Stimulation of Phosphatidylinositol Phosphorylation in the Sarcoplasmic Reticular Ca\textsuperscript{2+}-Transport ATPase by Vanadate

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Z. Naturforsch. 41c, 310–314 (1986); received October 16, 1985

Sarcoplasmic Reticulum ATPase, Vanadate, Phosphatidylinositol, Phosphorylation

Vanadate increases the initial phosphatidylinositol phosphate formation rate as well as the steady state level of the above lipid phosphate when phosphatidylinositol associated with the isolated Ca\textsuperscript{2+} transport ATPase is phosphorylated either by the membrane bound endogenously present phosphatidylinositol kinase or by exogenously added lipid kinase. Employing an ultrasonicated mixture of pure phosphatidylinositol and Triton X 100 (without membrane proteins) no vanadate effect can be seen. This vanadate effect is probably not mediated through the lipid kinase activity, but more likely, through conformational changes of the Ca\textsuperscript{2+} transport ATPase protein. Such conformational changes would lead to a higher degree of phosphatidylinositol exposed on the Ca\textsuperscript{2+} transport ATPase and thus a higher substrate concentration. Consequently, the initial phosphatidylinositol phosphate formation rate and steady state level increase.

Introduction

Phosphatidylinositol 4-phosphate (PtdIns4P) is formed on the isolated Ca\textsuperscript{2+} transport ATPase from rabbit skeletal muscle sarcoplasmic reticulum (SR) when these membranes (above 2 mg/ml) are incubated with ATP/Mg\textsuperscript{2+} [1]. This observation shows that a phosphatidylinositol kinase (PtdIns-kinase, EC 2.7.1.67) is associated with the isolated Ca\textsuperscript{2+} transport ATPase.

Recently, it has been shown that phosphorylase kinase exhibits PtdIns-kinase activity utilizing Ca\textsuperscript{2+} transport ATPase bound phosphatidylinositol (PtdIns) as substrate [2].

In addition we have shown that phosphorylase kinase (EC 2.7.1.38) enhances the rate and also the steady state level of the above phospholipid phosphorylation [3].

Phosphorylase kinase is composed of four heterologous, tightly associated subunits, called $\alpha$, $\beta$, $\gamma$ and $\delta$ [4]. The $\alpha$ subunit of phosphorylase kinase and the pp60$^{v\text{src}}$, a tyrosine protein kinase, exhibit about 35% sequence identity in the first 17 N-terminal positions [5].

Furthermore, evidence has been presented that certain oncogene coded proteins like the purified pp60$^{v\text{src}}$ kinase can phosphorylate PtdIns, PtdIns4P and 1,2 diacylglycerol [6]. Macara et al. [7] demonstrated that pp60$^{v\text{src}}$, a tyrosine protein kinase coded by the ros oncogene of the avian sarcoma virus UR2, can phosphorylate PtdIns, but neither PtdIns4P nor 1,2 diacylglycerol.

Moreover, the insulin receptor tyrosine protein kinase may also be associated with PtdIns-kinase activity [8].

Recently, Brown and Gordon [9] have shown that micromolar sodium orthovanadate stimulates the pp60$^{v\text{src}}$ tyrosine protein kinase activity in transformed chicken embryo fibroblasts. The enhanced kinase activity is due to an autophosphorylation of the enzyme on a tyrosine residue in the NH$_2$-terminal fragment.

We now report that vanadate enhances PtdIns4P formation when PtdIns bound to the isolated Ca\textsuperscript{2+} transport ATPase is phosphorylated either by the membrane associated endogenously present or by the exogenously added PtdIns-kinase.

Employing a sonicated mixture of PtdIns and Triton X 100 as substrate, without membrane proteins, no effect of vanadate can be observed.

Materials and Methods

SR membranes were isolated according to De Meis and Hasselbach [10] and the Ca\textsuperscript{2+} transport ATPase as described by MacLennan [11]. Protein concentration was measured by the method of Lowry et al. [12]. Bound radioactivity was assayed according to Mans and Novelli [13]. Phosphorylase kinase was
prepared according to Cohen [14], as modified by Jennissen and Heilmeyer [15], and Hessová et al. [16]. Tyrosine specific protein kinase from rous sarcoma virus induced mice tumor was prepared as described by Glossmann et al. [17] and kindly provided by Peter Presek. The preparation used had a specific activity of 159 fmole/min x mg using anti pp60src IgG as substrate.

Incubation of phosphorylase kinase with pp60^src tyrosine kinase: after extensive dialysis of both kinases against 25 mM Tris-HCl, pH 7.5, 1.02 mg/ml phosphorylase kinase was incubated with 21.25 µg/ml pp60^src tyrosine kinase in presence of 20 mM Tris-HCl, pH 7.5, 20 mM MgCl2, 50 µM γ³²P-ATP and 140 µM vanadate at 30 °C for 45 minutes. Under the same condition also phosphorylase kinase alone was incubated which resulted in an autophosphorylated enzyme. As positive control for the tyrosine phosphorylating activity of the pp60^src as substrate anti pp60src IgG was used. Assays for PtdIns-kinase activity of the above modified kinases and that of the pp60src kinase were carried out by using a sonicated mixture of PtdIns (Sigma) and Triton X 100 as substrate as described in the Legend of Fig. 2.

Preparation of probes for thin layer chromatography (TLC) was carried out as described by Varsányi et al. [1] with the slight modification that after phosphorylation the membranes of the isolated Ca²⁺ transport ATPase (ca. 500—800 µg) were precipitated by perchloric acid at a final concentration of 7% in presence of 6—7 mg/ml bovine serum albumin. TLC was carried out as described by Shaigh and Palmer [18].

Results

A representative time course of the ³²P incorporation into PtdIns associated with the isolated Ca²⁺ transport ATPase is shown in Fig. 1 when the reaction is catalyzed either by the endogeneously present (A) or exogeneously added (B) PtdIns-kinase. Before vanadate addition the ³²P incorporation is linear with time only for 20 to maximally 40 minutes depending on the membrane preparation, thereafter the ³²P incorporation rate decreases continuously and ceases completely. Depending on the ATPase preparation the initial phosphorylation rate varies between 50—200 pmole/min x mg for the phosphorylation with the ATPase associated endogeneous lipid kinase and between 200—500 pmole/min x mg for that with the exogeneously added PtdIns-kinase. Upon addition of vanadate a further immediate increase of ³²P incorporation occurs. In both cases depending on the ATPase preparation the initial phos-

![Fig. 1. Effect of vanadate on PtdIns phosphorylation catalyzed by the membrane associated endogenously present (A) and by the exogeneously added PtdIns-kinase (B). As substrate PtdIns associated with the isolated Ca²⁺ transport ATPase was used. Isolated Ca²⁺ transport ATPase (5 mg/ml reaction mixture) was incubated in a total volume of 600 µl at pH 7.5, at 26 °C containing 100 mM KCl, 100 mM Tris-HCl, 10 mM EDTA, 1 mM EGTA, 10 mM γ³²P ATP, 21 mM MgCl2 in absence (A) and presence (B) of 1 mg/ml phosphorylase kinase as described by Varsányi et al. [1]. At the 60th minute vanadate at a final concentration of 250 µM was added to the incubation mixture. Phosphate incorporation contributed by autophosphorylation of phosphorylase kinase was substracted to give the values shown. Identification of the phosphorylated product before (1) and after (2) vanadate addition was made as described in Materials and Methods.](image-url)
phorylation rates are 1.5—2 fold higher than in absence of vanadate; the level of the total incorporated amount of $^{32}$P is 4—6 fold enhanced again depending on the ATPase preparation.

After phosphorylation in the presence of vanadate approximately 95% of the ATPase bound radioactivity can be extracted by chloroform/methanol/concentrated HCl (100:100:0.6, v/v). This shows that also in presence of vanadate the enhanced amount of bound phosphate has been incorporated into phospholipid. TLC of the above organic extract reveals that Ptdlns4P is the main radioactive labeled product (Fig. 1 inset). The lysophosphatidylcholine are artificial products of the acidic extraction procedure.

The phosphorylated protein after lipid extraction (ca. 5% of the total bound radioactivity) has been subjected to partial acid hydrolysis in presence of 6 N HCl [1]. Autoradiography following separation of the liberated amino acids shows phosphoserine as the only labeled aminoacid both in absence and presence of vanadate (not shown).

Vanadate is without any effect when a sonicated mixture of PtdlNs and Triton X 100 is phosphorylated by PtdIns-kinase endogenously present in the phosphorylase kinase preparation. Again the reaction product is Ptdlns4P (Fig. 2); lysophosphatidylcholine are generated during the acidic isolation. The specific Ptdlns-kinase activity in the phosphorylase kinase preparations varies between 0.8—1.5 nanomole/min/mg of protein. In the experiment presented in Fig. 2 approximately 25% of the PtdIns has been converted into Ptdlns4P, however, repeated addition of PtdIns-kinase was necessary. The inactivation of PtdIns kinase in the phosphorylase kinase during the long incubation time seems to correlate with the autophosphorylation of the phosphorylase kinase itself which parallels to the Ptdlns4P formation (personal observation, not shown). Addition of sodium vanadate does neither influence the Ptdlns4P formation rate nor the total amount formed.

**Discussion**

Endogeneous PtdIns-kinase is associated with the isolated SR Ca$^{2+}$ transport ATPase. Depending on preparation this lipid kinase activity varies between 50—200 pmole/min/mg which is in good agreement with the PtdIns-kinase activity in muscle microsomal fractions reported by Harwood and Hawthorne [19]. In presence of sodium metavanadate an enhanced PtdIns4P formation on the isolated Ca$^{2+}$ transport ATPase can be observed (Fig. 1 A and B). It can either be due to an enhancement of the PtdIns-kinase activity or to the inhibition of an endogeneous phosphoinositide phosphatase(s) present in the isolated Ca$^{2+}$ transport ATPase or to an effect on the substrate, the PtdIns, associated with the Ca$^{2+}$ transport ATPase.

Vanadate in micromolar concentration is known to be a potent inhibitor of tyrosine protein phosphatase [20]. Therefore, in the described system vanadate could selectively inhibit a dephosphorylation of phosphotyrosine present on the phospholipid kinase. It would require that only a phosphotyrosine containing phospho-
phospholipid kinase is active and that a tyrosine phosphorylation occurs after vanadate addition (see Fig. 1). However, the stimulatory effect of vanadate can be observed immediately; no lag phase is detected; it argues against such an indirect vanadate action through tyrosine dephosphorylation and rephosphorylation. Furthermore, no phosphotyrosine has been detected after acid hydrolysis during PtdIns phosphorylation in presence of vanadate following a longer preincubation period without vanadate (see Fig. 1 A and B).

We have extensively searched for tyrosine kinase activity in phosphorylase kinase or in the isolated Ca\(^{2+}\) transport ATPase preparation but have found no evidence for either.

Additionally, upon preincubation of the PtdIns-kinase (present in the phosphorylase kinase) with pp60\(^{src}\) tyrosine kinase (see Materials and Methods) no phosphotyrosine formation is detectable. Subsequent employment of the above kinase as enzyme in PtdIns phosphorylation reaction shows a 50% decrease, rather than increase in PtdIns4P formation rate (data not shown). The same decrease is observed with autophosphorylated phosphorylase kinase: this autophosphorylation occurs also in the presence of pp60\(^{src}\) kinase. In summary, no indications have been found for a protein phosphorylation which would enhance the catalytic activity of the PtdIns-kinase in the presence of vanadate. The activity of an endogeneous phosphoinositide phosphatase present in the isolated SR Ca\(^{2+}\) transport ATPase which is responsible for the PtdIns4P degradation can not be inhibited by vanadate (personal observation, not shown).

The above conclusions are further supported by the fact that a mixture of pure PtdIns with Triton X 100 as substrate shows no vanadate effect in contrast to the employment of PtdIns associated with the membranes of the Ca\(^{2+}\) transport ATPase. The lack of vanadate effect with pure phospholipid as substrate leads to the conclusion that membrane protein component(s) of the Ca\(^{2+}\) transport ATPase are responsible for this vanadate effect.

Assuming 9—10 mol PtdIns/mol ATPase [21, 22], a \(K_m\) value of 2 mm PtdIns for the PtdIns-kinase endogenously present in the phosphorylase kinase preparation has been determined using as substrate PtdIns associated with the isolated Ca\(^{2+}\) transport ATPase (Georgoussi and Heilmeyer, personal communication). The phosphorylations in our system were carried out at a PtdIns concentration of 0.5 mm. Therefore, the enhancement of the initial PtdIns phosphorylation rate by vanadate could be a consequence of the increase of substrate concentration, i.e. increase in PtdIns concentration upon addition of vanadate.

Vanadate has been shown to exhibit a great number of metabolic effects (for review see Ramasarma, [23]). Vanadium compounds affect systems that utilize ATP [24—26] and phosphorylation-dephosphorylation reactions [20, 27—30]. The activity of the SR Ca\(^{2+}\) transport ATPase decreases by vanadate [31—35]. Later studies have shown that vanadate causes conformational changes in the SR membranes. Recently, Hasselbach et al. [36] and Medda and Hasselbach [37, 38] have demonstrated that in lipid-reconstituted preparations as well as in native membranes vanadate induces the disappearance of external high affinity and simultaneously the appearance of internal low affinity calcium binding sites on the Ca\(^{2+}\) transport ATPase protein.

Therefore, the observed increase of PtdIns phosphorylation rate and PtdIns4P formation level in presence of vanadate could be due to a vanadate induced conformational change of the Ca\(^{2+}\) transport ATPase. This conformational change of the protein would lead to a higher degree of PtdIns exposed on the membranes resulting in an apparent increase of the substrate, PtdIns, concentration. Consequently, the initial PtdIns phosphorylation rate and PtdIns4P formation level increases.

**Acknowledgements**

We are very grateful to Professor L. M. G. Heilmeyer Jr. for his helpful discussions and valuable suggestions. We also thank U. Siemen for his excellent technical assistance.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft, SFB 168/C1.

This publication contains parts of the PhD Thesis of G. B. and M. S.


