Inhibition of 2′-phosphodiesterase by cAMP-dependent protein kinase

Involvement of phosphorylation of protein inhibitor

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2′-Phosphodiesterase from NIH 3T3 cells was purified about 530-fold. Treatment of the cell lysate with the cAMP-dependent protein kinase causing the 2′-phosphodiesterase inhibition did not result in phosphorylation of the enzyme itself. The kinase was found to phosphorylate a specific 18-kDa protein, the phosphorylated form of this protein being the inhibitor of 2′-phosphodiesterase.

cAMP-dependent protein kinase *2′-Phosphodiesterase* *2′-Phosphodiesterase protein inhibitor*

1. INTRODUCTION

2′-Phosphodiesterase (2′-PDE), the enzyme which hydrolyzes 2′,5′-oligoadenylates (2′,5′-oligo(A)) to ATP and AMP, is involved in the regulation of biological processes associated with variations of the intracellular 2′,5′-oligo(A) level including interferon action and alterations in the proliferative status of cells [1].

We have previously shown that 2′-PDE activity in the NIH 3T3 cells decreases significantly as a result of the elevation of the intracellular cAMP level. The treatment of the cell lysates with the homogeneous preparation of the catalytic subunit of the cAMP-dependent protein kinase (in the presence of ATP) causes the inhibition of 2′-PDE activity as well [2].

The present results show that the inhibition of 2′-PDE by cAMP-dependent protein kinase involves a specific 18-kDa protein inhibitor of 2′-PDE. Phosphorylation of this protein in cell lysate results in the enhancement of its inhibitory activity. It is suggested that the previously described cAMP-induced decrease in 2′-PDE activity in NIH 3T3 cells is mediated by the protein inhibitor.

2. METHODS

2.1. *Cell culture*

NIH 3T3 cells were cultivated in the mixture of media Eagle's: 199 (1:1) containing 10% calf serum (Serva, FRG). Rapidly growing cells (≥24 h before confluence) were used for homogenate preparation.

2.2. *Treatment of the cell homogenate with protein kinase*

The highly purified preparation of the catalytic subunit of cAMP-dependent protein kinase from pig brain was a kind gift of Dr S.N. Kochetkov (Institute of Molecular Biology, USSR Academy of Sciences, Moscow). The solution of the catalytic subunit (100 μl, 0.4 mg/ml, 10 mM $MgCl_2$, 20 mM Tris–HCl, pH 7.2) was added to 1 ml cell homogenate simultaneously with [γ-^{32}P]ATP (10^{-6} M, 10^7 cpm/ml) and the mixture was incubated for 1 h at 37°C. Then unlabeled ATP (10^{-4} M) was added and the incubation was continued for 1 h. The treated homogenate, as well as the non-treated (control) homogenate, were used for DEAE-cellulose chromatography and subsequent procedures (see below).

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2.3. *Isolation of 2'-PDE*

The isolation of 2'-PDE was carried out as in [3] with modifications.

The cells (approx. 2.0×10^8) were detached from the glass with standard 0.02% Versene solution, collected by centrifugation, resuspended in buffer 1 (5 mM $MgCl_2$, 1 mM dithiothreitol, 10% glycerol, 20 mM Tris–HCl, pH 7.5), homogenized using a Potter homogenizer, and the homogenate obtained was centrifuged at $8000 \times g$ for 6 min.

The supernatant (10 ml, approx. 2 mg protein/ml) was applied to a DEAE–cellulose DE-32 (Whatman, England) column (1×5 cm) and eluted with a linear gradient (2×30 ml) of KCl (25–150 mM) based on buffer 1. The active fractions (eluted with 60–80 mM KCl) were applied to a phosphocellulose P-11 (Whatman) column (1×3 cm) and eluted with the linear gradient (2×20 ml) of KCl (25–800 mM) based on buffer 2 (1 mM dithiothreitol, 10% glycerol, 20 mM Tris–HCl, pH 6.7).

Active fractions (eluted with approx. 400 mM KCl) were diluted with 4 vols of buffer 2 containing 25 mM KCl, applied to a phosphocellulose P-11 column (0.3×1 cm) and eluted with 1 ml of buffer 2 containing 800 mM KCl and 10 mM $MgCl_2$ (this stage was absent in the original procedure [3]). The material obtained was applied to the Sephadex G-100 column (1×50 cm) (instead of chromatography on hydroxyapatite proposed originally [3]) and eluted with buffer 3 (150 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, 20 mM Tris–HCl, pH 7.5). The active fractions were collected, dialyzed against water and lyophilized.

2.4. *2'-PDE assay*

The 2'-PDE activity was assayed as in [2].

2.5. *Partial purification of 2'-PDE inhibitor*

The inhibitor-containing fractions were collected after elution of proteins of the cell homogenate treated with protein kinase, from DEAE-cellulose (see above). The collected fractions were applied to a Sephadex G-100 column (1×50 cm) and eluted with buffer 3. Inhibitor-containing fractions were collected, the bovine serum albumin (0.3 mg/ml) was added, the protein was precipitated with 10% trichloroacetic acid, washed with acetone and then used for electrophoretic analysis.

To assay the inhibitor activity, the fraction of non-phosphorylated protein eluted from the DEAE-cellulose column with 60–80 mM KCl (fig.1) was used as the partially purified enzyme preparation. The samples containing 10 μl of this enzyme preparation, 20 μl of 2',5'-[^{3}H]oligo(A) solution (approx. 2×10^3 cpm per sample) and 20 μl of the inhibitor fraction were incubated and 2'-PDE activity was assayed as usual [2].

Electrophoresis [4] and stain of the gels with $AgNO_3$ [5], were performed as described.

3. RESULTS AND DISCUSSION

3.1. *Isolation of 2'-PDE*

The treatment of the cell homogenate with the homogeneous catalytic subunit of cAMP-dependent protein kinase resulted in a 60% decrease in 2'-PDE activity as compared with the control homogenate which was incubated without protein kinase.

Separation of cell homogenate proteins using the DEAE-cellulose revealed two peaks of 2'-PDE activity, which were eluted at 60–80 mM (peak I) and 100–130 mM (peak II) KCl (fig.1). When the proteins, pretreated with protein kinase, were separated, peak I was significantly smaller and shifted to the right (fig.1). This indicated that the decrease in 2'-PDE activity after protein kinase treatment of the homogenate was associated, at

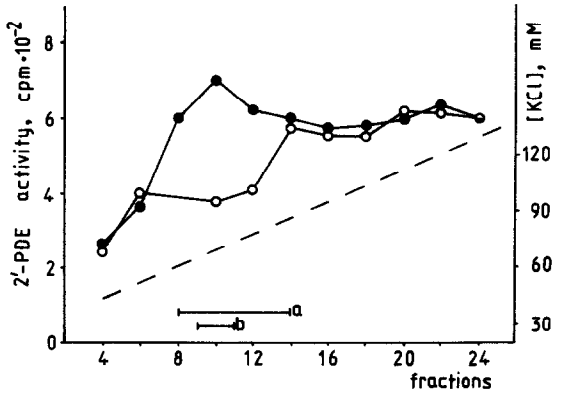

Fig.1. Separation of 2'-PDE from the cell homogenate treated (○) and not treated (●) with cAMP-dependent protein kinase. (– – –) KCl concentration; bracket a indicates the fractions collected for 2'-PDE purification, bracket b the fractions collected for inhibitor purification.

Table 1

Purification of 2′-PDE from NIH 3T3 cells

Stage	Protein (%)	General activity (%)	Specific activity (%)
Homogenate	100	100	1.0
DE-32	24.8	167	6.7
P-11	0.5	18	36
G-100	0.03	16	533

least partially, with the enzyme form which corresponds to peak I. To clarify the mechanism of the inhibition, we carried out the isolation of this form of 2′-PDE, referred to here as form I.

The result of purification of 2′-PDE from homogenate not treated with protein kinase is shown in table 1. The degree of 2′-PDE purification was about 530, similar to the value reported for L-cells [3]. Electrophoresis of the preparation obtained showed that this represented a virtually homogeneous protein of 47 kDa (fig.2). A similar homogeneous preparation of 2′-PDE was obtained from the homogenate treated with protein kinase. However, [^{32}P]phosphate was not detected in the 2′-PDE preparation. Thus, inhibition of 2′-PDE caused by the homogenate treatment with protein kinase did not involve direct phosphorylation of 2′-PDE form I.

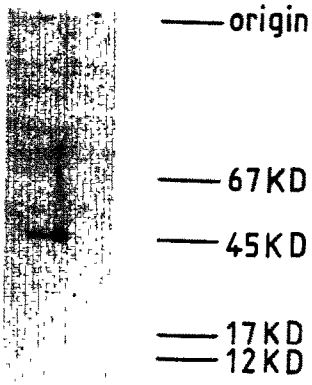

Fig.2. Electrophoresis of the 2′-PDE preparation. Positions of marker proteins (67 kDa, bovine serum albumin; 45 kDa, ovalbumin; 17 kDa, myoglobin; 12 kDa, cytochrome *c*) are indicated. KD, kDa.

3.2. *Partial purification of 2′-PDE inhibitor*

Since the 2′-PDE molecules were found to be non-phosphorylated we supposed that treatment of the cell homogenate with the protein kinase resulted in activation of a protein inhibitor of 2′-PDE, eluted from DEAE-cellulose at 50–70 mM KCl, that caused the observed difference in the elution profiles (fig.1) of 2′-PDE from the protein kinase-treated, as compared to the non-treated homogenate. To check this suggestion, we carried out the 2′-PDE assay in the presence of the hypothetical inhibitor (i.e., fractions 9–11, fig.1) from the protein kinase-treated homogenate. The results demonstrated that these fractions contain the 2′-PDE inhibitor (table 2); the activity in sample 2 is higher than in sample 1 because fractions 9–11 of the non-treated homogenate contain active 2′-PDE.

The gel-filtration of the inhibitor-containing fraction on Sephadex G-100 showed that the peak of inhibitory activity corresponded to 20 kDa (fig.3). One of the peaks of ^{32}P radioactivity coincided with the peak of inhibitory activity. Hence, there was good reason to suggest that direct phosphorylation of this protein with cAMP-dependent protein kinase resulted in an enhancement of its inhibitory activity.

Electrophoresis followed by autoradiography of the inhibitor fraction from the DEAE-cellulose column showed that the fraction contained three predominant phosphoproteins of 70, 32 and 18 kDa. The fraction of inhibitor obtained from the Sephadex G-100 column contained a single phosphoprotein of 18 kDa (fig.4).

Table 2

Activity of the partially purified preparation of 2′-PDE in the presence of the protein inhibitor fraction

Sample	2′-PDE activity (cpm)
1. 2′-PDE preparation + buffer 1	596
2. 2′-PDE preparation + inhibitor fraction from the homogenate not treated with protein kinase	870
3. 2′-PDE preparation + inhibitor fraction from the homogenate treated with protein kinase	233

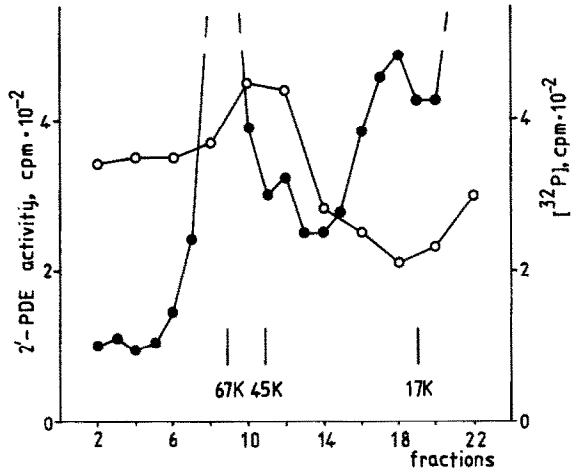

Fig.3. Separation of the inhibitory fraction on the Sephadex G-100 column. The ^{32}P radioactivity (●) and 2′-PDE activity in the presence of the fractions obtained (○) are represented. Positions of marker proteins are indicated. K, kDa.

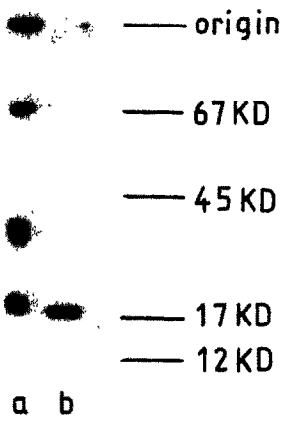

Fig.4. Autoradiogram of electrophoresis of the inhibitor preparation before (a) and after (b) purification on the Sephadex G-100 column. The positions of marker proteins are indicated. KD, kDa.

These data allow us to suggest that the 2′-PDE inhibition caused by the treatment of the cell homogenate with the catalytic subunit of the cAMP-dependent protein kinase, is not due to the direct phosphorylation of the enzyme itself. This effect appears to involve the phosphorylation of a 18-kDa protein, the phosphoform of this protein being the inhibitor of 2′-PDE.

It should be noted that the data obtained do not exclude the possibility of some other mechanism for 2′-PDE inhibition by the cAMP-dependent protein kinase. Further investigations are necessary to clarify whether or not the mechanism described is involved in the process of 2′-PDE inhibition in NIH 3T3 cells which is caused by the increase in the intracellular cAMP level [2]. Interestingly, however, a similar mechanism involved in the regulation of the phosphatase 1 [6] was found both in vitro and in vivo: the inhibitor of this enzyme (referred to as inhibitor 1) is activated by cAMP-dependent phosphorylation [6]. Our results allow us to suggest an analogous hypothetical scheme of regulation for the 2′-PDE.

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