

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.

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

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.

2. MATERIALS AND METHODS

2.1. Materials

Chemicals and calmodulin-deficient bovine PDE were obtained from Sigma; [$8\text{-}^3\text{H}$] cAMP (26.0 Ci/mmol) and [$8\text{-}^3\text{H}$] cGMP (18.3 Ci/mmol) from Amersham; [$8\text{-}^3\text{H}$] 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

with 2% sucrose, were each inoculated with 5×10^7 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 μ M CaCl₂, 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 $12000 \times g$ for 15 min. The combined supernatants were centrifuged at $113000 \times g$ 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 $12000 \times g$. 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 μ M Ca²⁺.

2.4. Phosphodiesterase assays

The reaction mixture contained, in a final volume of 100 μ l: 100 mM Tris-HCl (pH 7.4); 25 μ M CaCl₂; 5 mM MgCl₂; an appropriate amount of enzyme, plus any additions. For substrate concentrations higher than 2.0 μ M, 50 pmol of ³H-labelled substrate were added, and the amount of unlabelled substrate was varied. For lower substrate concentrations, the amount of ³H-labelled substrate was varied, and no unlabelled substrate was added. Unless otherwise stated, the substrate used was 200 μ M cAMP. Reaction mix-

tures were incubated at 25°C for 10 min, substrate added and incubation continued at 25°C. Reactions were stopped by adding 400 μ l of methanol, and 15 μ l 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 $12000 \times g$ for 5 min to remove precipitated protein, and 3 μ l 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 Ca²⁺.

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 Ca²⁺, 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 Ca²⁺ (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 Ca²⁺, but PDE assayed with an 8-fold molar excess of EGTA, activity was

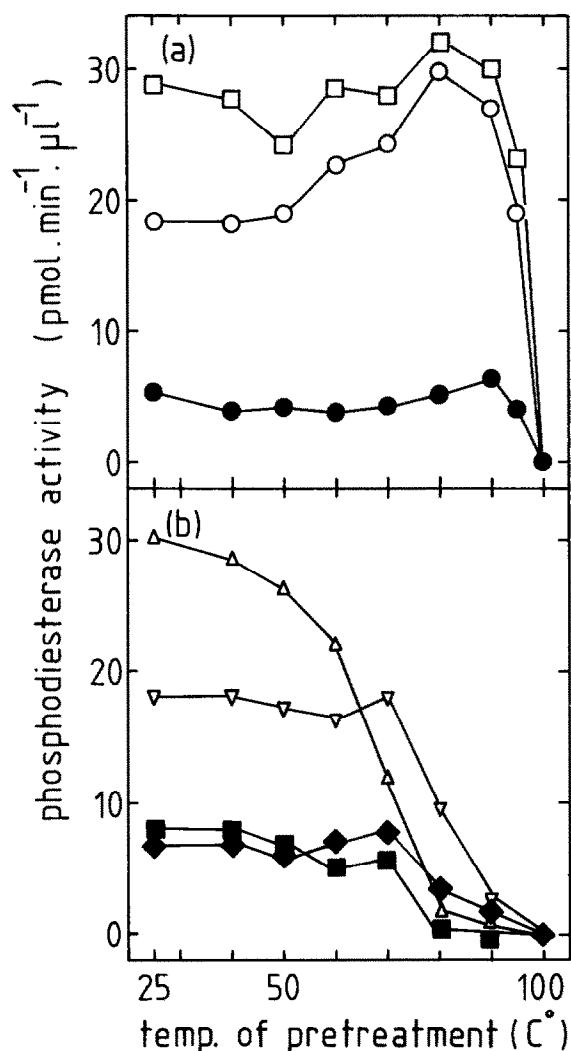


Fig.1. Effect of heating on the soluble PDE activity of *Neurospora*. (a) The enzyme extract was heated at the indicated temperatures in 100 μM Ca^{2+} . Extracts were then assayed in: 25 μM Ca^{2+} (●—●); or 25 μM Ca^{2+} and 8-fold molar excess EGTA — 200 μM (○—○); or 25 μM Ca^{2+} and 40-fold molar 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

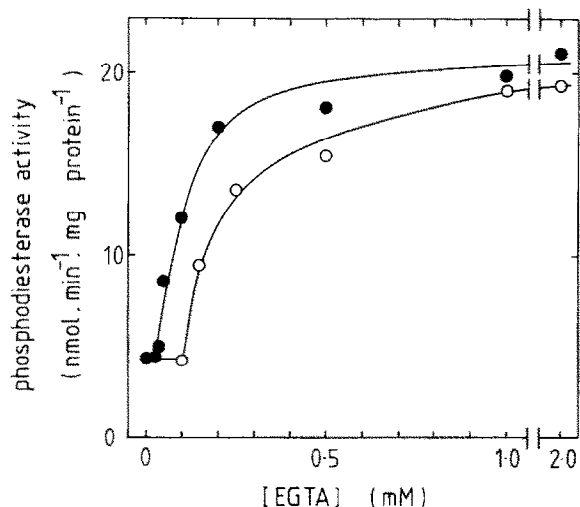


Fig. 2. Activation by EGTA of the heat-stable PDE of *Neurospora*. The assay mixture was preincubated in the presence of the EGTA concentrations indicated for 10 min, and the incubations started by the addition of substrate. The Ca^{2+} concentrations in the assays were 25 μM (●—●) or 125 μM (○—○).

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 block-

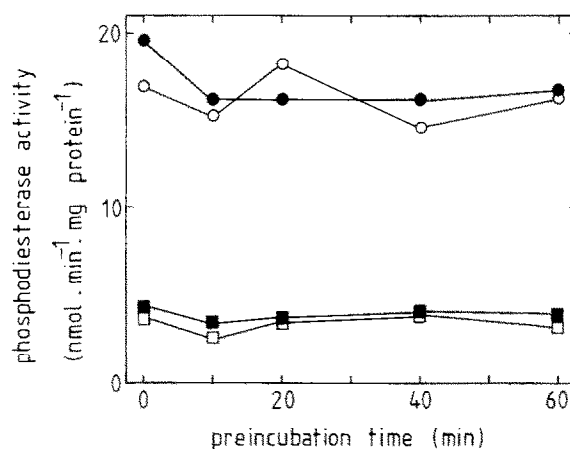


Fig. 3. Effect of preincubation time in the presence of a molar excess of Ca^{2+} or EGTA, on the activity of the heat-stable PDE. Assay was carried out in 200 μM EGTA, 25 μM Ca^{2+} with preincubation in 40 μM Ca^{2+} (○—○); or in 40 μM Ca^{2+} , 290 μM EGTA (●—●); or assay was carried out at 320 μM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

ed 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 μ M EGTA to chelate the Ca²⁺ (25 μ 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 μ M Ca²⁺ and 200 μ M EGTA. All incubation mixtures contained 5 mM Mg²⁺. No stimulation or inhibition of PDE activity by Mg²⁺ 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 Ca²⁺-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 Ca²⁺, 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 Ca²⁺-inhibited adenylate cyclases have been reported (e.g., [16]), and a heat-stable PDE is present in bovine pineal gland [17]. Many PDEs are Ca²⁺-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 Ca²⁺ and a further example of the interaction of Ca²⁺ with cyclic nucleotide systems. This work also raises the question of whether there are similar Ca²⁺-inhibited PDEs in, for example, animal tissues.

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