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

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Vanadate increases the initial phosphatidylinositolphosphate formation rate as well as the steady state level of the above lipid phosphate when phosphatidylinositol associated with the isolated Ca^{2+} transport ATPase is phosphorylated either by the membrane bound endogeneously present phosphatidylinositol-kinase or by exogeneously 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^{2+} transport ATPase protein. Such conformational changes would lead to a higher degree of phosphatidylinositol exposed on the Ca^{2+} transport ATPase and thus a higher substrate concentration. Consequently, the initial phosphatidylinositolphosphate formation rate and steady state level increase.

Introduction

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

Recently, it has been shown that phosphorylase kinase exhibits PtdIns-kinase activity utilizing Ca^{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 α, β, γ and δ [4]. The α 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 $pp68^{v\text{-}ros}$, 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^{2+} transport ATPase is phosphorylated either by the membrane associated endogeneously present or by the exogeneously 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^{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

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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 fmolexmin^{-1}xmg^{-1} using anti pp60^{src}-IgG as substrate.

Incubation of phosphorylase kinase with pp60$^{v\text{-}src}$ 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$^{v\text{-}src}$ tyrosine kinase in presence of 20 mM Tris HCl, pH 7.5, 20 mM $MgCl_2$, 50 μM γ ^{32}P-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$^{v\text{-}src}$ as substrate anti pp60^{src} IgG was used. Assays for PtdIns-kinase activity of the above modified kinases and that of the pp60^{src} 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 Ca^{2+} 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 ^{32}P incorporation into PtdIns associated with the isolated Ca^{2+} 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 ^{32}P incorporation is linear with time only for 20 to maximally 40 minutes depending on the membrane preparation, thereafter the ^{32}P incorporation rate decreases contineously and ceases completely. Depending on the ATPase preparation the initial phosphorylation rate varies between 50–200 pmolexmin^{-1}xmg^{-1} for the phosphorylation with the ATPase associated endogeneous lipid kinase and between 200–500 pmolexmin^{-1}xmg^{-1} for that with the exogeneously added PtdIns-kinase. Upon addition of vanadate a further immediate increase of ^{32}P incorporation occurs. In both cases depending on the ATPase preparation the initial phos-

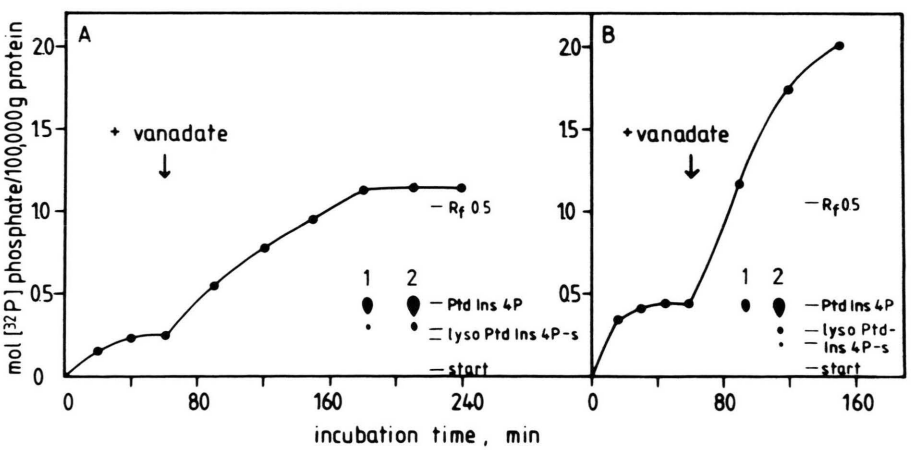

Fig. 1. Effect of vanadate on PtdIns phosphorylation catalyzed by the membrane associated endogenously present (A) and by the exogeneously added PtdIns-kinase (B). As substrate PtdIns associated with the isolated Ca^{2+} transport ATPase was used. Isolated Ca^{2+} transport ATPase (5 mg/ml reaction mixture) 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 γ ^{32}P ATP, 21 mM $MgCl_2$ 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.

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 PtdIns4P is the main radioactive labeled product (Fig. 1 inset). The lysoforms 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 PtdIns and Triton X 100 is phosphorylated by PtdIns-kinase endogeneously present in the phosphorylase kinase preparation. Again the reaction product is PtdIns4P (Fig. 2); lysoforms are generated during the acidic isolation. The specific PtdIns-kinase activity in the phosphorylase kinase preparations varies between 0.8–1.5 nanomolex$min^{-1}xmg^{-1}$. In the experiment presented in Fig. 2 approximately 25% of the PtdIns has been converted into PtdIns4P, 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 PtdIns4P formation (personal observation, not shown). Addition of sodium vanadate does neither influence the PtdIns4P formation rate nor the total amount formed.

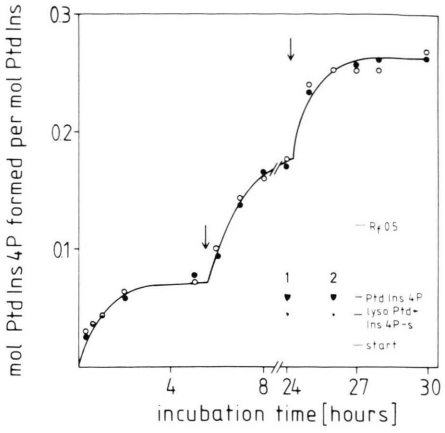

Fig. 2. Phosphorylation of an ultrasonicated mixture of PtdIns and Triton × 100 in absence and presence of vanadate. 2.5 mg PtdIns (SIGMA) was suspended in 500 µl, 100 mM KCl, 100 mM Tris-HCl pH 7.5, 2.4% Triton × 100. The suspension was sonicated for 1 hour at 40–50 °C. Two aliquots of this suspension (final PtdIns concentration 1 mM) were preincubated at 4 °C for 15 minutes in a total volume of 240 µl containing 100 mM KCl, 100 mM Tris-HCl pH 7.5, 10 mM EDTA, 1 mM EGTA, 1 mM DTE, 0.4% Triton × 100, 750 µg/ml phosphorylase kinase in absence (○) and presence (●) of 250 µM vanadate. After raising the temperature to 36 °C and after addition of 10 mM γ ^{32}P ATP to the preincubated mixture two aliquots of 15 µl were taken and added each to 300 µl chloroform/methanol, 2:1, v/v (solution 1) to determine the nonlipid radioactivity extracted into the organic phase. Phosphorylation was started by addition of 22 µl 400 mM $MgCl_2$. During the phosphorylation samples of 15 µl were removed, given to 300 µl solution 1 and stored on ice. Determination of lipid bound radioactivity was carried out as described by Schacht (39). Arrows indicate the time points at which further 475 µg/ml phosphorylase kinase was readded. Inset shows the autoradiograms of the TLC plates after PI phosphorylation in absence (1) and presence (2) of vanadate.

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 $pmolexmin^{-1}xmg^{-1}$ 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 phos-

pholipid 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 endogeneously 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.

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