleotide phosphodiesterase (PDE). Both unheated and heat-stable PDE activities were inhibited by micromolar concentrations of $Ca^{2+}$. This inhibition was reversed by EGTA or EDTA in molar excess of the $Ca^{2+}$ concentration. Calmodulin was not involved in the $Ca^{2+}$ inhibition, nor was $Ca^{2+}$ inhibition of the heat-stable PDE due to cleavage inactivation of the enzyme by a $Ca^{2+}$-stimulated protease. In addition to $Ca^{2+}$, several other cations inhibited the activity of the heat-stable enzyme. Cyclic AMP and cGMP, but not 2'3'cAMP were substrates for both unheated and heat-stable PDEs. This is the first report of a PDE which is inhibited by micromolar concentrations of $Ca^{2+}$.

Neurospora crassa *Cyclic nucleotide phosphodiesterase* *$Ca^{2+}$ inhibition* *Heat stability*

## 1. INTRODUCTION

*Neurospora crassa* contains cAMP, cGMP [1], adenylate cyclase [2], and cAMP-dependent protein kinase [3,4]. Cyclic nucleotide phosphodiesterase (PDE) (EC 3.1.4.17) in *Neurospora* has not been extensively studied. In [5] it was reported that a particulate cAMP PDE was inhibited by EDTA in a crude mycelial extract of *Neurospora*, and recently the authors in [6] reported the partial purification of two forms of soluble PDE from the same organism.

We are studying the possible interaction of cyclic nucleotides and blue light-induced carotenogenesis in *Neurospora* [7], and as part of this work we required more data on the PDEs in this organism. The present paper reports some properties of a heat-stable $Ca^{2+}$-inhibited PDE from *Neurospora*. This is the first report of a PDE which is inhibited by micromolar concentrations of $Ca^{2+}$. Calmodulin, the $Ca^{2+}$-dependent regulatory protein, which mediates the activation of PDE in many animal tissues (for reviews see [8,9]), has also been characterised in *Neurospora* [10]. However, calmodulin is not involved in the $Ca^{2+}$ inhibition of heat-stable *Neurospora* PDE.

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*Abbreviations:* PMSF, phenylmethylsulfonyl fluoride; EGTA, ethyleneglycol-bis-(β-aminoethyl ether) *N,N'*-tetraacetic acid; TLC, thin-layer chromatography

## 2. MATERIALS AND METHODS

### 2.1. *Materials*

Chemicals and calmodulin-deficient bovine PDE were obtained from Sigma; [8-$^3$H] cAMP (26.0 Ci/mmol) and [8-$^3$H] cGMP (18.3 Ci/mmol) from Amersham; [8-$^3$H] 2'3'cAMP (21.2 Ci/mmol) from New England Nuclear; TLC plates from E. Merck (Darmstadt); and scintillation fluid (Ultrafluor) was from National Diagnostics.

### 2.2. *Growth of* N. crassa

Erlenmeyer flasks (4 l), each containing 2 l of Vogel's [11] Minimal Medium N supplemented

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with 2% sucrose, were each inoculated with $5 \times 10^7$ conidia of wild-type *N. crassa*, strain St. Lawrence 74A, Fungal Genetics Stock Center (FGSC) number 987, obtained from the FGSC, Humboldt State University Foundation (Arcata, CA). Cultures were shaken at 175 revolutions/min in the dark at 18°C for 5 days. Mycelia were harvested by filtration, washed with deionized water, frozen at −70°C, and freeze-dried.

### 2.3. *Preparation of phosphodiesterase*

Lyophilized mycelia were ground in a mortar, and extracted twice for 30 min with 10 ml/g ice-cold buffer solution containing 100 mM Tris–HCl (pH 7.4), 100 μM $CaCl_2$, 1 mM PMSF and 2 mM benzamidine. Subsequent steps were done between 4°C and 6°C unless otherwise stated. After each extraction the homogenate was centrifuged at 12000 × *g* for 15 min. The combined supernatants were centrifuged at 113000 × *g* for 1 h. The resulting clear supernatant was termed the soluble phosphodiesterase extract. The soluble PDE extract was heat-treated by bringing it to the desired temperature in a water bath, and incubating it at that temperature for 5 min. The extract was then cooled and centrifuged for 15 min at 12000 × *g*. The resulting clear supernatants were stored at −20°C and thawed before use in the assays. The PMSF concentration of the 80°C-treated supernatant, which was used in the majority of the work was readjusted to 1 mM after heating. PMSF has a half-life of 55 min at pH 7.5 and 25°C [12], and we assumed that all the PMSF initially present in the extraction buffer was destroyed by the heat treatment. The term 'heat-stable PDE' refers to the enzyme prepared by heating at 80°C in buffer containing 100 μM $Ca^{2+}$.

### 2.4. *Phosphodiesterase assays*

The reaction mixture contained, in a final volume of 100 μl: 100 mM Tris–HCl (pH 7.4); 25 μM $CaCl_2$; 5 mM $MgCl_2$; an appropriate amount of enzyme, plus any additions. For substrate concentrations higher than 2.0 μM, 50 pmol of $^3$H-labelled substrate were added, and the amount of unlabelled substrate was varied. For lower substrate concentrations, the amount of $^3$H-labelled substrate was varied, and no unlabelled substrate was added. Unless otherwise stated, the substrate used was 200 μM cAMP. Reaction mixtures were incubated at 25°C for 10 min, substrate added and incubation continued at 25°C. Reactions were stopped by adding 400 μl of methanol, and 15 μl of TLC carrier solution containing 25 mM each of 5′AMP, cAMP, adenosine, and inosine (for assays in which cAMP was used as substrate); 5′GMP, cGMP and guanosine (cGMP used as substrate); or 3′AMP, 2′3′cAMP, adenosine and inosine (2′3′cAMP used as substrate). Incubation tubes were then centrifuged at 12000 × *g* for 5 min to remove precipitated protein, and 3 μl of the supernatant spotted for TLC. Reaction substrate and products were separated by TLC [13] with the following modifications: 50 mM KCl was used as solvent for assays in which cAMP or 2′3′cAMP was used as substrate and 100 mM KCl for cGMP. One half ml of KCl (1 M) was put in each scintillation vial with the nucleotide or nucleoside spots cut out from the TLC sheets, the vials shaken, and 5 ml of scintillation fluid added. Radioactivity was determined by scintillation counting.

### 2.5. *Preparation of 'crude'* Neurospora *calmodulin*

The soluble PDE extract was heated to 100°C for 10 min, cooled and centrifuged to remove precipitated protein. The resulting supernatant contained no PDE activity but activated calmodulin-deficient bovine PDE in the presence of $Ca^{2+}$.

### 2.6. *Analytical methods*

Proteins were determined as in [14] using bovine serum albumin as a standard.

## 3. RESULTS AND DISCUSSION

Fig.1 shows the effect of pretreatment at various temperatures on the soluble PDE activity of *Neurospora*. The heat treatments shown in fig.1a were done in the presence of $Ca^{2+}$, whereas those in fig.1b were done in a molar excess of EGTA. When the extract was heated and PDE assayed in the presence of $Ca^{2+}$ (fig.1a), activity was maximal after heating up to 80–90°C. Activity was decreased by heating at higher temperatures and was zero after heating at 100°C. When the extract was heated in the presence of $Ca^{2+}$, but PDE assayed with an 8-fold molar excess of EGTA, activity was

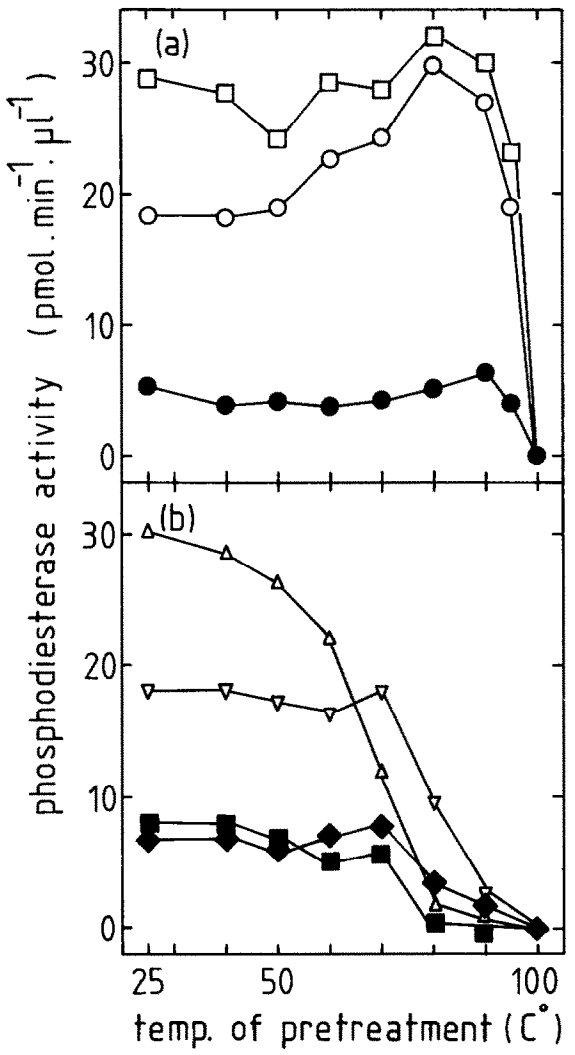


Fig.1. Effect of heating on the soluble PDE activity of *Neurospora*. (a) The enzyme extract was heated at the indicated temperatures in 100 µM $Ca^{2+}$. Extracts were then assayed in: 25 µM $Ca^{2+}$ (●—●); or 25 µM $Ca^{2+}$ and 8-fold molar excess EGTA – 200 µM (○—○); or 25 µM $Ca^{2+}$ and 40-fold molar excess EGTA – 1000 µM (□—□); (b) The enzyme extract was heated at the indicated temperatures in: 14-fold molar excess of EGTA – 700 µM EGTA, 50 µM $Ca^{2+}$ – and assayed in a molar excess of $Ca^{2+}$ – 525 µM $Ca^{2+}$, 200 µM EGTA (◆—◆); 14-fold molar excess of EGTA – 700 µM EGTA, 50 µM $Ca^{2+}$ – and assayed in an 8-fold molar excess of EGTA – 200 µM EGTA, 25 µM $Ca^{2+}$ (▽—▽); 40-fold molar excess of EGTA – 2000 µM EGTA, 50 µM $Ca^{2+}$ – and assayed in a molar excess of $Ca^{2+}$ – 2500 µM $Ca^{2+}$, 1000 µM EGTA (■—■); 40-fold molar excess EGTA – 2000 µM EGTA, 50 µM $Ca^{2+}$ – and assayed in a 40-fold excess of EGTA – 1000 µM EGTA, 25 µM $Ca^{2+}$ (△—△).

at least 3-times higher for all temperatures studied except at 100°C. Maximum stimulation was seen after heating up to 80–90°C. When the concentration of EGTA used in the assay was increased from an 8-fold to a 40-fold molar excess, the activity of the unheated (25°C) extract almost doubled, whereas the PDE activity in the extracts heated between 70°C and 95°C only increased slightly. These results showed that the enzyme was inhibited by $Ca^{2+}$, and that the $Ca^{2+}$ binding site may have been denatured at a lower temperature than the catalytic site, since:

(i) activity increased slightly after heating up to 80–90°C in $Ca^{2+}$ when the enzyme was then assayed in $Ca^{2+}$; and

(ii) less EGTA was required to give near maximum stimulation of the enzyme after it had been heated.

Similar results (fig.1b) were seen when the extract was heated with a molar excess of EGTA, and either assayed with a molar excess of EGTA or $Ca^{2+}$. However, activity was decreased by heating at temperatures above 70°C, indicating that the enzyme was more heat-stable in the presence of $Ca^{2+}$. Addition of EDTA (200 µM) produced stimulation similar to that produced by EGTA of both unheated (25°C) and heated (80°C) PDE (not shown).

Fig.2 shows stimulation by EGTA of the heat-stable PDE. At both $Ca^{2+}$ concentrations used, 25 µM and 125 µM, a molar excess of EGTA was required for any reversal of inhibition, and an approximate 8-fold molar excess of EGTA was needed to give stimulation approaching maximum. This showed that the inhibition was mainly due to $Ca^{2+}$, not some other metal present in the extract, and that a large molar excess of EGTA was required for full activation of the enzyme. When the enzyme was extracted in buffer to which no $Ca^{2+}$ had been added, the trace amounts of $Ca^{2+}$, or other metals present, were sufficient to fully inhibit the enzyme. This inhibition could be reversed by EGTA or EDTA.

To determine whether the $Ca^{2+}$ inhibition was due to the presence of a $Ca^{2+}$-stimulated protease in the heated extract, two experiments were done. In the first, the heat-stable PDE was preincubated and assayed in the presence of $Ca^{2+}$, with and without an additional protease inhibitor, leupeptin (10 µM). The resulting activities were the same. In

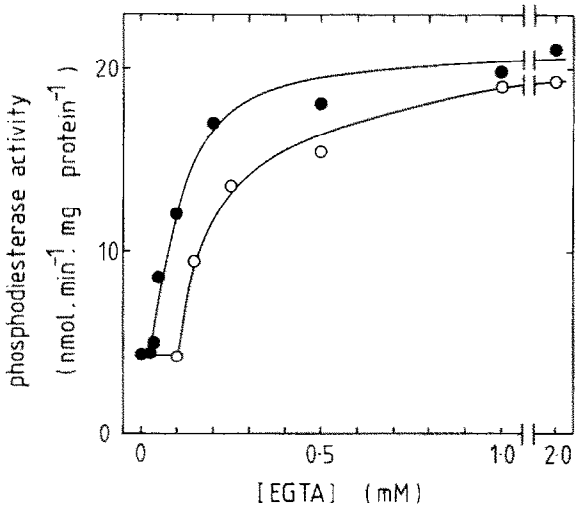


Fig.2. Activation by EGTA of the heat-stable PDE of *Neurospora*. The assay mixture was preincubated in the presence of the EGTA concentrations indicated for 10 min, and the incubations started by the addition of substrate. The $Ca^{2+}$ concentrations in the assays were 25 $\mu$M (●—●) or 125 $\mu$M (○—○).

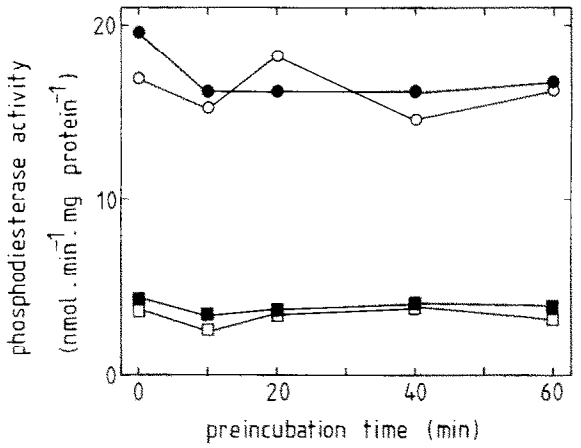


Fig.3. Effect of preincubation time in the presence of a molar excess of $Ca^{2+}$ or EGTA, on the activity of the heat-stable PDE. Assay was carried out in 200 $\mu$M EGTA, 25 $\mu$M $Ca^{2+}$ with preincubation in 40 $\mu$M $Ca^{2+}$ (○—○); or in 40 $\mu$M $Ca^{2+}$, 290 $\mu$M EGTA (●—●); or assay was carried out at 320 $\mu$M $Ca^{2+}$, 200 $\mu$M EGTA with preincubation in 40 $\mu$M $Ca^{2+}$ (□—□); or in 40 $\mu$M $Ca^{2+}$, 290 $\mu$M EGTA (■—■).

the second experiment the enzyme was preincubated in EGTA or $Ca^{2+}$ for various times up to 1 h, before assay in either excess EGTA or $Ca^{2+}$. The results (fig.3) showed that preincubation in $Ca^{2+}$ or EGTA had no effect on the activity of the enzyme when it was subsequently assayed. If a $Ca^{2+}$-stimulated protease were present, the activity of the enzyme would be expected to decline in proportion to the length of preincubation in the presence of $Ca^{2+}$, and not decline when preincubated with EGTA. These results provide evidence that the $Ca^{2+}$ inhibition is not caused by a $Ca^{2+}$-stimulated protease.

Calmodulin is required for the regulation of $Ca^{2+}$-stimulated PDEs [8,9], and thus it was possible that the $Ca^{2+}$ inhibition observed with *Neurospora* PDE could be mediated by calmodulin. Chlorpromazine is a member of the phenothiazine family of antipsychotic drugs which binds calmodulin in a $Ca^{2+}$-dependent manner and thus blocks the $Ca^{2+}$-activation of PDE [15]. The heat-stable PDE preparation contained calmodulin, but there was no effect of chlorpromazine (10 mM) on the inhibition of the heat-stable enzyme by $Ca^{2+}$. As a control the activation of calmodulin-dependent bovine PDE by a crude preparation of *Neurospora* calmodulin was blocked by the same concentration of chlorpromazine. These results showed that calmodulin does not mediate the $Ca^{2+}$-dependent inhibition of *Neurospora* PDE. However, calmodulin in the cell might indirectly control the activity of the $Ca^{2+}$-inhibited PDE by binding free $Ca^{2+}$.

Table 1 shows the effects of several cations on the heat-stable PDE activity. The enzyme was inhibited by cations other than $Ca^{2+}$. A common feature of those cations which produced inhibition of equal magnitude to that of $Ca^{2+}$ was that they (and $Ca^{2+}$) have one or two electrons in the outer (N) shell.

The above results are consistent with the following model. *Neurospora* has a heat-stable PDE which is inhibited by calcium and certain other cations. Calcium binds to the enzyme, or to a regulatory protein which then binds to the enzyme, and causes inhibition. The affinity of the $Ca^{2+}$-binding site for $Ca^{2+}$ is high, as shown by the amount of EGTA needed to cause maximum stimulation. $Ca^{2+}$ confers increased heat stability on the enzyme, and the $Ca^{2+}$-binding site is more susceptible to thermal inactivation than the catalytic site.

Both unheated and heat-stable PDE used both cAMP and cGMP, but not 2'3'cAMP as

Table 1

Effect of various cations on the heat-stable PDE from *Neurospora*

| Addition | PDE activity (nmol.min$^{-1}$.mg protein$^{-1}$) |
|---|---|
| Control | 17.0 |
| $Ca^{2+}$ | 3.9 |
| $Mn^{2+}$ | 3.2 |
| $Co^{2+}$ | 2.6 |
| $Ni^{2+}$ | 2.2 |
| $Cu^{2+}$ | 3.4 |
| $Zn^{2+}$ | 2.7 |
| $Sn^{2+}$ | 13.7 |
| $Cs^{+}$ | 15.0 |
| $Ba^{2+}$ | 9.0 |
| $La^{3+}$ | 10.6 |

The reaction mixture was preincubated for 10 min in 200 $\mu$M EGTA to chelate the $Ca^{2+}$ (25 $\mu$M) present. The cations (1 mM) were then added as chloride salts and incubated for another 10 min. At the end of the second 10 min, substrate was added. No cation was added to the control which contained 25 $\mu$M $Ca^{2+}$ and 200 $\mu$M EGTA. All incubation mixtures contained 5 mM $Mg^{2+}$. No stimulation or inhibition of PDE activity by $Mg^{2+}$ was observed. The results are the mean of 3 determinations

substrates. EGTA increased the enzyme activity when either cAMP or cGMP was used as substrate. The protein concentration of the unheated extract was 7.0 mg.ml$^{-1}$, whereas that of the heated (80°C) extract was 1.3 mg.ml$^{-1}$. At these concentrations both extracts were stable to freezing at −20°C. The unheated, but not the heated enzyme extract contained 5′ nucleotidase.

From a practical standpoint the results have several important implications. The heat-stable $Ca^{2+}$-stimulated enzyme is present in unheated extracts and may have been previously studied [6]. In the same investigation, EDTA was present in the enzyme extracts, and we have shown that kinetic results will be affected by the concentration of $Ca^{2+}$, EGTA, or EDTA used in assays. Also the procedure often employed by investigators to stop enzyme reactions (heating in a boiling water bath), may give erroneous results in *Neurospora* PDE studies if the temperature of the reaction mixture is not instantaneously brought to 100°C. The error would be especially high for short incubation times.

Several $Ca^{2+}$-inhibited adenylate cyclases have been reported (e.g., [16]), and a heat-stable PDE is present in bovine pineal gland [17]. Many PDEs are $Ca^{2+}$-activated, and this effect is mediated by calmodulin. However, this paper is the first report of a PDE which is inhibited by micromolar concentrations of $Ca^{2+}$ and a further example of the interaction of $Ca^{2+}$ with cyclic nucleotide systems. This work also raises the question of whether there are similar $Ca^{2+}$-inhibited PDEs in, for example, animal tissues.

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