Inositol 1,4-bisphosphate is an allosteric activator of muscle-type 6-phosphofructo-1-kinase

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The allosteric effects of various inositol bisphosphate (InsP_2) isomers and other inositol phosphates, of glycerophosphoinositol phosphates (GroPInsP_x) and of phosphoinositides (PtdInsP_x) on muscle-type 6-phosphofructo-1-kinase (PFK) were investigated. The binding of these substances to PFK was indirectly estimated by their ability to stabilize the tetrameric enzyme. At near-physiological concentrations of other allosteric effectors, muscle PFK was activated AMP-dependently by $\text{Ins}(1,4)\text{P}_2$ ($K_a = 43 \mu\text{M}$), $\text{Ins}(2,4)\text{P}_2$ ($K_a = 70 \mu\text{M}$) and $\text{GroPIns}4\text{P}$ ($K_a = 20 \mu\text{M}$). These compounds activated PFK by a mechanism similar to that established for activating hexose bisphosphates. Indirect binding experiments indicated minimal $K_{d,\text{app}}$ values of about $5 \mu\text{M}$ for the binding of $\text{Ins}(1,4)\text{P}_2$ in the presence of 0.1 mM -AMP at pH 7.4. This apparent affinity was comparable with that of fructose 1,6-bisphosphate and glucose 1,6-bisphosphate at identical conditions. The enzyme was also found to interact specifically with $\text{PtdIns}4\text{P}$ ($K_{d,\text{app}} = 37 \mu\text{M}$), the inositol phospholipid carrying $\text{Ins}(1,4)\text{P}_2$ as its head group. The regulatory behaviour of muscle-type PFK *in vitro* and the concentrations of $\text{Ins}(1,4)\text{P}_2$ *in vivo* (between 4 and $> 50 \text{ nmol/g}$ wet wt. of tissue) are consistent with the hypothesis that there is a functional interaction *in vivo*. Furthermore, a role of $\text{PtdIns}4\text{P}$ in membrane compartmentation of PFK is suggested. Comparative experiments with liver PFK indicate that these regulatory properties may be relatively specific for the muscle isoform. Unlike muscle PFK, the liver isoform was slightly activated by sub-micromolar concentrations of $\text{Ins}(1,4,5)\text{P}_3$.

INTRODUCTION

A number of doubly phosphorylated sugars are potent allosteric activators of eukaryotic 6-phosphofructo-1-kinase (PFK), an archetype of a multi-modulated key glycolytic enzyme (see [1] for a review; [2]). The C-terminal half domains of eukaryotic PFK subunits appear to be mainly responsible for the binding of these substances [2,3]. These regulatory domains have evolved by a prokaryotic gene duplication and divergent mutations from catalytic sites. The main effects of doubly phosphorylated sugars on vertebrate PFK are (i) a substantial lowering of the $s_{0.5}$ for Fru6P, (ii) a stabilization of the tetrameric active form of the enzyme or of higher oligomers, (iii) an antagonization of the allosteric inhibition by ATP, and (iv) a synergistic interaction with AMP, another potent allosteric activator (reviewed in [1–4]). Members of this class of allosteric activators are the product of PFK, Fru(1,6) P_2 [5,6], Glc(1,6) P_2 [7], which might be involved in glycolysis regulation in muscle [8,9] and other tissues [9], Man(1,6) P_2 , which is probably important in the regulation of erythrocyte PFK [10], and sedoheptulose 1,7-bisphosphate [11]. The most potent activator, a new isomer of fructose bisphosphate, Fru(2,6) P_2 , was discovered in Hers' laboratory in 1980 [12]. Although subsequent studies showed that each of the isolated eukaryotic isoforms of PFK was most potently activated by Fru(2,6) P_2 *in vitro* [1,13,14], the importance

of Fru(2,6) P_2 *in vivo* is as yet far from clear in most tissues [2,15]. Only in liver does Fru(2,6) P_2 appear to play a crucial role in switching from gluconeogenesis to glycolysis [14–16].

Among homotetrameric forms of vertebrate PFK consisting of M-type (muscle-type), L-type (liver-type) or F-type (fibroblast-type, also referred to as P- or C-type) subunits, the M_4 isoenzyme appears to bind hexose bisphosphates other than Fru(2,6) P_2 best [2,17–19]. A structural comparison of natural and synthetic bisphosphorylated substances effectively activating M_4 -type PFK [20,21] indicates that their main similarity is the ability of the two phosphate groups to adopt a distance of about 0.9 nm ($9 \text{ \AA}$). On the other hand, striking differences exist in the structures of the phosphate-bearing moieties. In particular, the presence of a furanose ring seems not to be obligatory, since hexitol 1,6-bisphosphate [20], butanediol 1,4-bisphosphate [20], Glc(1,6) P_2 and Man(1,6) P_2 [10] are also potent activators. However, if an activator is to be active below micromolar concentration, the furanose ring and the *trans*-orientation of its two free hydroxy groups appear to be necessary [22].

When building models of these effectors, I encountered a steric resemblance between β -D-Glc(1,6) P_2 and β -D-Man(1,6) P_2 , and the D- and L-enantiomorph, respectively, of *myo*-Ins(1,4) P_2 . This similarity concerns the adoptable P–P distances and, furthermore, the orientation of some

Abbreviations used: PFK, 6-phosphofructo-1-kinase; GroPIns, GroPIns4P, GroPIns(4,5) P_2 , glycerophosphoinositol, its 4-phosphate and its 4,5-bisphosphate; InsP, Ins P_2 , Ins P_3 , Ins P_4 , Ins P_5 and Ins P_6 , D-enantiomorphs (unless otherwise indicated) of *myo*-inositol mono-, bis-, tris-, tetraakis-, pentakis- and hexakis-phosphate, with positional isomerism of phosphomonoesters as indicated in parentheses; PtdIns, PtdIns4P, PtdIns(4,5) P_2 , phosphatidylinositol, its 4-phosphate and its 4,5-bisphosphate; Fru6P, D-fructose 6-phosphate; Glc6P, D-glucose 6-phosphate; PEP, phosphoenolpyruvate; Fru(1,6) P_2 , D-fructose 1,6-bisphosphate; Fru(2,6) P_2 , D-fructose 2,6-bisphosphate; Glc(1,6) P_2 , D-glucose 1,6-bisphosphate; Man(1,6) P_2 , D-mannose 1,6-bisphosphate; DTE, dithioerythritol.

of the hydroxy groups. In many cells and tissues, including heart and smooth muscle, the D-isomer of this substance is generated upon stimulation by agonists which activate the phosphoinositide signalling pathway (for review see [23]). Striated muscle, and, to a lesser extent, numerous other tissues and cells express the M-type PFK subunits [24,25], which appear to be most sensitive towards hexose bisphosphates other than Fru(2,6) P_2 . Therefore, potential actions of inositol bisphosphates and their analogues on this enzyme were studied. A potential role of phosphatidylinositol phosphates in the previously shown membrane binding [26] of PFK was also investigated here.

MATERIALS AND METHODS

Chemicals

Fru(1,6) P_2 , ATP (low-vanadate grade), NADH, AMP, PtdIns4P and PtdIns(4,5) P_2 were from Boehringer (Mannheim, Germany). Fru6P, Glc(1,6) P_2 , Fru(2,6) P_2 , sedoheptulose 1,7-bisphosphate, Ins2P and PtdIns were from Sigma (Taufkirchen, Germany).

Enzymes

Fructose-bisphosphate aldolase, triosephosphate isomerase, glycerol-3-phosphate dehydrogenase, pyruvate kinase and lactate dehydrogenase, all from rabbit skeletal muscle, were purchased as crystal suspensions in $(\text{NH}_4)_2\text{SO}_4$ from Boehringer. They were dialysed before the enzyme assays in order to decrease the concentration of $(\text{NH}_4)_2\text{SO}_4$ appropriately. M-type PFK from rabbit skeletal muscle was isolated as described in [27]. The enzyme was stored and depleted of ATP as described in [28]. The concentration of M-type PFK was determined spectrophotometrically by using a value of $A_{279,1\%} = 10.7$ [29] and a subunit (protomer) M_r of 84000. A crude preparation of L-type PFK (16 units/mg; pH 8.0, 30 °C, optimal assay) from rabbit liver was purchased from Sigma.

Inositol phosphates [Ins1P, Ins4P, Ins(1,4) P_2 , Ins(2,4) P_2 , Ins(4,5) P_2 , Ins(1,4,5) P_3], GroPIns, GroPIns4P and GroPIns(4,5) P_2 were prepared from bovine brain phosphoinositide lipids as described in [30]. The purity and identity of these substances were assessed by paper chromatography [30], ^1H -n.m.r. spectroscopy using the instrumentation and measuring conditions as described in [31], and the recently developed 'h.p.l.c.-m.d.d.' technique, an h.p.l.c. analysis with post-column 'metal-dye detection' of phosphorylated substances [32]. Phosphoinositides were analysed by t.l.c. [33]. All inositol compounds were over 97% pure, except Ins1P, which contained up to 15% of Ins2P. The purity of Fru(2,6) P_2 was checked by paper chromatography [30], and that of Fru(1,6) P_2 and Glc(1,6) P_2 by h.p.l.c.-m.d.d. The concentrations of these substances were determined by their total phosphate content [30].

PFK activity was determined spectrophotometrically at 366 nm in either a Fru(1,6) P_2 - or an ATP-coupled enzyme system. The latter assay was performed with diluted enzyme (0.1–1 $\mu\text{g}/\text{ml}$ final concn.) in a near-physiological incubation medium at 30 °C. For assays of M-type PFK this medium had a pH of 7.3, and was composed of 100 mM-KCl, 50 mM-Hepes/KOH, 5 mM-MgCl₂, 4 mM-ATP, 0.5 mM-P_i, 0.7 mM-PEP, 0.2 mM-NADH, 2.5 mM-DTE, 0.1 mM-EDTA, 1 mM- $(\text{NH}_4)_2\text{SO}_4$, 50 μM -Fru6P and 25 μg each of pyruvate kinase and

lactate dehydrogenase/ml unless otherwise indicated. L-type PFK was assayed at pH 7.4 in medium containing 0.1 M-KCl, 40 mM-Hepes/KOH, 5 mM-MgCl₂, 3 mM-ATP, 1 mM-P_i, 0.6 mM-PEP, 0.2 mM-NADH, 2.5 mM-DTE, 0.2 mM-citrate, 0.1 mM-EDTA, 1 mM- $(\text{NH}_4)_2\text{SO}_4$, 0.2 mM-AMP, 60 μM -Fru6P and 10 μg each of pyruvate kinase and lactate dehydrogenase/ml. After a 15 min preincubation period in a thermostatically controlled aluminium block, quartz cuvettes containing 1 ml of reaction medium were placed in a Perkin-Elmer Lambda 5 spectrophotometer and the reaction was started by adding 5 μl of PFK, pre-diluted to 20–200 $\mu\text{g}/\text{ml}$ in 100 mM-potassium phosphate (pH 8.0)/1 mM-EDTA/5 mM-DTE. Only the initial decrease in the u.v. absorbance was taken for activity calculations.

'Optimal assays' of samples of preincubated enzyme, performed to determine the fraction of (active) tetrameric enzyme by the residual activity, were performed as described in [34], but with 5 μM -Fru(2,6) P_2 included in the assay.

The binding of allosteric activators to sites of PFK which stabilize the active tetrameric state was indirectly measured by a 'tetramer stabilization assay' [34]. In this two-step assay, initially tetrameric enzyme at 1 μM protomer concentration was incubated in the absence of substrates but in the presence of effectors. During the incubation, diluted samples were analysed for the amount of tetrameric enzyme still present. The experiments were performed as follows: PFK was extensively dialysed against 100 mM-potassium phosphate (pH 8.0)/1 mM-EDTA/5 mM-DTE in order to remove ATP and $(\text{NH}_4)_2\text{SO}_4$, adjusted to a concentration of 8.4 mg/ml (100 μM protomers), and ultracentrifuged (40000 g, 20 min) to remove aggregates. Under these conditions, PFK has been shown to be present as an active four-subunit enzyme (termed 'tetrameric' [34]). A series of incubation buffers containing 100 mM-KCl, 40 mM-Hepes/KOH, 1 mM-MgCl₂, 5 mM-DTE and 0.2 g of NaN_3/l was prepared by adjusting the pH values to between 6.7 and 7.8 (routinely 7.2) with KOH (at 30 °C) and the final conductivity to a constant value of 15.5 mS by adding KCl. To 500 μl of this buffer supplemented with the desired effector(s), after a 20 min period of preincubation, 5 μl of the PFK solution was added. At intervals up to 200 min, 10 μl samples were analysed for residual activity as described above and for P_i.

RESULTS

AMP-dependent activation of muscle PFK by Ins(1,4) P_2 and GroPIns4P

Effects of inositol phosphates on M₄-type PFK were investigated under near-physiological conditions, which have been demonstrated previously to allow an allosteric activation by micromolar concentrations of sugar bisphosphates [2,4,17]. For that, AMP, a ligand known to act synergistically with sugar bisphosphates [4,13,17,35–37], was included at a concentration of 100 μM . A basal specific activity of 12 units/mg was thus obtained in absence of any sugar bisphosphate. As shown in Fig. 1(a), Fru(1,6) P_2 exerted a half-maximal activation of PFK at 2.2 μM (these concentrations were defined as K_a values) under these conditions. At concentrations above 20 μM , the activity decreased again from its maximal value, as demonstrated previously for L-type PFK [13,38]. All three sugar bisphosphates tested, Fru-

Fig. 1. Allosteric activation of M_4 -type PFK by inositol phosphates and analogues

Allosteric assays were performed as described in the Materials and methods section, except that 0.1 mM-AMP was included in all assays. The concentration of PFK was 0.2 μ g/ml. Allosteric activators varied in concentration were: in (a): $\circ$, Fru(1,6) P_2 ; $\bullet$, Ins(1,4) P_2 ; $\triangle$, Ins(1,4) P_2 in presence of 0.5 μ M-Fru(1,6) P_2 ; $\blacksquare$, Ins(2,4) P_2 ; $\blacktriangledown$, Ins1P; $\square$, Ins(1,4,5) P_3 ; in (b): $\circ$, Glc(1,6) P_2 ; $\bullet$, GroPIns4P; $\square$, GroPIns(4,5) P_2 ; $\blacktriangledown$, GroPIns.

(1,6) P_2 , Fru(2,6) P_2 and Glc(1,6) P_2 (see Table 1 for K_a values), gave a maximal activity of about 80 units/mg. Among the inositol phosphates tested (Fig. 1, Table 1), two isomers, Ins(1,4) P_2 and, less effectively, Ins(2,4) P_2 , in fact activated the enzyme, with K_a values of 43 and 70 μ M respectively. However, the highest extents of activation obtained with millimolar concentrations of both substances were significantly lower than those observed with the sugar bisphosphates. Up to 1 mM-Ins(4,5) P_2 had no effect. As also shown in Fig. 1, the stimulatory effect on PFK by Ins(1,4) P_2 is additive with the effect of Fru(1,6) P_2 . As a consequence thereof, concentrations of Fru(1,6) P_2 that only slightly increased the basic activity caused a significant leftward shift of the Ins(1,4) P_2 -activation curve.

Neither inositol monophosphate (Table 1, Fig. 1) had a significant effect on PFK at up to millimolar concentrations. Ins(1,4,5) P_3 had no activating effect, but, conversely, was inhibitory at concentrations above 20 μ M (Fig. 1). Isomeric mixtures of Ins P_4 , Ins P_5 and Ins P_6 also

acted as inhibitors, but only at concentrations above 50 μ M.

An analogue of Ins(1,4) P_2 containing a 1-glycero-phospho group at position 1 of the inositol ring, GroPIns P , was a still more potent allosteric activator of PFK ($K_a = 20$ μ M) than Ins(1,4) P_2 (Fig. 1b). The maximal activation obtained by GroPIns4P (70 units/mg) was higher than that obtained by Ins(1,4) P_2 . GroPIns(4,5) P_2 , the corresponding analogue of Ins(1,4,5) P_3 , was a weak allosteric activator (Fig. 1b, Table 1), unlike the parent compound. Though only at millimolar concentrations, GroPIns was also slightly activating (Table 1).

Many of the inositol phosphates and analogues were prepared as carrier-free ammonium salts of pH 7. Therefore an effect of the NH_4^+ counter-ions in the allosteric assay had to be excluded. Total NH_4^+ up to 3.5 mM had no effect on the basal PFK activity, whereas higher concentrations of NH_4^+ ions led to an activation of PFK (to maximally 70 units/mg) with a K_a of 8.2 mM. As assessed by conductivity measurements, none of the inositol phosphate preparations contained excess salt over its counter-ions. Therefore, an activation by the NH_4^+ ions imported by up to 1 mM-inositol bisphosphates or analogues could be excluded.

The experiments shown in Fig. 2 demonstrate that Ins(1,4) P_2 , like sugar bisphosphates, acts synergistically with AMP in activating PFK. The half-maximal activation by AMP, which in the absence of Ins(1,4) P_2 was attained at 74 μ M, was shifted to 48 μ M- and 32 μ M-AMP when 20 μ M- and 40 μ M-Ins(1,4) P_2 , respectively, were present.

AMP-enhanced binding of Ins(1,4) P_2 and analogues thereof to muscle PFK

In the assays described above, strongly diluted enzyme was incubated under conditions selected to mimic the concentrations *in vivo* of several other allosteric effectors of PFK, such as P_i , NH_4^+ , H^+ , PEP, ADP, Fru6P and ATP [1,4]. Concentrations of inositol phosphates leading to half-maximal effects on PFK under these conditions often differ from the dissociation constants which are obtained when measuring the binding to more concentrated enzyme in the absence of most other effectors [2]. Such a binding assay had to cope with three demands: (i) use of non-radioactively labelled inositol phosphates and analogues, because of the lack of many of them as radioligands; (ii) absence of most other allosteric effectors, in particular of those rapidly interconverted by PFK; (iii) near-physiological concentrations of enzyme. An indirect binding assay, based on tetramer stabilization, was successfully used previously [34] to study the binding of ligands which stabilize or destabilize the tetrameric form of PFK. As the sugar bisphosphates have been shown to stabilize tetrameric PFK [1,4,5,39–42], this type of assay appeared to be as well suited to estimate $K_{d,app}$ values of inositol phosphates and analogues thereof.

Fig. 3(a) shows the effect of increasing concentrations of Ins(1,4) P_2 on the stabilization of initially tetrameric PFK. In the absence of AMP, 160 μ M-Ins(1,4) P_2 was required to effect half-maximal tetramer stabilization, whereas in the presence of 0.1 mM-AMP 15 μ M-Ins(1,4) P_2 was sufficient (see Table 1 for $K_{d,app}$ values). This substantial leftward shift indicates a strong enhancement of the binding of Ins(1,4) P_2 by AMP. A corresponding effect of AMP was found for the tetramer stabilization

Table 1. Allosteric and tetramer-stabilizing effects of inositol bisphosphates and analogues hereof on M_4 -type PFK

K_a and K_i values designate concentrations of effector giving half-maximal allosteric effect at pH 7.3; $K_{d,app}$ values designate concentrations giving half-maximal tetramer stabilization at pH 7.2 (see the Materials and methods section for conditions). The amount of tetramers was routinely estimated from residual activities. Abbreviations: act., activation; inh., inhibition; n.d., not determined; l.t.stab., long-term tetramer stabilization; destab., destabilization.

Effector varied	AMP (μM)	Allosteric action			Tetramer stabilization	
		Effect	K_a (μM)	K_i (μM)	Effect	$K_{d,app}$ (μM)
Ins1P	100	act.	> 500		none	—
GroPIns	100	act.	> 500		none	—
Ins4P	100	none	—		none	—
Ins(1,4) P_2	0	act.	260		l.t.stab.†	160
Ins(1,4) P_2	20	n.d.	n.d.		l.t.stab.†	42
Ins(1,4) P_2	100	act.	43		l.t.stab.†	15
Ins(1,4) P_2	100*	act.	28		n.d.	—
GroPIns4P	100	act.	20		tr.stab.‡	17
Ins(2,4) P_2	100	act.	70		l.t.stab.†	45
Ins(4,5) P_2	100	none	—		l.t.stab.†	450
Ins(1,4,5) P_3	100	inh.		74	l.t.stab.†	670
GroPIns(4,5) P_2	100	act.	112		none	—
Ins P_4	100	inh.		n.d.	l.t.stab.§	> 1000
Ins P_5	100	inh.		n.d.	l.t.stab.§	950
Ins P_6	100	inh.		n.d.	l.t.stab.§	510
PtsIns	100	n.d.	n.d.		destab.	—
PtdIns4P	100	n.d.	n.d.		l.t.stab.§	37
PtdIns(4,5) P_2	100	n.d.	n.d.		destab.	—
Glc(1,6) P_2	100	act.	12		l.t.stab.†	11
Fru(1,6) P_2	100	act./inh.	2.1	> 100	l.t.stab.†	6
Fru(2,6) P_2	100	act.	0.13		l.t.stab.†	0.03

* Presence of 0.5 μM -Fru(1,6) P_2 when starting the reaction.

† $K_{d,app}$ estimated from residual activities after 200 min incubation.

‡ Only transient stabilization (see Fig. 1b); $K_{d,app}$ estimated from initial rates of tetramer dissociation.

§ $K_{d,app}$ value determined from residual activities after 100 min of incubation.

Fig. 2. Synergistic interaction between AMP and Ins(1,4) P_2 in the activation of M_4 -type PFK

Experimental conditions were as described for allosteric assays, except that the concentration of P_1 was 1 mM. The concentrations (μM) of Ins(1,4) P_2 are indicated in the Figure.

caused by Fru(1,6) P_2 , Fru(2,6) P_2 and Glc(1,6) P_2 (Table 1, see also Fig. 6(b) below and [42]). Direct binding measurements also have shown an enhancement of hexose bisphosphate binding by AMP [2]. The synergistic interaction of AMP with Ins(1,4) P_2 and the essential role of the bisphosphate in tetramer stabilization are further substantiated by the experiments shown in Fig. 3(b). In the absence of any bisphosphorylated compound, up to 1 mM-AMP was unable to stabilize the tetrameric enzyme permanently. However, when Ins(1,4) P_2 was present during incubation, AMP exerted a strong tetramer-stabilizing effect, which in the presence of 40 μM -Ins(1,4) P_2 was half-maximal at 23 μM -AMP.

Fig. 4 shows the effects of analogues of Ins(1,4) P_2 on tetramer stabilization. Ins(2,4) P_2 , as observed in the allosteric assays (above), was a somewhat weaker binding effector. Its apparent binding affinity was one-third that of Ins(1,4) P_2 (Table 1), and also the maximal degree of long-term tetramer stabilization was lower. Among other inositol phosphates and glycerophosphoinositol phosphates tested, no substance exhibited comparable $K_{d,app}$ values, although some exerted weak long-term tetramer-stabilizing effects (Fig. 4 and Table 1). GroPIns4P had no such effect. The initial decrease in residual activity, however, was in fact strongly decreased by GroPIns4P as compared with control experiments

Fig. 3. Tetramer stabilization of M-type PFK by $\text{Ins}(1,4)\text{P}_2$ and AMP

The concentration of PFK was $1 \mu\text{M}$. The final incubation buffer (see the Materials and methods section) had a pH of 7.2 and contained 1 mM-P_i . The residual activities measured after 200 min of incubation are depicted. The initial specific activity was 116 units/mg . (a): ●, without AMP; ○, □, in presence of indicated concentrations of AMP. $\text{Ins}(1,4)\text{P}_2$ was varied. (b): ●, no $\text{Ins}(1,4)\text{P}_2$; △, ○, □, indicated concentrations of $\text{Ins}(1,4)\text{P}_2$. AMP was varied.

($K_{d,app} = 17 \mu\text{M}$). This indicates that GroPIns4P in fact bound to the tetrameric enzyme and also decelerated its dissociation. However, it was apparently unable to stabilize tetramers permanently. No evidence for a dephosphorylation of GroPIns4P during the incubation was detected by phosphate analyses. However, a slow cleavage of the inositol-glycerol phosphodiester bridge could not be excluded. GroPIns and $\text{GroPIns}(4,5)\text{P}_2$ were completely ineffective.

pH-dependence of the binding of $\text{Ins}(1,4)\text{P}_2$ to muscle PFK

In order to find out whether $\text{Ins}(1,4)\text{P}_2$, like the sugar biphosphates [40–42], is much more strongly bound at alkaline pH, tetramer stabilization experiments were performed at pH values between 6.7 and 7.8. Fig. 5(a) clearly confirms the expected behaviour. The $K_{d,app}$ values, derived from data obtained in the presence of 0.1 mM-AMP , greatly decreased between pH 6.7 and 7.4 from 100 to $5.5 \mu\text{M}$, reaching a minimum at pH 7.8 ($K_{d,app} = 3.7 \mu\text{M}$). In Fig. 5(b), plots of $\text{p}K_{d,app}$ versus pH obtained for $\text{Ins}(1,4)\text{P}_2$ in the absence and the presence

Fig. 4. Long-term tetramer stabilization of M_4 -type PFK by phosphoinositol compounds

Conditions were as described in Fig. 3 legend; pH was 7.2, and 0.1 mM-AMP was included in all incubations. ●, $\text{Ins}(1,4)\text{P}_2$; ■, $\text{Ins}(2,4)\text{P}_2$; ▲, $\text{Ins}(4,5)\text{P}_2$; ○, $\text{Ins}(1,4,5)\text{P}_3$; □, GroPIns4P .

of 0.1 mM-AMP are compared with corresponding data obtained for $\text{Fru}(1,6)\text{P}_2$ and $\text{Fru}(2,6)\text{P}_2$. Whereas the maximal slope of this plot for $\text{Fru}(1,6)\text{P}_2$ has a value of 1, for both $\text{Ins}(1,4)\text{P}_2$ and $\text{Fru}(2,6)\text{P}_2$ it is close to 2. This indicates that the increase in the apparent binding affinity of $\text{Fru}(1,6)\text{P}_2$ with pH requires one essential deprotonation reaction (on the enzyme or the ligand), whereas for $\text{Ins}(1,4)\text{P}_2$ and $\text{Fru}(2,6)\text{P}_2$ two groups must be deprotonated. Since the increase in the apparent binding affinity caused by 0.1 mM-AMP (indicated by the upward shifts of the plots) is by far largest in $\text{Ins}(1,4)\text{P}_2$ (+1.1 compared with between +0.5 and +0.7 pK units), the effectiveness of $\text{Ins}(1,4)\text{P}_2$ becomes comparable with that of $\text{Fru}(1,6)\text{P}_2$ if sufficient AMP is present and the pH is above 7.2.

Interaction of PtdIns4P with muscle PFK

GroPIns4P has been shown (see above) to activate PFK better than $\text{Ins}(1,4)\text{P}_2$. As this substance also represents the head group of PtdIns4P , the question arose whether PtdIns4P could also be a target of PFK. To test that, the tetramer-stabilization technique was again used. Problems encountered with potential interactions of concentrated phospholipid with any of the numerous factors present in allosteric assays were thus largely avoided. The effect of increasing concentrations of PtdIns , PtdIns4P and $\text{PtdIns}(4,5)\text{P}_2$ on the stabilization of tetrameric PFK is shown in Fig. 6. With PtdIns and $\text{PtdIns}(4,5)\text{P}_2$ no stabilization, but a further dissociation of the small amount of tetramers present in the control (K_i 30 and $>100 \mu\text{M}$ respectively), was observed. PtdIns4P , however, in fact led to an increased tetramer stabilization in the concentration range 10 – $100 \mu\text{M}$ ($K_{d,app} = 37 \mu\text{M}$). In the absence of AMP, no tetramer stabilization was detectable by up to 0.2 mM-PtdIns4P (results not shown). The low degree of tetramer stabilization (maximally about 10% residual activity) and the reversal of this effect above $100 \mu\text{M-PtdIns4P}$, like the negative effects of PtdIns and $\text{PtdIns}(4,5)\text{P}_2$, could have their rationale in an inhibitory interaction with PFK of

Fig. 5. pH-dependent interactions of Ins(1,4)P₂ and hexose biphosphates with M₄-type PFK

(a) Dependence of tetramer stabilization by Ins(1,4)P₂ on the pH. Conditions were as described in Fig. 3 legend, except that the pH values were 6.7, 7.1, 7.4 and 7.8. [AMP] was 0.1 mM. (b) Plots of pK_{d,app.} versus pH for Ins(1,4)P₂, Fru(1,6)P₂ and Fru(2,6)P₂. K_{d,app.} values were obtained as in (a). ○, ●, Ins(1,4)P₂; □, ■, Fru(1,6)P₂; △, ▲, Fru(2,6)P₂; ○, □, △, no AMP; ●, ■, ▲, 0.1 M-AMP present.

Fig. 6. Tetramer stabilization of M₄-type PFK by PtdIns4P

Conditions were as described in Fig. 3 legend, except that residual activities after 100 min of incubation are depicted. The pH was 7.2. Phosphoinositides dried from chloroform/methanol were dissolved in incubation buffer by sonication. From fresh stock solution (2.5 mM) the desired dilutions were made. [AMP] was 0.1 mM.

the hydrophobic diacyl moieties of the phospholipids. T.l.c. analyses excluded a dephosphorylation or phosphodiester cleavage during incubation. However, up to 10% unesterified fatty acids were present in the incubation mixture. Earlier reports about a binding of fatty acids to L₄-type PFK with a concomitant loss of activity by tetramer dissociation are in line with this interpretation [43].

Actions of inositol phosphates on liver PFK

A partially purified PFK from liver was employed in order to test whether inositol phosphates have allosteric effects on the L₄-type isoenzyme. Fig. 7 shows that up to

Fig. 7. Allosteric effects of Ins(1,4)P₂ and Ins(1,4,5)P₃ on L₄-type PFK

Assay conditions are described in the Materials and methods section. Activities (*v*) are given relative to V_{max}, the optimal activity at pH 8.0 in the presence of 1 μM-Fru(2,6)P₂; 0.2 mM-AMP was present. Effectors varied: ○, Fru(1,6)P₂; ●, Ins(1,4)P₂; □, Ins(1,4,5)P₃.

100 μM-Ins(1,4)P₂ caused only a slight activation, and distinctly higher concentrations of effector were required for a stronger activation (*K*_a = 160 μM). Interestingly, Ins(1,4,5)P₃, unlike with M₄-type PFK, had a weak (about 2-fold) activating effect on L₄-type PFK below micromolar concentrations (*K*_a = 0.4 μM); as observed for M₄-type PFK, it was inhibitory above micromolar concentrations (*K*_i = 20 μM).

DISCUSSION

There is ample evidence that Ins(1,4)P₂ is the prevailing inositol biphosphate *in vivo*. After agonist stimulation,

this isomer is generated both by dephosphorylation of $\text{Ins}(1,4,5)\text{P}_3$ and by an eventual phosphodiesteratic cleavage of $\text{PtdIns}4\text{P}$ [44,45]. Therefore, not only from the structural similarity, but also from the aspect of abundance *in vivo*, $\text{Ins}(1,4)\text{P}_2$ was the most likely candidate as an effector of PFK. In fact, under all experimental conditions employed, only $\text{Ins}(1,4)\text{P}_2$, $\text{GroPIns}4\text{P}$ and $\text{Ins}(2,4)\text{P}_2$ behave similarly to the allosteric effectors of the hexose bisphosphate type in activating M_4 -type PFK. Half-maximal effects on M_4 -type PFK under conditions resembling those in muscle were observed in the 20–50 μM concentration range. Indirect binding data showed, however, that under more favourable conditions (higher pH, higher concentrations of AMP, low concentrations of ATP) dissociation constants are substantially lower. These conditions may prevail in smooth muscle and many non-muscle cells [46,47]. Furthermore, the effect of $\text{Ins}(1,4)\text{P}_2$ on the enzyme is additive to that of hexose bisphosphates. Preliminary experiments with a crude preparation of liver PFK (L_4 isoenzyme) indicate that the substantial activation by $\text{Ins}(1,4)\text{P}_2$ is relatively specific to the muscle isoenzyme.

Determinations by h.p.l.c. of absolute concentrations of $\text{Ins}(1,4)\text{P}_2$ in various cells and tissues, including heart and skeletal muscle, are at present in progress (see [32] for the technique employed). Preliminary results from these studies indicate that, starting from basal values of about 5 nmol/g wet wt., more than 10-fold concentration rises can rapidly occur after stimulation by Ca^{2+} -mobilizing agonists in most tissues; in skeletal muscles, the resting concentrations are already above 10 nmol/g. In cells and tissues rich in M -type PFK subunits, these concentrations of $\text{Ins}(1,4)\text{P}_2$ may be sufficient to induce an allosteric activation of PFK when the pH value and the concentrations of synergistic activators, AMP or ADP [1,3,4], are high enough simultaneously (see above). As the plasma membrane is the main source for formation of $\text{Ins}(1,4)\text{P}_2$, a diffusion- and dephosphorylation-controlled gradient of $\text{Ins}(1,4)\text{P}_2$ might build up towards the inner cytoplasmic space, thus restricting this kind of activation of PFK and glycolysis to the sub-plasmalemmal region. In fact, such a compartmentation of glycolysis has been shown for smooth muscle [48].

High basal concentrations of $\text{Ins}(1,4)\text{P}_2$ could lead to a 'priming' of M -type PFK, rendering its AMP- or ADP-induced activation independent of an increase in $\text{Fru}(1,6)\text{P}_2$ [37] or $\text{Glc}(1,6)\text{P}_2$ [7,8]. On the other hand, the binding of $\text{Ins}(1,4)\text{P}_2$ to PFK could prevent a rapid dephosphorylation of this inositol phosphate under resting conditions. Every large increase in activating hexose bisphosphates, ligands competing for the same binding sites on PFK, would release the bound $\text{Ins}(1,4)\text{P}_2$ and thus indirectly enhance its dephosphorylation.

An important potential functionality arises from the finding *in vitro* that M_4 -type PFK binds to and is activated by the head group of $\text{PtdIns}4\text{P}$. By this mechanism, the long-known but hardly understood binding of a significant amount of PFK to the plasma membrane ([26,49], reviewed in [50]) could be explained. Several functional implications would result *in vivo*. Firstly, the degree of membrane compartmentation and of concomitant activation of PFK would become dependent on PtdIns metabolism. Secondly, the release of PFK from the membranes would be coupled to the activity of phospholipase C, which in many cells is under agonist control. Thirdly, the activation of PFK by released

$\text{Ins}(1,4)\text{P}_2$ might be facilitated by this kind of membrane binding. Fourthly, a binding of PFK to $\text{PtdIns}4\text{P}$ present in cell membranes should influence the size of the accessible $\text{PtdIns}4\text{P}$ pool, its interaction with other enzymes such as $\text{PtdIns}4\text{P}$ kinase, phospholipase C and $\text{PtdIns}4\text{P}$ phosphomonoesterase, and thus its functional dynamics and turnover. The concentration of $\text{PtdIns}4\text{P}$ in plasma membranes has been shown to be sufficient by far to allow for such an interaction with PFK [51,52]. From these findings, some degree of coupling between the glycolytic activity, the phosphoinositide metabolism and the cytoplasmic Ca^{2+} (see also [45]) may be expected. Very interesting in this context are recent findings of linked oscillations of the cytoplasmic free Ca^{2+} concentration and the glycolytic activity [53], our own previous findings of binding of $\text{Ins}(1,4,5)\text{P}_3$ to the subsequent glycolytic enzyme, aldolase [30], and previous reports of inhibition of $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphatase by glycolytic intermediates [54].

Furthermore, it appears reasonable to consider and investigate in further studies the possibility that the repeatedly reported, but mechanistically unexplained, α -adrenergic activation of PFK in heart muscle [55,56] and adipose tissue [57], as well as hitherto unexplained regulatory facets of the liver glycolytic-gluconeogenic system (reviewed in [58]), might be related to the phenomena demonstrated here *in vitro*.

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