Glycine-enhanced inhibition of rat liver nucleotide pyrophosphatase/phosphodiesterase-I by EDTA: a full account of the reported inhibition by commercial preparations of acidic fibroblast growth factor (FGF-1)

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Abstract The earlier reported inhibition of rat liver nucleotide pyrophosphatase/phosphodiesterase I (EC 3.1.6.9/EC 3.1.4.1; NPP/PDE) by culture-grade acidic fibroblast growth factor (FGF-1) correlates with a low-M_r contaminant. ^{1}H-NMR analyses revealed EDTA in the total-volume fractions of a gel-filtration experiment, where all the inhibitory activity of the FGF-1 preparation was recovered. NPP/PDE inhibition by EDTA (and by unfractionated FGF-1 or the EDTA-containing fractions) was time-dependent, blocked by the substrate *p*-nitrophenyl-dTMP, and strongly enhanced by glycine. The use of glycine buffers in earlier work was critical to the apparent inhibition by FGF-1. The results point to a conformational change favored by glycine that may be relevant to the biological role of NPP/PDE.

Key words: Nucleotide pyrophosphatase; Phosphodiesterase I; Fibroblast growth factor, acidic; Glycine; EDTA; Rat liver

1. Introduction

Nucleotide pyrophosphatase/phosphodiesterase I (EC 3.1.6.9/EC 3.1.4.1; NPP/PDE) enzymes hydrolyze both phosphoanhydrides and phosphodiesters of 5′-nucleotides. Much attention is centered on a group of mammalian NPP/PDE identical [1–5] or closely related [6–8] to the mouse plasma-cell differentiation antigen PC-1 [9,10], a membrane glycoprotein also expressed in other tissues including liver [11]. PC-1 and homolog NPP/PDE enzymes are capable of autophosphorylation and autoadenylylation in a threonine residue [3,4,12], which may represent regulatory mechanisms and/or catalytic intermediates [5,6,12,13]. These enzymes appear linked to the pathogenesis of insulin resistance [14], tumor metastasis [6] and the modulation of physiologic and pathologic bone and cartilage mineralization [15].

Concerning NPP/PDE regulation, FGF-1 was reported to affect bovine and rat liver PC-1 differently: slight activation [3] and strong inhibition [4], respectively. Recently, we (and others [16]) reinvestigated the effect of FGF-1 on rat liver NPP/PDE and found that the commercial FGF-1 reported to inhibit rat PC-1 [4] contains a low-M_r, non-protein contaminant identified as an NPP/PDE inhibitor. While Stefan et al. hypothesized that it could be EDTA but could not characterize it [16], we have demonstrated that: (i) the inhibitory FGF-1 preparation did indeed contain EDTA, which accounted for the inhibition, (ii) potent NPP/PDE inhibition by EDTA required glycine which, used as a buffer in [4], was critical for the observation of inhibition by EDTA-containing FGF-1. Glycine-enhanced inhibition by EDTA revealed indirectly a novel effect of the amino acid on the conformation of NPP/PDE.

2. Materials and methods

2.1. Materials

Rat liver NPP/PDE was purified by an abridged version of a published procedure [17]. In short, membranes were isolated from a rat liver homogenate prepared in isotonic sucrose. NPP/PDE was extracted from the membranes with Triton X-100, solubilized by limited trypsinization, adsorbed to a DEAE-cellulose column in 20 mM Tris-HCl, pH 8.7, 5 mM $MgCl_2$ (buffer A), eluted with a 0–400 mM NaCl linear gradient in buffer A, concentrated by ultrafiltration in a PM30 membrane (Amicon), chromatographed in a Sephacryl S-200 column in buffer A, concentrated again, and buffer exchanged to 5 mM sodium phosphate, pH 8.25, in a Sephadex G-25 column (PD10, Pharmacia). The specific activity was 13 U/mg (assayed as in Section 2.2). The enzyme, supplemented with BSA (1 mg/ml) and stored at −20°C, was stable for at least 1 year. Once thawed, each enzyme aliquot was kept at 4°C and was stable for at least 1 month. Prior to use, the enzyme was diluted about 50-fold in 50 mM Tris-HCl, pH 9 (37°C).

p-Nitrophenyl-dTMP (ammonium salt) was from Sigma. Glycine, Tris, BSA (fraction V) and culture-grade FGF-1 (ref. 1033476; also named endothelial cell growth factor; the one reported to inhibit rat PC-1 [4,16]) were from Boehringer. EDTA (disodium salt) was from Merck. All other reagents were of analytical grade from standard commercial suppliers.

2.2. NPP/PDE assay

In all cases, NPP/PDE was assayed by its PDE activity at 37°C, under conditions of linearity with incubation time and amount of enzyme, in 50 mM Tris-HCl, pH 9, 1 mM *p*-nitrophenyl-dTMP and other additions as indicated. The reaction was started by the addition of substrate (from a 50 mM stock solution) and stopped with 5 volumes of 0.2 M NaOH. The formation of *p*-nitrophenol was measured at 405 nm ($\varepsilon = 18.5$ mM^{-1} cm^{-1}). Blanks without enzyme and/or substrate were processed in parallel. One unit enzyme activity was defined as 1 μmol of substrate/min.

2.3. Treatment of NPP/PDE with EDTA or FGF-1

Treatment of NPP/PDE with EDTA or FGF-1 was carried out in the reaction mixtures described above, prior to the addition of the substrate. When indicated, 5 mM glycine (from a 100 mM stock solution adjusted to pH 9 at 37°C with NaOH) was also included in these mixtures. The addition of enzyme (about 4–8 mU/ml of reaction mixture) initiated the so-called preincubation period, of varia-

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Abbreviations: FGF-1, acidic fibroblast growth factor; NPP/PDE, nucleotide pyrophosphatase/phosphodiesterase I

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ble length, which was ended by the addition of substrate starting the NPP/PDE assay.

3. Results

3.1. Presence of EDTA in the culture-grade preparation of FGF-1 which inhibited rat liver NPP/PDE

All the NPP/PDE-inhibitory activity present in culture-grade FGF-1 was recovered in the total volume ($M_r = 4000$) of a Sephadex G-100 column, well after the expected elution volume of FGF-1 ($M_r = 15\,000$–$17\,000$) (results not shown, and [16]). Stefan et al. have recently suggested that this low-M_r inhibitor could be EDTA, because its inhibitory activity was counteracted by Mg^{2+} in the same way as the inhibition caused by authentic EDTA [16], but they could not chemically characterize it. We have recorded a ^{1}H-NMR spectrum of the chromatographic fractions containing the inhibitor, which showed two major resonance signals with identical chemical shifts (δ 2.49 and δ 1.91 ppm) and the same ratio of the integrated areas (1.9) as the two proton signals of authentic EDTA under the same conditions. From these spectroscopic results, it was estimated that culture-grade FGF-1 contained around 40 nmol EDTA/μg of protein.

3.2. Time dependence and potentiation by glycine of the inhibition of rat liver NPP/PDE by EDTA

The effect of EDTA on NPP/PDE was initially studied in Tris buffer and so in the absence of glycine, as usual in our laboratory and in others [1,3,6–8,18,19]. The enzyme was thus inhibited by EDTA in a concentration- and time-dependent manner (Fig. 1A,B). Stefan et al. have reported that 6 μM EDTA inhibited the PDE activity of rat liver PC-1 by 70% after 2 min preincubation [16]. However, under these conditions, the results of Fig. 1A,B showed very little or no inhibition. Comparison of our assay conditions with those of Stefan et al. [16] revealed a major difference in the use of, respectively, 50 mM Tris or 100 mM glycine as buffer at pH 9. The results of Fig. 1C,D, versus those of Fig. 1A,B, indicate that NPP/PDE inhibition by EDTA was strongly enhanced by glycine, which by itself (up to 25 mM) was not inhibitory. In the presence of glycine, inhibition was little affected by preincubation (Fig. 1D).

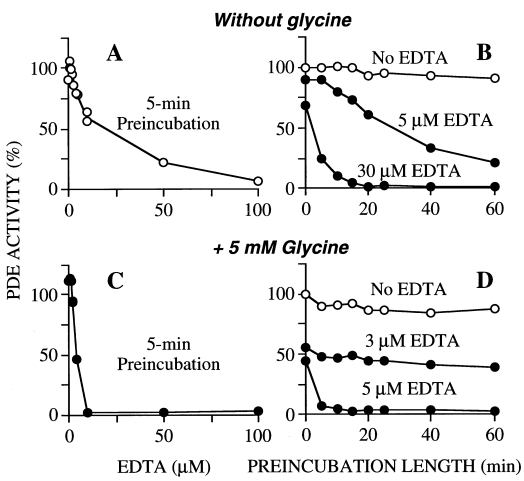

Fig. 1. Inhibition of rat liver NPP/PDE by EDTA: time dependence and enhancement by glycine. Before assay of PDE activity (Section 2.2), the enzyme was preincubated (Section 2.3) for the length of time and with the additions indicated in each plot. Activities are expressed as percentages of the control activity measured without preincubation and in the absence of EDTA and glycine.

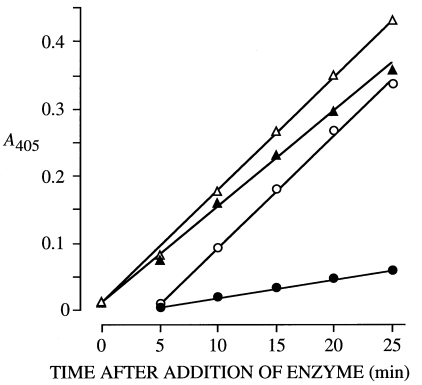

Fig. 2. Blocking by substrate of the time-dependent inhibition of rat liver NPP/PDE by EDTA. The plots represent the reaction courses of *p*-nitrophenyl-dTMP hydrolysis in the absence (open symbols) or in the presence of 30 μM EDTA (closed symbols). Time 0 corresponds in all cases to the addition of a standard amount of NPP/PDE to reaction mixtures with(out) EDTA. The addition of the substrate *p*-nitrophenyl-dTMP was done either immediately before the addition of NPP/PDE (triangles) or 5 min after it (circles). For explanation, see Section 3.3.

NPP/PDE inhibition by unfractionated culture-grade FGF-1 or by the low-M_r inhibitor isolated from it by gel filtration (Section 3.1) showed the same time dependence and potentiation by glycine (results not shown) as the inhibition by authentic EDTA.

3.3. The time-dependent inhibition of NPP/PDE by EDTA was blocked by the substrate p-nitrophenyl-dTMP

The time-dependent NPP/PDE inhibition by EDTA, seen in the absence of glycine, was blocked by the substrate *p*-nitrophenyl-dTMP (Fig. 2). In the presence of 30 μM EDTA, when substrate was added without enzyme preincubation (i.e. preincubation length = 0 min), the little-inhibited rate thus measured was linear with time for at least 25 min despite the presence of EDTA in the assay mixture (Fig. 2, closed triangles). In the same experiment, delaying the addition of substrate for as little as 5 min (i.e. preincubation length = 5 min) resulted in a strongly inhibited, though again linear, reaction rate (Fig. 2, closed circles). In contrast, 5 min of preincubation without EDTA did not affect NPP/PDE activity (Fig. 2, open circles).

4. Discussion

The results prove that, as recently hypothesized [16], the apparent inhibition of rat liver NPP/PDE by culture-grade FGF-1 was actually caused by EDTA. In addition, the results revealed a feature of NPP/PDE inhibition by EDTA which was previously unrecognized: the inhibitory potency of the metal chelator was strongly increased by glycine, at millimolar concentrations of the amino acid. From the EDTA content of culture-grade FGF-1 estimated by NMR (40 nmol/μg of protein) and from the experimental conditions employed by those who studied the inhibition of rat PC-1 [4,16], one can infer that the concentrations of EDTA present in experiments showing 50% PC-1 inhibition by culture-grade FGF-1 [4] were 0.5–2 μM. Interestingly, that study of rat liver PC-1

was carried out at pH 9, with 100 mM glycine as a buffer. Our results indicate that, if it were not for the enhancing effect of glycine, the low micromolar concentrations of EDTA and the very short, 2 min preincubation applied in [4] and [16] would be insufficient to inhibit NPP/PDE appreciably (see Fig. 1A,B).

From a practical point of view, the results of this work are a warning that, when studying NPP/PDE enzymes, one should bear in mind that micromolar traces of EDTA, which would not normally affect enzyme activity, may be strongly enhanced by addition of amino acids or other agents having a similar effect.

The influence of divalent cations on NPP/PDE conformation is supported by experiments showing that EDTA favors thermal inactivation and proteolytic degradation of PC-1, whereas divalent cations have the opposite effect [19]. The time dependence of the NPP/PDE inhibition by EDTA in the absence of glycine (Fig. 1B) points to the presence of slowly exchangeable metal ions that are necessary for activity. The protection exerted by the substrate seems to indicate that they may be close to (part of) the active site and/or that a conformational change of NPP/PDE (hindered by substrate binding) is needed for the exchange to take place. The enhancing of EDTA inhibition and the loss of its time dependence, both elicited by glycine, support the occurrence of a conformational change induced or stabilized by the amino acid. This effect on NPP/PDE conformation may be relevant to the regulation of the enzyme, independently of the presence of metal chelators. This does not mean necessarily a direct effect of the amino acid on the catalytic activity of NPP/PDE. Although glycine inhibits rat liver NPP/PDE at high concentrations (100 mM; [20] and data from our laboratory), at the concentrations used in this work, glycine by itself did not affect the PDE activity of NPP/PDE (results not shown). However, it is possible that NPP/PDE has biological functions not dependent on its enzymatic activity, and the change promoted by glycine may affect them. In this connection, it is interesting that the inhibition of insulin receptor phosphorylation is elicited both by wild-type PC-1 and by a mutant devoid of PDE activity [14,21].

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