Inhibition of nucleoside diphosphate kinase (NDPK/nm23) by cAMP analogues

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Received 27 October 1996

Abstract Nucleoside diphosphate kinase (NDPK/nm23) ATP/GDP phosphotransferase activity and serine autophosphorylation is inhibited by N^6-mbcAMP, 8-ClcAMP and 8-BrcAMP. Inhibition of the enzymatic activity largely depends on the concentration of ATP and becomes significant at ATP concentrations up to 0.5 mM and at effector concentrations measured in C6 cells stimulated with 1 mM cAMP analogue. N^6-mbcAMP is a substrate of the enzyme. DbcAMP and O'^2-mbcAMP, cAMP analogues with a modified O'^2-ribose, did not affect the NDPK activity. Cyclic AMP is only a moderate inhibitor of NDPK even at low ATP concentrations. Possible inhibitory effects of cAMP and cAMP analogues on reported extra- and intracellular functions of NDPK/nm23 are discussed.

Key words: cAMP; 8-Chloro cAMP; Nucleoside diphosphate kinase; nm23; Breast cancer

1. Introduction

Nucleoside diphosphate kinase (NDPK, EC 2.7.4.6) catalyzes the transphosphorylation of a terminal phosphate of triphosphate nucleotides to nucleoside diphosphates. In higher eucaryotes two distinct polypeptide chains have been identified. The acidic NDPK-A (β) and the basic NDPK-B (α) chain are encoded by the nm23-H1 and nm23-H2 genes, respectively [1–5].

The enzyme is present in most tissues and is the main extramitochondrial enzyme involved in the conversion of GDP into GTP at the expense of ATP (reviewed in [6]). The enzyme acts by formation of an unstable phosphohistidine intermediate [7], but recently a stable serine phosphorylation has also been detected that is not involved in the phosphotransferase reaction [8,9]. NDPK is located in several cell compartments and has even been found associated with the cell surface, suggesting that it may have several distinct intra- and extracellular functions. The enzyme may be functional in the GDP-GTP conversion on GTP-binding proteins [10,11] and has been proposed to be involved in lymphocyte and neuroblastoma proliferation [12,13] and in the inhibition of myeloid leukemia differentiation [14]. In human breast carcinoma the expression of the nm23-H1 gene has been inversely related with the metastatic phenotype [15], has been correlated with the inhibition of cell motility in response to serum, PDGF and IGF-I [16], and its overexpression leads to synthesis of basement proteins and to growth arrest [17]. Serine autophosphorylation of nm23-H1 has been related to its potential to suppress metastasis [9]. The nm23-H2 gene has been identified as the c-myc transcription factor PuF which does not require NDPK activity for its DNA binding [18]. These data clearly point to a function of the nm23 genes in the mechanism of cell proliferation and differentiation.

In this communication we present evidence that the phosphotransferase activity of NDPK/nm23 can be differentially inhibited intra- and extracellularly, and in normal and tumoral cells by some cAMP analogues. The inhibition by these effectors raises new perspectives on the elucidation of additional functions of NDPK/nm23 and on the use of cAMP analogues as anticancer agents.

2. Materials and methods

2.1. Materials

Rat C6 glioma (ATCC no. CCL 107) was obtained from Flow Laboratories (UK) and maintained in monolayer culture as described [19]. Soybean trypsin inhibitor (STI), aprotinin, ATP, GDP, AMP, cAMP, dbcAMP, O'^2-mbcAMP, N^6-mbcAMP, and 8-BrcAMP were obtained from Sigma Chemical Co. (USA); [γ-^{32}P]ATP, [β-^{32}P]GDP and 8-ClcAMP were from ICN Biomedicals (USA).

2.2. NDPK phosphotransferase assay

The standard NDPK phosphotransferase assay was performed in buffer A (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM $MgCl_2$), in the presence of different concentrations of ATP and GDP, and 0.1 Ci/ml [γ-^{32}P]ATP or [β-^{32}P]GDP as indicated in the figure legends. Incubation was at 37°C for 2.5, 5, 7.5 and 10 min. The reaction was stopped by addition of 50 mM EDTA, 1 μl of the assay mixture applied on a polyethyleneimine cellulose plate (Merck, Germany) and the nucleotides separated in 750 mM KH_2PO_4 (pH 3.65) by ascending chromatography. The radioactive spots were excised and counted. The reaction rates were calculated from the slope of the linear regression of the phosphotransfer at different time points. The standard deviation on the reaction rate is the error on the slope.

2.3. Serine autophosphorylation of NDPK

Autophosphorylation was performed in buffer B (25 mM HEPES (pH 7.3), 140 mM NaCl, 1 mM $MgCl_2$, 0.8 mM $CaCl_2$, 5 mM KCl, 5 mM EDTA) and in the presence of 20 nM [γ-^{32}P]ATP (4500 Ci/mmol). Incubation was at 37°C for 30 min. The reaction was stopped by addition of SDS-PAGE sample buffer to a final concentration of 0.5% (w/v) SDS, 10% (v/v) glycerol, 0.5% (v/v) 2-mercaptoethanol, 6 mM Tris-HCl (pH 6.8). After boiling of the samples for 5 min, they were analyzed by SDS-PAGE on a 20% (w/v) polyacrylamide gel. The gel was Coomassie stained (30 min in 0.1% (w/v) CBB-R250, 7.5% (v/v) acetic acid, 40% (v/v) methanol) and destained for 16 h by successive washes in 10% (v/v) acetic acid, 40% (v/v) methanol. After dehydration in 50% ethanol the gel was dried and autoradiographed.

2.4. Phosphoamino acid analysis

Identification of the autophosphorylated residue was carried out using a modified procedure of Boyle et al. [20]. Briefly, NDPK was

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Abbreviations: 8-BrcAMP, 8-bromo cAMP; 8-ClcAMP, 8-chloro cAMP; dbcAMP, N^6,O'^2-dibutyryl cAMP; IGF, insulin-like growth factor; N^6-mbcAMP, N^6-monobutyryl cAMP; O'^2-mbcAMP, O'^2-monobutyryl cAMP; PDGF, platelet-derived growth factor; TLC, thin-layer chromatography.

PII S0014-5793(96)01358-0

autophosphorylated as described, separated by SDS-PAGE and blotted onto an Immobilon P membrane. The labelled NDPK was excised and hydrolyzed in 6 N HCl at 110°C for 1 h. Two-dimensional phosphoamino acid analysis was performed by TLC.

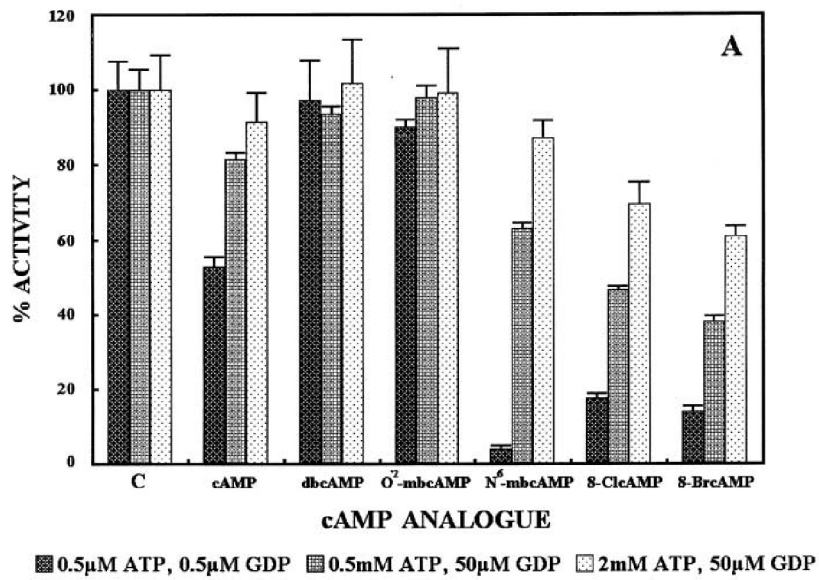
2.5. Purification of NDPK from C6

C6 cells were grown in serum-free chemically defined medium [19]. The cells were harvested in 50 mM EDTA in PBS, homogenized in a Potter Elvejhem (30 strokes, 1000 rpm, 4°C) alternated by sonication (15 min, 4°C). The homogenization buffer contained 20 mM Tris-HCl (pH 7.2), 10 mM KCl, 1 mM EDTA, 20 μg/ml STI and 5 μl/ml aprotinin. The cell extract was dialyzed against 50 mM Tris-HCl (pH 7.1) and insoluble material removed by centrifugation at 20000×*g* for 30 min. The homogenate was successively applied on a heparin-Sepharose CL 6B (210×10 mm; Pharmacia LKB, Sweden) and a DEAE-5PW column (75×7.5 mm; Waters, USA). Most NDPK was recovered from the flow through of both columns, of which the proteins were concentrated by binding to Bio-Gel-HTP hydroxyapatite (Bio-Rad Lab., USA) [19]. Further purification was by affinity chromatography on a cAMP-agarose column using a modified procedure of Weber et al. [21]. Briefly, the cyanogen bromide activated C-8 cAMP-agarose column (500×10 mm; Sigma Chemical Co., USA)) was equilibrated with starting buffer (SB) (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10% glycerol (w/v), 1 mM DTT). Aspecifically bound proteins were removed by successive washing with SB containing 2 M NaCl and 1 mM AMP. NDPK was eluted with 1 mM cAMP in SB. The presence of NDPK in the different fractions was evaluated by immunoblotting using an NDPK-B antibody (CRS-B), kindly provided by Dr. M. Véron (Institut Pasteur, Paris). Cyclic AMP and EDTA were removed by binding to a Sep-Pak Light Waters Accell Plus QMA Cartridge (Waters, USA).

3. Results

NDPK is located in several cell compartments and has also been detected in the extracellular medium of cells [14]. We detected the presence of NDPK in C6 conditioned medium, in human bone marrow plasma and human sera (Willems, R., Van Bockstaele, D., and Slegers, H., unpublished data).

The effect of cAMP analogues on NDPK activity has been investigated in the presence of different concentrations of ATP, e.g. 2 mM representing the physiological cellular concentration [22–26], 0.5 mM representative of some pathological situations [22,23,27,28] and 0.5 μM representing the extracellular ATP concentration in plasma and in the synaptic cleft



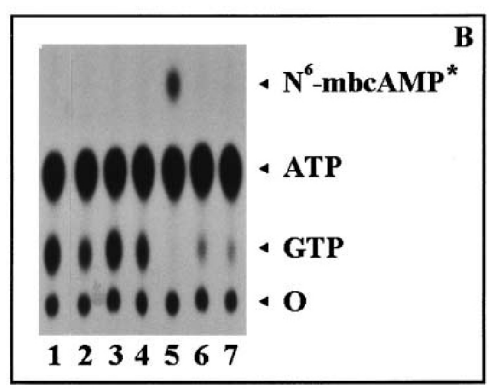


Fig. 1. Inhibition of the NDPK phosphotransferase activity by cAMP and cAMP analogues. (A) NDPK was assayed in the absence or presence of 1 mM of the indicated effector and in the presence of 2 mM ATP/50 μM GDP (0.1 mCi/ml [β-^{32}P]GDP), 0.5 mM ATP/50 μM GDP (0.1 mCi/ml [β-^{32}P]GDP), or 0.5 μM GDP/0.5 μM ATP (0.1 mCi/ml [γ-^{32}P]ATP) as described in Section 2. Data are expressed as % activity. The reaction rate in the absence of effector is taken as 100%. C, control. (B) Autoradiograph of the TLC plate of assays in the presence of 0.5 μM GDP/0.5 μM ATP, and in the absence (lane 1) or presence of 1 mM cAMP (lane 2), dbcAMP (lane 3), O'^2-mbcAMP (lane 4), N^6-mbcAMP (lane 5), 8-ClcAMP (lane 6), 8-BrcAMP (lane 7). N^6-mbcAMP*, phosphorylated N^6-mbcAMP; O, origin.

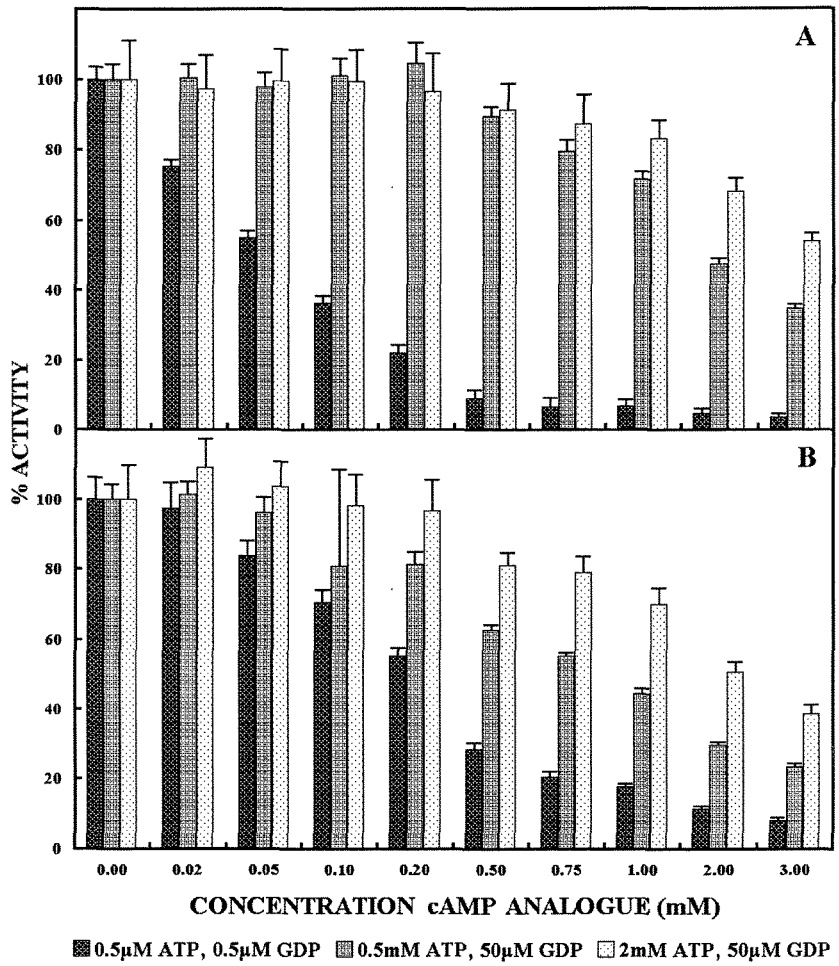


Fig. 2. Inhibition of the NDPK phosphotransferase activity as a function of the concentration of N^6-mbcAMP and 8-ClcAMP. The phosphotransferase activity of NDPK was determined in the presence of a varying concentration of N^6-mbcAMP (A) and 8-ClcAMP (B) as described in Section 2. ATP and GDP concentrations are as described in the legend to Fig. 1. Data are expressed as % activity. The reaction rate in the absence of effector is taken as 100%.

[23,29–31]. The physiological cellular GDP level is reported to be 20 μM [25]. To avoid GDP inhibition of NDPK and its depletion within the reaction time used, a GDP working concentration of 50 μM seemed to be a reasonable estimate to represent the intracellular environment [32]. To our knowledge, the GDP level in extracellular fluids has not been reported and in the experiments using 0.5 μM ATP, an equimolar GDP concentration was used.

In the presence of 2 mM ATP and 50 μM GDP and at an effector concentration of 1 mM, none of the assayed cAMP analogues induced inhibition of the ATP/GDP phosphotransferase activity greater than 40% (Fig. 1). However, when the ATP concentration was lowered to 0.5 mM the inhibition by 1 mM N^6-mbcAMP, 8-ClcAMP and 8-BrcAMP already increased to 40, 50 and 60%, respectively. At 0.5 μM ATP the inhibition by the former effectors increased to more than 80% and also cAMP inhibited the NDPK ATP/GDP phosphotransferase reaction by 50%. DbcAMP and O'^2-mbcAMP did not affect the NDPK activity under all conditions assayed (Fig. 1). At 0.5 μM ATP N^6-mbcAMP was a more potent inhibitor than 8-ClcAMP, e.g. the concentration of effector resulting in 50% inhibition (I_{50}) was 50 and 200 μM for N^6-mbcAMP and 8-ClcAMP, respectively. On the other hand, at 0.5 and 2 mM ATP, the inhibition of N^6-mbcAMP was lower than those of 8-ClcAMP (Fig. 2A). Analysis by TLC of the reaction mixtures at low ATP/GDP concentrations demonstrated that N^6-mbcAMP is a substrate of NDPK and becomes phosphorylated in the enzymatic reaction (Fig. 2B).

NDPK autophosphorylation assays were performed in the absence of GDP. The latter induces rapid dephosphorylation of the autophosphorylated intermediate. In the absence of GDP and in the presence of EDTA a stable serine phosphoenzyme intermediate was obtained. This autophosphorylation can be inhibited by those cAMP analogues which are also able to inhibit the phosphotransferase activity (Fig. 3B).

4. Discussion

Cyclic AMP is implicated in the regulation of cell growth and differentiation by activation of two isoforms of PKA, e.g. PKA-I which relates to cell proliferation and PKA-II primarily involved in differentiation [33]. Chloro cAMP, a strong growth inhibitor of cancer cells in culture and in vivo [34–37], has been proposed to restore the normal balance of the PKA isoforms in tumor cells and has been included in preclinical trials I/II for the treatment of human breast cancers [38].

In this communication we present evidence that NDPK/

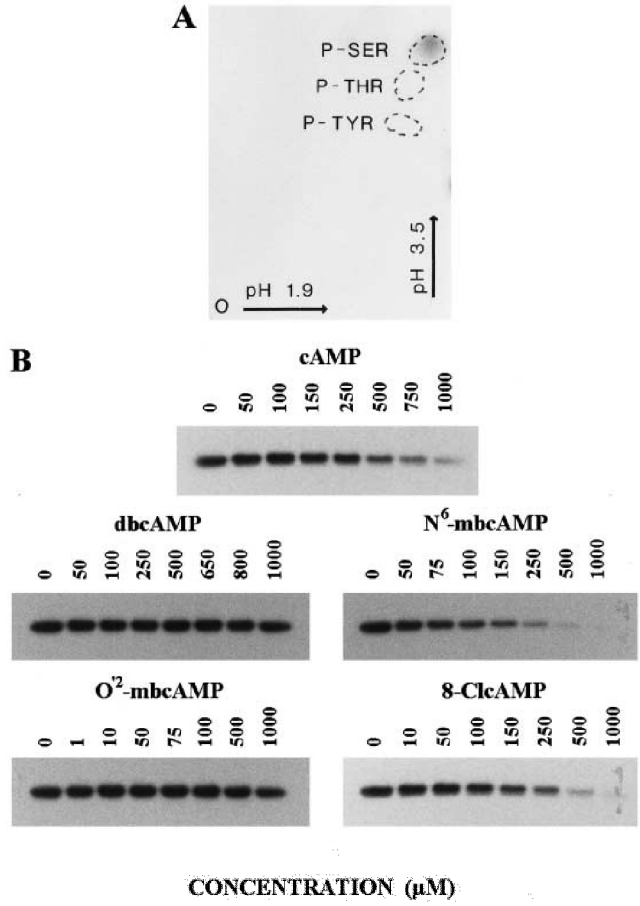


Fig. 3. Autophosphorylation of NDPK and its inhibition by cAMP and cAMP analogues. (A) Identification of the phosphorylated amino acid. The acid- and heat-stable phosphoamino acid was identified by two-dimensional TLC. Phosphotyrosine, -threonine and -serine markers were added to the sample before TLC separation. (B) NDPK autophosphorylation was performed as described in Section 2 in the presence of the indicated concentration of effector. The autoradiographs shown are representative of at least two independent experiments.

nm23 is an additional target of cAMP analogues and may be involved in the growth inhibition of cancer cells.

Inhibition of erythrocyte NDPK by cAMP has already been described by Mourad and Parks [39]. More recently, crystallographic data of the cAMP-NDPK complex have been reported indicating a possible physiological function for the cAMP-mediated inhibition of NDPK [40]. We have evaluated the effect of cAMP and cAMP analogues on the NDPK ATP/GDP phosphotransferase activity and its autophosphorylation at ATP concentrations approximating the concentration in normal (2 mM) and tumoral (0.5 mM) cells and approximating the extracellular concentration (0.5 μM). Cyclic AMP was only a weak inhibitor of the NDPK phosphotransferase activity with an I_{50} of approx. 1 mM at an ATP concentration of 0.5 μM. In comparison, the cAMP analogues 8-ClcAMP, 8-BrcAMP and N^6-mbcAMP were more efficient effectors and inhibited the NDPK activity by more than 80% at an ATP concentration of 0.5 μM. Under the latter conditions, an I_{50} of approx. 50 and 200 μM has been measured for N^6-mbcAMP and 8-ClcAMP, respectively. The presented data demonstrate that the extracellular NDPK activity detected in sera and conditioned medium of cells can be completely inhibited by the former cAMP analogues. Furthermore, in tumor cells the intracellular NDPK activity can also be inhibited taking into account: (i) an intracellular concentration of N^6-mbcAMP and 8-ClcAMP in the millimolar range, e.g. 18.0 and 10.6 fmol/cell (1.6 μl/10^6cells), respectively, after stimulation of rat C6 glioma for 48 h with 1 mM effector ([41], Anciaux, K., Van Dommelen, K., Nicolaï, S., Van Mechelen, E. and Slegers, H. unpublished data); and (ii) a reduction of the intracellular ATP concentration in tumor cells by 30–70% [22,23]. N^6-mbcAMP is a substrate of the enzyme and as such a competitive inhibitor of NDPK. DbcAMP and O'^2-mbcAMP did not affect the NDPK phosphotransferase activity. However, in rat C6 glioma dbcAMP is able to increase the intracellular concentration of cAMP to 0.6 mM, resulting in moderate inhibition of NDPK [42]. Based on crystallographic data, Strelkov et al. [40] proposed a competition between ATP and cAMP for binding in the active site of the enzyme. Cyclic AMP binds in the catalytic cleft mainly by its ribose moiety. The O'^2 atom of the latter interacts with Ile^{111} [40] indicating that cAMP analogues with a modified O'^2 atom like dbcAMP and O'^2-mbcAMP are unable to bind in the active site of NDPK.

Besides its phosphotransferase activity involving a phosphohistidine intermediate, NDPK can be autophosphorylated on a serine [43]. The latter autophosphorylation is low in comparison with the histidine phosphorylation [43,44] but has been inversely related to the potential of NDPK-A/nm23-H1 to suppress metastasis [9]. In order of potency, the autophosphorylation of NDPK is inhibited by N^6-mbcAMP, 8-ClcAMP and cAMP and closely parallels the inhibition of the phosphotransferase activity.

The fact that cAMP can bind to NDPK but is only a weak inhibitor of its phosphotransferase activity and autophosphorylation suggests that other activities are probably regulated by cAMP, e.g. its transcription factor (PuF) activity for the c-myc gene [18]. Although inhibition by cAMP and cAMP analogues of the latter transcriptional activity remains to be determined, the presented data demonstrated that effectors like N^6-mbcAMP and 8-ClcAMP can be used to elucidate the still unknown extra- and intracellular function(s) of NDPK/nm23.

In addition, the differential inhibition of NDPK by 8-ClcAMP as a result of the ATP difference in normal and tumoral cells may provide new perspectives on its use as an anticancer agent [34,37].

Acknowledgements: We thank Dr. P. Cras for his interest in our work. This work was supported by grants from the Belgian National Fund of Scientific Research (FGWO) and the Concerted Research Action 'Control mechanisms of cell proliferation in eukaryotes'. We thank Dr. M. Véron (Institut Pasteur, Paris) for the gift of anti-NDPK-B antibody. K.A. is a fellow of the Belgian National Fund for Scientific Research (NFWO, 'Kom op tegen Kanker'). R.W. is a fellow of the Belgian Institute for Scientific Technology (IWT). D.R. is a fellow of the Concerted Research Action '96.

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