

Two diverse effects of poly(L-lysine) on rabbit skeletal muscle phosphorylase kinase: stimulation of autophosphorylation and inhibition of its activity

Akira Negami, Kuniyasu Sakai⁺, Tomoko Kobayashi, Hikaru Tabuchi[†], Shun-ichi Nakamura and Hirohei Yamamura*

Department of Biochemistry, Fukui Medical School, Matsuoka, Fukui 910-11, Japan

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Polylysine greatly stimulated the autophosphorylation of phosphorylase kinase from rabbit skeletal muscle. When fully autophosphorylated, about 14 mol of phosphate per tetramer ($\alpha\beta\gamma\delta$) were incorporated in the presence of polylysine, which was twice as much as those observed without polylysine. In contrast to this stimulatory effect of polylysine on the autophosphorylation, polylysine strongly inhibited the conversion reaction of phosphorylase *b* to *a*. The inhibition is competitive with a K_i of 2.3 $\mu\text{g/ml}$. No effects of polylysine were observed on the activities of phosphorylase and cAMP-dependent protein kinase.

<i>Glycogen phosphorylase kinase</i>	<i>Autophosphorylation</i>	<i>Polylysine</i>	<i>Rabbit skeletal muscle</i>
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1. INTRODUCTION

Glycogen phosphorylase kinase, one of the major rate-limiting enzymes of glycogen metabolism, is well known to be regulated by phosphorylation-dephosphorylation mechanism [1,2]. Various protein kinases have been described in the literature which catalyze the phosphorylation of phosphorylase kinase [3] and in particular cAMP-dependent protein kinase (protein kinase A) has been studied in considerable detail and is believed to be a key step in the hormonal regulation of glycogen meta-

bolism [1]. Although other phosphorylation reactions have not yet been fully studied in point of regulation, the autophosphorylation reaction of phosphorylase kinase has also been considered to be of physiological significance [4,5]. To date, various effectors upon phosphorylase kinase have been reported. However, all the effectors seem to have a similar effect on autophosphorylation [2]. Although authors in [6] described briefly that polylysine could stimulate the autophosphorylation, the exact mechanism of polylysine has not been clarified. Here, it is found that polylysine has two different influences on phosphorylase kinase, that is, stimulation of autophosphorylation and inhibition of its activity.

2. EXPERIMENTAL PROCEDURES

2.1. Materials and chemicals

Purified phosphorylase kinase [7], purified phosphorylase *b* [8], a catalytic subunit of protein kinase A [9] and its inhibitor protein [10] were prepared from rabbit skeletal muscle. Whole

⁺ Present address: Department of Pediatrics, Kobe University School of Medicine, Kobe 650, Japan

[†] Present address: Department of Internal Medicine, Kobe University School of Medicine, Kobe 650, Japan

* To whom correspondence should be addressed

Abbreviations: protein kinase A, cAMP-dependent protein kinase; SDS, sodium dodecyl sulfate; buffer A, 50 mM α -glycerophosphate (pH 6.8), 2 mM EDTA and 10 mM 2-mercaptoethanol; Hepes, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid

histone [11] was prepared from calf thymus. [γ - ^{32}P]ATP was prepared as in [12]. α -D-[U- ^{14}C]Glucose 1-phosphate (303 mCi/mmol) was a product of the Radiochemical Centre, Amersham. Poly(L-lysine) hydrobromide (type 1-B) was obtained from Sigma. Other chemicals were obtained from commercial sources.

2.2. Enzyme assay and determination

Autophosphorylation of phosphorylase kinase was assayed by measuring the amount of ^{32}P incorporated into phosphorylase kinase itself. The reaction mixture (0.1 ml) contained 5 μmol Hepes at pH 7.5, 1 μmol magnesium acetate, 3 μmol 2-mercaptoethanol, 20 nmol CaCl_2 , 0.3 μmol [γ - ^{32}P]ATP ($3\text{--}10 \times 10^3$ cpm/nmol), protein kinase A inhibitor (13 μg), phosphorylase kinase (50 μg) and various concentrations of polylysine as indicated in each experiment. The incubation was usually carried out for 5 min at 30°C. The reaction was stopped by the addition of 10% trichloroacetic acid, and acid-precipitable materials were collected on a glass filter (Whatman GF 83). The activity of phosphorylase kinase was measured by the amount of ^{32}P incorporated into rabbit skeletal muscle phosphorylase *b* as in [13]. Protein kinase A was similarly assayed using phosphorylase kinase (14 μg) or whole histone (100 μg) as substrate. Phosphorylase was assayed by measuring the amount of [^{14}C]glycogen produced as in [14]. Protein was determined using Protein Assay (Bio-Rad) with ovalbumin as a reference protein. SDS-PAGE was carried out as in [15] on 7.5% gels. The radioactivity in each protein band was determined by transverse sectioning of the gel into 1.5 mm-width and their Cerenkov radiation measured with a liquid scintillation spectrometer. Each assay was done in duplicate or triplicate.

3. RESULTS

Fig.1 shows a stimulatory effect of polylysine on the autophosphorylation of phosphorylase kinase. In the absence of EGTA, polylysine stimulated the incorporation of ^{32}P into the enzyme about 2-fold. At the time of the 30 min incubation about 14 and 7 mol of phosphate were incorporated into phosphorylase kinase ($\alpha\beta\gamma\delta$) in the presence and absence of polylysine, respectively. In the presence of EGTA small amounts of phosphate were incor-

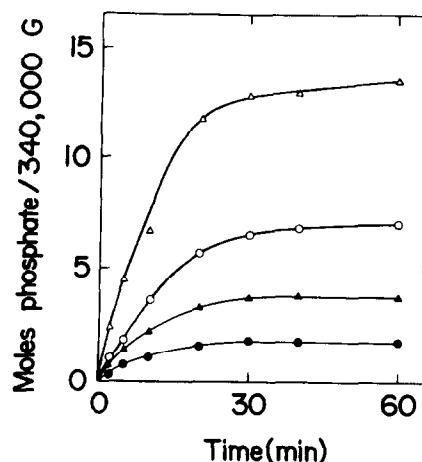


Fig.1. Activation of autophosphorylation by polylysine. Assay conditions are described in the text. Polylysine (50 $\mu\text{g}/\text{ml}$) and EGTA (4 mM) were added where indicated. (▲, △) Incubation with polylysine in the presence and absence of EGTA; (●, ○) incubation without polylysine in the presence and absence of EGTA.

porated into the enzyme both in the presence and absence of polylysine. Half maximal stimulatory concentration of polylysine was about 15 $\mu\text{g}/\text{ml}$ and maximal stimulation was obtained at a concentration of 40 $\mu\text{g}/\text{ml}$. Similar stimulation was observed by the addition of polyarginine, but polyalanine, polyaspartic acid, putrescine, spermidine, and spermine (up to 100 $\mu\text{g}/\text{ml}$) had no effects (not shown). These basic polypeptides themselves were not phosphorylated in this reaction.

In order to investigate the sites of phosphorylation by autophosphorylation of phosphorylase kinase, an enzyme sample with polylysine was autocatalytically phosphorylated prior to SDS gel electrophoresis. Fig.2 shows the time course of phosphorylation of the subunits during autophosphorylation. Only α - and β -subunits were phosphorylated, and no phosphorylation was detected in γ - and δ -subunits. There was no initial lag both in the presence and absence of polylysine in phosphorylation of α - and β -subunits. The ratio of phosphate in α - and β -subunits was 2.2–2.8:1 both in the presence and absence of polylysine.

Next experiments were performed whether the autophosphorylation stimulated by polylysine was accompanied with an increase in phosphorylase

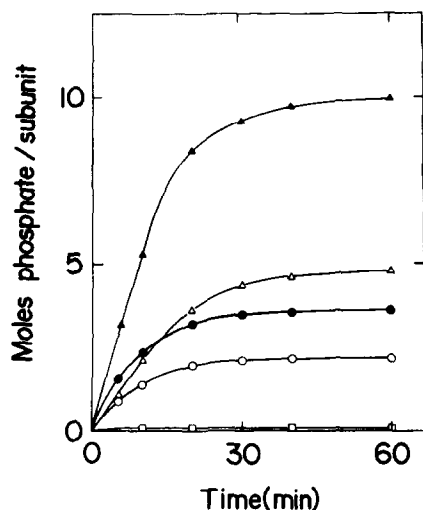


Fig.2. Time course of the phosphorylation of the subunits of phosphorylase kinase. Reaction conditions are described in the text. Aliquots containing about 20 μg protein each time were subjected to SDS-gel electrophoresis to separate the enzyme into its subunits. The assay of subunit phosphorylation was carried out as described in the text. The value represents the mean of two different preparations of phosphorylase kinase. (\blacktriangle , \triangle) Subunit α with and without polylysine; (\bullet , \circ) subunit β with and without polylysine; (\square) subunits γ and δ with and without polylysine.

kinase activity. Fig.3 shows that when phosphorylase kinase was preincubated with ATP- Mg^{2+} in the presence of polylysine, the enzyme activity was completely inhibited at 60 min. While the enzyme was preincubated without polylysine, the activity was stimulated more than 5-fold at 60 min. The inhibition by polylysine was not dependent on pH. Polylysine also inhibited the non-activated and protein kinase A-activated phosphorylase kinase activities (not shown). The double-reciprocal plots of polylysine concentration vs phosphorylase kinase activity indicated that the inhibition was competitive with a K_i of 2.3 $\mu\text{g}/\text{ml}$. The activities of phosphorylase and protein kinase A were not affected by the polylysine added (up to 40 $\mu\text{g}/\text{ml}$). These results strongly suggest that polylysine bound to phosphorylase kinase and stimulated autophosphorylation of phosphorylase kinase, but inhibited the phosphorylase kinase activity.

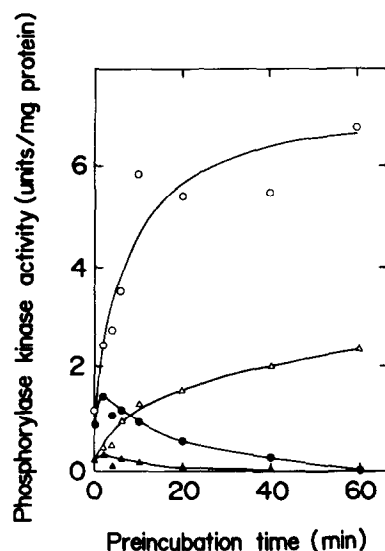


Fig.3. The effect of polylysine on the activity of phosphorylase kinase. Reaction mixtures containing 50 mM Hepes (pH 7.4), 10 mM Mg^{2+} -acetate, 16.5 mM 2-mercaptoethanol, 0.2 mM CaCl_2 , 0.2 mM EDTA, 3 mM ATP, 0.15 μg protein kinase inhibitor, 25 mM NaF, 250 μg phosphorylase kinase with (\bullet , \blacktriangle) or without (\circ , \triangle) 25 μg polylysine in a total volume of 100 μl . The reaction mixture was preincubated at 30°C. An aliquot at each time was diluted 1 : 19 in cold buffer A containing 400 $\mu\text{g}/\text{ml}$ BSA, 1 $\mu\text{g}/\text{ml}$ protein kinase inhibitor and 25 mM NaF. All samples were then assayed for phosphorylase kinase at pH 6.8 (Δ , \blacktriangle) and pH 8.2 (\circ , \bullet) as described in section 2.

4. DISCUSSION

Although authors in [14] have briefly reported that highly basic proteins, such as polylysine and histones may stimulate the autophosphorylation of phosphorylase kinase, the exact role of basic proteins has not been studied in detail. The results presented in this paper show that 7 mol phosphate/ $\alpha\beta\gamma\delta$ are incorporated in the absence of polylysine. However, in the presence of polylysine about 14 mol phosphate are incorporated into the enzyme ($\alpha\beta\gamma\delta$) and the ratio of phosphate in α - and β -subunits was about 2.2–2.8:1. No phosphorylation has been found in the γ - and δ -subunit. The autophosphorylation by polylysine is also dependent on Ca^{2+} , although its dependency is not complete. As shown in fig.3, though polylysine stimulates the autophosphorylation,

phosphorylase kinase could not be stimulated but was inhibited. The inhibition by polylysine is competitive. The activity of neither phosphorylase nor protein kinase A was affected by polylysine. Also, polylysine inhibited the activities of non-activated and protein kinase A-activated phosphorylase kinase. These results suggest that the polylysine could bind the phosphorylase kinase and stimulate the autophosphorylation and inhibit the phosphorylase kinase activity.

Authors in [17] proposed that phosphorylase and troponin were phosphorylated by two different catalytic centers of phosphorylase kinase. More recently, authors in [18] found that 3 different classes of substrates were distinguished in the phosphorylase kinase reaction and they proposed that there were at least two types of catalytic sites on phosphorylase kinase which showed different, but not absolute, specificities for different substrates. Our results may suggest that the autocatalytic site is different from the phosphorylase catalytic site in the phosphorylase kinase.

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