Identification of the glycogenic compound 5-iodotubercidin as a general protein kinase inhibitor

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Addition of micromolar concentrations of the adenosine derivative 5-iodotubercidin (Itu) initiates glycogen synthesis in isolated hepatocytes by causing inactivation of phosphorylase and activation of glycogen synthase [Flückiger-Isler and Walter (1993) Biochem. J. **292**, 85–91]. We report here that Itu also antagonizes the effects of saturating concentrations of glucagon and vasopressin on these enzymes. The Itu-induced activation of glycogen synthase could not be explained by the removal of phosphorylase *a* (a potent inhibitor of the glycogen-associated synthase phosphatase). When tested on purified enzymes, Itu did not affect the activities of the major Ser/Thr-specific protein phosphatases (PP-1, PP-2A, PP-2B and PP-2C), but it inhibited various Ser/Thr-specific protein kinases as well as the tyrosine kinase activity of the insulin receptor (IC_{50} between 0.4 and 28 μM at 10–15 μM ATP). Tubercidin, which did not affect glycogen synthase or phosphorylase in liver cells, was 300 times less potent as a protein kinase inhibitor. Kinetic analysis of the inhibition of casein kinase-1 and protein kinase A showed that Itu acts as a competitive inhibitor with respect to ATP, and as a mixed-type inhibitor with respect to the protein substrate. We propose that Itu inactivates phosphorylase and activates glycogen synthase by inhibiting phosphorylase kinase and various glycogen synthase kinases. Consistent with the broad specificity of Itu *in vitro*, this compound decreased the phosphorylation level of numerous phosphopolypeptides in intact liver cells. Our data suggest that at least some of the biological effects of Itu can be explained by an inhibition of protein kinases.

INTRODUCTION

Glycogen metabolism is mainly controlled by the phosphorylation state of glycogen synthase and phosphorylase [1–4]. While a host of protein kinases can contribute to the inactivation of glycogen synthase, phosphorylase is only phosphorylated and activated by phosphorylase kinase. In the liver, the activities of glycogen synthase and phosphorylase are tightly coupled, since the active *a*-form of phosphorylase is a potent allosteric inhibitor of the glycogen-associated synthase phosphatase. Hence the prior inactivation of phosphorylase facilitates the activation of glycogen synthase by the latter phosphatase. This coupling may explain, for example, why glucose, which binds to phosphorylase *a* and turns it into a better substrate for protein phosphatases, only promotes the activation of glycogen synthase when present at concentrations that allow for the near-complete dephosphorylation of phosphorylase [2,4]. Nucleosides and purine derivatives, which bind to an inhibitory purine site in phosphorylase *a* [3], also enhance the inactivation of phosphorylase *in vitro* [2]. Accordingly, caffeine has been shown in isolated hepatocytes to accelerate the glucose-induced inactivation of phosphorylase and to advance the subsequent activation of glycogen synthase [5]. In contrast, amino acids such as glutamine and asparagine stimulate glycogen synthesis by a different mechanism, which involves cell swelling [6]; the associated decrease in intracellular Cl^- and increases in glutamate and aspartate increase the synthase phosphatase activity without affecting phosphorylase phosphatase [7].

Initial interest in tubercidin (7-deaza-adenosine) and its derivatives [8] stemmed from their potential use as anti-tumour and anti-viral agents, resulting from their interference in numerous cellular processes including mitochondrial respiration and synthesis of purines, nucleic acids and proteins [8,9]. At variance with tubercidin, the derivative 5-iodotubercidin (Itu) is a potent inhibitor of adenosine kinase [10,11]. Furthermore, Itu (but not tubercidin) causes muscle relaxation and hypothermia when injected intraperitoneally into mice [10,11]. Recently it was found that Itu also promotes glycogen synthesis in liver cells [12]. This glycogenic action was associated with an inactivation of phosphorylase and an activation of glycogen synthase. The effects of Itu on glycogen metabolism could not be explained by changes in the concentration of metabolites such as cyclic AMP, AMP or ATP, and were not associated with cell swelling [12].

We conclude here that the glycogenic action of Itu is not explained by a putative binding to the purine site in phosphorylase *a*. Our data show that this compound acts by inhibiting a broad range of protein kinases, and decreases the phosphorylation level of many phosphopolypeptides in liver cells.

EXPERIMENTAL

Materials

Itu was obtained from both ICN Biomedicals (Costa Mesa, CA, U.S.A.) and RBI (Natick, MA, U.S.A.). It was suspended in water at a concentration of 3.5 mM and kept for up to 3 weeks at 4 °C. Longer storage of the suspension resulted in a loss of glycogenic potency. For each experiment, fresh dilutions were made from the stock solution in 50 mM Tris/HCl, pH 7.5. Tubercidin, histone IIA, histone IIIS, vasopressin, poly(GluNa,Tyr) 4:1 and the catalytic subunit of protein kinase A from beef heart were obtained from Sigma. Glucagon was purchased from Novo, yeast hexokinase from Boehringer and

Abbreviations used: Itu, 5-iodotubercidin; PP, protein phosphatase; PP-1_C, catalytic subunit of protein phosphatase-1; PP-$2A_C$, catalytic subunit of protein phosphatase-2A.

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rabbit skeletal muscle pyruvate kinase from Calbiochem. γ-^{32}P-labelled ATP and radioactive P_i were purchased from Amersham.

Phosphorylase *b* [13] and phosphorylase kinase [14] were prepared from rabbit skeletal muscle. Phosphorylase *b* was converted to radiolabelled phosphorylase *a* as described [15]. Casein kinases-1 and -2 were isolated from pig spleen [16], and protein kinase C was isolated from rat liver [17]. The recombinant tyrosine kinase domain of the human insulin receptor was expressed in insect cells and purified as described [18,19]. The insulin receptor kinase was activated by autophosphorylation in the presence of 30 μg/ml protamine, 0.5 mM ATP and 5 mM magnesium acetate during 30 min at 30 °C, and then extensively diluted prior to assay with poly(Glu,Tyr). Glycogen synthase was isolated from dog liver [20]. Casein was prepared according to the procedure of Mercier et al. [21] and phosphorylated by the catalytic subunit of protein kinase A [22]. Except for PP-2C, which was prepared from rat liver [23], the protein phosphatases (PP) were purified from rabbit skeletal muscle. These include the catalytic subunits of PP-1 ($PP\text{-}1_C$) [24] and PP-2A ($PP\text{-}2A_C$) [25], PP-2A holoenzymes [26] and PP-2B [27].

Hepatocytes and liver extracts

Hepatocytes were prepared from the liver of normally fed male Wistar rats [28]. Unless indicated otherwise, the cells (5×10^6/ml) were incubated in a Krebs–Henseleit medium supplemented with 13.5 mM lactate, 1.5 mM pyruvate, 0.2 mM glycerol and 10 mM glucose. Samples for two-dimensional electrophoresis and for the assays of glycogen synthase and phosphorylase were immediately diluted with a buffer containing kinase and phosphatase inhibitors, and frozen in liquid nitrogen [28].

Assays

Glycogen synthase and phosphorylase were assayed as described before [20,28]. Glucokinase, hexokinase, fructokinase, triokinase, glycerol kinase and pyruvate kinase were assayed spectrophotometrically [29,30] at a final ATP concentration of 50 μM. Glucokinase, triokinase and glycerol kinase were assayed in liver cytosol [29], while fructokinase was assayed after partial purification from rat liver, until after precipitation with poly(ethylene glycol) [30].

The activities of purified protein kinases were calculated from the linear rate of incorporation of phosphate from [γ-^{32}P]ATP (10–15 μM, unless stated otherwise) into a protein substrate (1–2 mg/ml) at 30 °C. The labelled products were separated from radioactive ATP by adsorption to P81 phosphocellulose paper and quantified by liquid scintillation counting. The substrates were histone IIA for protein kinase A, histone IIIS for protein kinase C, poly(Glu,Tyr) for the insulin receptor, and casein for the casein kinases. Extra additions for the assay of protein kinase C included 0.15 mg/ml phosphatidylserine, 0.015 mg/ml diolein, 0.1 mM $CaCl_2$ and 13 mM $MgCl_2$. The assay of phosphorylase kinase was performed in the presence of 0.1 mM $CaCl_2$ and 2.5 mM $MgCl_2$. The other protein kinases were assayed in the presence of 1 mM $MgCl_2$ (protein kinase A) or 5 mM $MgCl_2$ (casein kinases and insulin receptor kinase). One unit of protein kinase incorporates 1 nmol of phosphate/min into the substrate.

Protein phosphatase activities were measured with glycogen synthase *b*, phosphorylase *a* or phosphocasein as substrates, as appropriate [20,22]. The purified protein phosphatases were assayed with phosphorylase *a* (PP-1, PP-2A) or phosphocasein (PP-2B, PP-2C) as substrate. The intracellular free calcium concentration was estimated by Fura-2 fluorescence [31].

For the assay of the specific radioactivity of the γ-phosphate of ATP (see legend to Figure 7), cells incubated with [^{32}P]P_i were sedimented by centrifugation and extracted with 1 M $HClO_4$, and the neutralized extracts were used as a source of ATP for the complete phosphorylation of purified phosphorylase *b* (monitored by activity measurements) by purified phosphorylase kinase [15]. Phosphorylase *a* was separated from other ^{32}P-labelled compounds by SDS/PAGE (7.5% gels), visualized by Coomassie Blue staining as well as autoradiography, and the incorporated radioactivity was quantified by measurement of Čerenkov radiation in a liquid scintillation spectrometer.

Two-dimensional electrophoresis

To the frozen cell samples (see above) was added 1 vol. of a mixture containing 6 M urea, 2 mM 2-mercaptoethanol, 4% (v/v) Pharmalyte, 2% (v/v) glycerol and 2% (v/v) Triton X-100. In the first dimension the polypeptides were separated according to their pI, using Immobiline strips (Pharmacia-LKB) for isoelectric focusing between pH 4 and 7. In the second dimension they were separated according to molecular mass, using 8–18% polyacrylamide gradient gels in the presence of 0.4% SDS (ExcelGel SDS; Pharmacia-LKB).

RESULTS

Independent effects of Itu on the dephosphorylation of phosphorylase and synthase

When hepatocytes were incubated in the presence of 10 mM glucose, phosphorylase was progressively inactivated, and after 20 min the activation of glycogen synthase started (Figure 1a). Addition of Itu (40 μM) enhanced the rate of phosphorylase inactivation and shortened the lag before the activation of glycogen synthase (Figure 1a). This is roughly in keeping with a caffeine-like effect of Itu, owing to its putative binding to the purine site in phosphorylase *a*. However, the actual rate of activation of glycogen synthase was also increased 5-fold by 40 μM Itu, and this cannot be explained by an exclusive effect of Itu on the concentration of phosphorylase *a*, which inhibits the glycogen-associated synthase phosphatase. Further, in the pres-

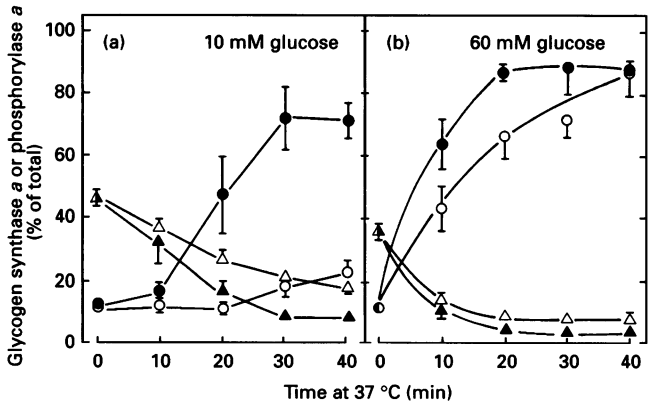

Figure 1 Effect of Itu on the activities of glycogen synthase and phosphorylase

Hepatocytes were incubated with 10 mM (**a**) or 60 mM (**b**) glucose in the absence (○, △) or presence (●, ▲) or 40 μM Itu. At the indicated times, samples were taken for the assays of phosphorylase (△, ▲) and glycogen synthase (○, ●) activities. Results represent means ± S.E.M. ($n = 4$).

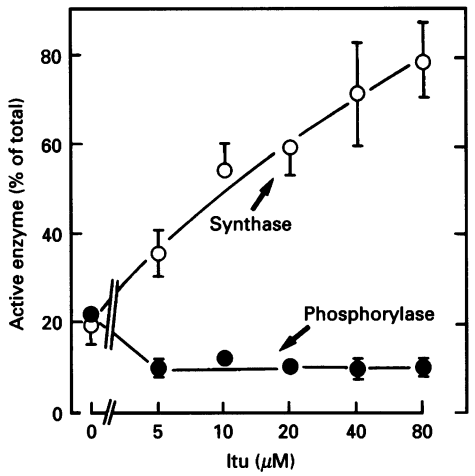

Figure 2 Concentration-dependence of the effects of Itu on glycogen synthase and phosphorylase

Hepatocytes were incubated with 10 mM glucose in the presence of the indicated concentrations of Itu (on a log scale). After 30 min, samples were taken for the assays of phosphorylase (●) and glycogen synthase (○) activities. Results represent the means ± S.E.M. ($n = 4$). The residual concentration of phosphorylase *a* at 30 min reflects correctly the rate of phosphorylase inactivation.

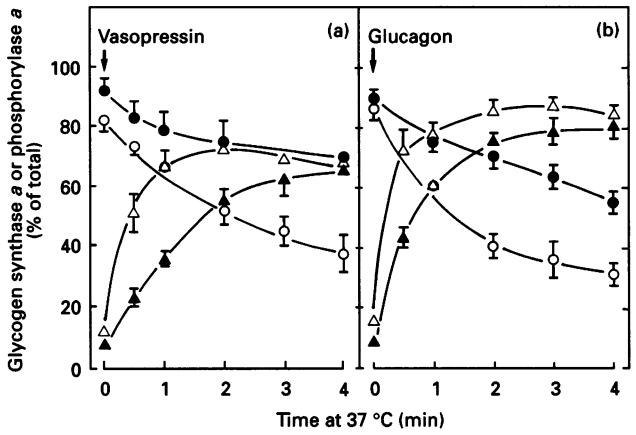

Figure 3 Itu antagonizes the effects of glucagon and vasopressin on phosphorylase and glycogen synthase

Hepatocytes were preincubated for 30 min with 60 mM glucose in the absence (○, △) or presence (●, ▲) of 50 μM Itu. Then (arrows) either 0.1 μM vasopressin (**a**) or 0.1 μM glucagon (**b**) was added, and samples were taken at the indicated times for assays of phosphorylase (△, ▲) and glycogen synthase (○, ●) activities. Results represent the means ± S.E.M. ($n = 4$–8).

ence of a saturating (60 mM) concentration of glucose, 40 μM Itu had a minimal effect on the (rapid) inactivation of phosphorylase, but it still enhanced markedly the rate of activation of glycogen synthase (Figure 1b). The latter result also discounts the hypothesis that Itu could enhance the glucose-sensitivity of the inactivation of phosphorylase; moreover, when added to hepatocytes from fasted rats, 40 μM Itu was still able to enhance the inactivation of phosphorylase and to elicit some activation of glycogen synthase, in the total absence of glucose (results not shown). Comparison of dose–response curves for Itu in the presence of 10 mM glucose (Figure 2) clearly showed independent effects of Itu on the inactivation of phosphorylase

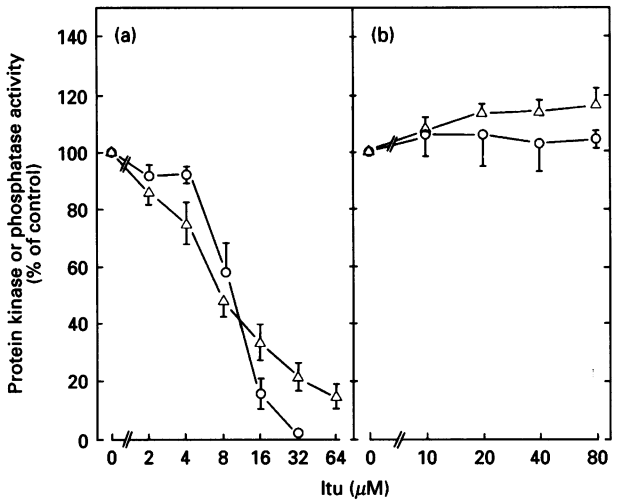

Figure 4 Effects of Itu on protein kinases and protein phosphatases involved in glycogen metabolism

The effects of the indicated concentrations of Itu (on a log scale) on the activities of protein kinases (**a**) and protein phosphatases (**b**) were measured as indicated in the Experimental section. The activities of the purified protein kinases were measured in the presence of 0.55 mM ATP. The protein phosphatase activities were measured in liver extracts. (**a**) ○, Protein kinase A; △, phosphorylase kinase; (**b**) ○, synthase phosphatase; △, phosphorylase phosphatase. The results represent the means ± S.E.M. for 3–11 experiments. A statistically significant effect on phosphorylase phosphatase activity was observed at all concentrations of Itu ($P < 0.04$).

(which was already maximal at 5 μM Itu) and on the activation of glycogen synthase (which was still increasing at 80 μM Itu). Tubercidin at concentrations up to 80 μM did not affect the activation state of phosphorylase or glycogen synthase (results not shown).

Itu antagonizes the effects of glucagon and vasopressin

After a preincubation of liver cells with 60 mM glucose (cf. Figure 1b), the addition of a saturating dose (0.1 μM) of either glucagon or vasopressin resulted in a rapid re-activation of phosphorylase and a re-inactivation of glycogen synthase (Figure 3). In the presence of 50 μM Itu these effects of glucagon and vasopressin occurred much more slowly. This shows that Itu opposes the effects of both cyclic AMP and calcium signalling pathways.

Previous investigations have shown that 50–100 μM Itu, paradoxically, causes a moderate increase in the concentration of cyclic AMP [11,12]. We found that 50 μM Itu did not affect the basal concentration of free calcium in single hepatocytes (results not shown).

Interaction of Itu with protein kinases

Taken together, the above data indicated that the glycogenic action of Itu is due to an inhibition of protein kinases and/or a stimulation of protein phosphatases that act on glycogen synthase and phosphorylase. When added to a liver extract, Itu did not affect the synthase phosphatase activity, and had only a slight (< 20%) stimulatory effect on the phosphorylase phosphatase activity (Figure 4b). In contrast, Itu potently inhibited two major protein kinases involved in glycogen metabolism, i.e. phosphorylase kinase and the catalytic subunit of cyclic AMP-dependent protein kinase (Figure 4a). A half-complete inhibition was obtained at 5–10 μM Itu (in the presence of 0.55 mM ATP).

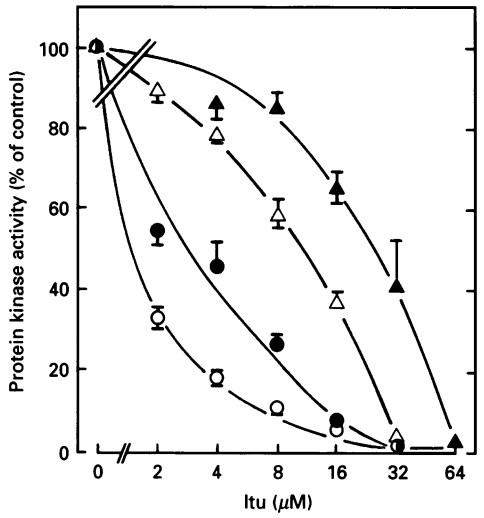

Figure 5 Differential sensitivity of purified protein kinases to inhibition by Itu

Casein kinase-1 (○), the activated insulin-receptor tyrosine kinase fragment (●), casein kinase-2 (△) and protein kinase C (▲) were assayed as described in the Experimental section with 10–15 μM ATP and the indicated concentrations of Itu (on a log scale). The results represent the means + S.E.M. for 4–6 experiments.

These findings prompted us to investigate the effect of Itu on other purified protein kinases. It turned out that all the protein kinases tested could be completely inhibited (Figure 5). At similar concentrations of ATP (10–15 μM), casein kinase-1 was most potently inhibited (IC_{50} 0.4±0.1 μM; $n = 6$), followed by the tyrosine kinase activity of the insulin receptor fragment (IC_{50} 3.5±0.7 μM; $n = 4$), casein kinase-2 (IC_{50} 10.9±2.6 μM; $n = 7$) and protein kinase C (IC_{50} 27.7±7.9 μM; $n = 4$). In addition, 50 μM Itu also inhibited completely the autophosphorylation of two protein kinases tested (results not shown): the insulin receptor kinase fragment, and a hepatic Thr-ectoprotein kinase related to the plasma cell differentiation antigen PC-1 [32]. Tubercidin was a poor inhibitor of the purified protein kinases (results not shown). For example, half-maximal inhibition of casein kinase-1 required 120±10 μM tubercidin ($n = 6$), which is 300-fold higher than the IC_{50} for Itu.

When tested on casein kinase-1, Itu inhibited competitively with respect to ATP (Figure 6a). With respect to casein (Figure 6b), Itu behaved as a mixed-type inhibitor: it decreased the $V_{max.}$ of the kinase, but increased its affinity for the protein substrate (Table 1). Similar results were obtained with protein kinase A (Table 1).

Effects of Itu on other kinases and on protein phosphatases

The broad inhibitory action of Itu on protein kinases and its known inhibitory effect on adenosine kinase [10,11] prompted us to investigate the effect of Itu on other phosphotransferases with an alcohol group as acceptor (Table 2). Itu (50 μM) did not significantly affect the activities of hepatic fructokinase, glycerol kinase, triokinase, yeast hexokinase or muscle pyruvate kinase in the presence of 50 μM ATP. It did, however, cause a 40% decrease in the activity of hepatic glucokinase.

With dog liver glycogen synthase *b* as substrate, the synthase phosphatase activity in a liver extract stems exclusively from type-1 protein phosphatases, whereas the phosphorylase

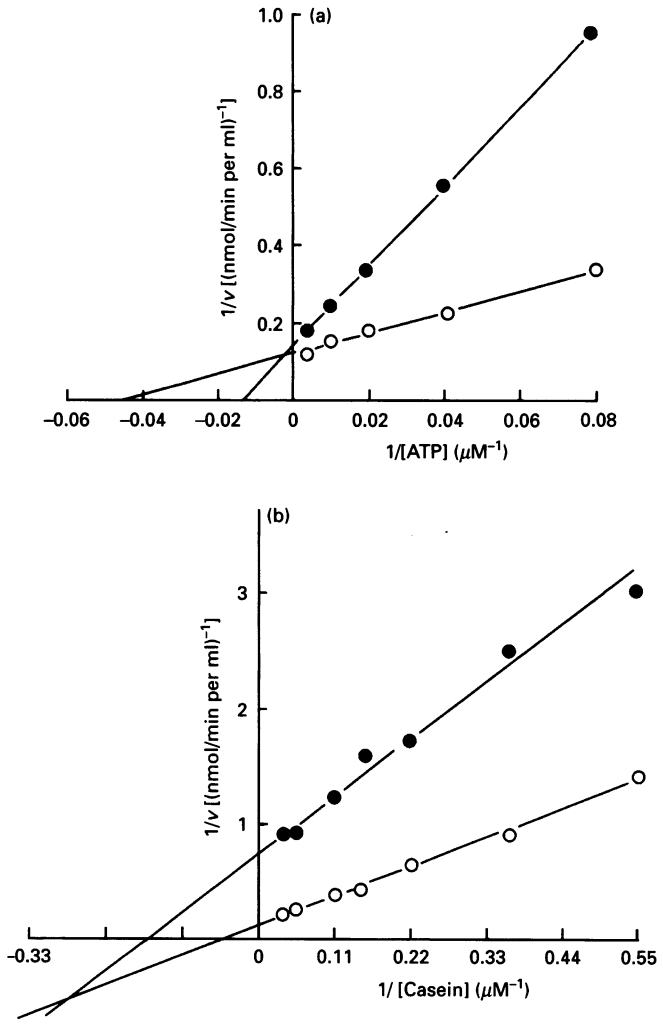

Figure 6 Kinetics of inhibition of casein kinase-1 by Itu

Casein kinase-1 was assayed in the absence (○) or presence (●) of 2 μM Itu, either with 1 mg/ml casein and various concentrations of ATP (**a**), or with 10 μM ATP and various concentrations of casein (**b**). (**a**) and (**b**) show Lineweaver–Burk plots of the means for seven and five experiments respectively.

Table 1 Kinetics of the inhibition of protein kinases by Itu

The results represent the means ± S.E.M. for 3–7 experiments. *Significantly different from the control without Itu ($P < 0.05$).

		Casein kinase I		Protein kinase A	
Substrate	Itu (μM)	$V_{max.}$ (nmol/min per ml)	K_m (μM)	$V_{max.}$ (nmol/min per ml)	K_m (μM)
ATP	0	7.7±1.0	22±1	2.6±1.0	16±3
	2	6.3±0.9	67±15*	–	–
	4	–	–	2.7±0.7	30±3*
Casein	0	7.4±1.2	22±6	–	–
	2	1.6±0.3*	5±1*	–	–
Histone IIA	0	–	–	3.3±0.6	6±2
	4	–	–	1.3±0.3*	3±1*

Table 2 Effect of Itu on the activities of kinases and protein phosphatases

The results represent the means ± S.E.M. for *n* observations. *Significantly different from the control without Itu ($P = 0.0013$). PCS, polycation-stimulated protein phosphatase; subscripts define the subunit composition [26].

Enzyme	*n*	Enzyme activity (% of control) in the presence of... 20 μM Itu	50 μM Itu	80 μM Itu
Kinases				
Glucokinase	3		63 ± 1*	
Hexokinase	2		105 ± 2	
Fructokinase	6		94 ± 14	
Triokinase	2		101 ± 10	
Pyruvate kinase	2		89 ± 4	
Glycerol kinase	2		105 ± 14	
Protein phosphatases				
PP-1_C	3	107 ± 5		103 ± 5
PP-$2A_C$	7	112 ± 11		125 ± 21
PP-2A: PCS_H	5	121 ± 16		119 ± 14
PCS_M	5	101 ± 9		114 ± 15
PCS_L	6	110 ± 12		97 ± 12
PP-2B	9	105 ± 4		91 ± 11
PP-2C	7	109 ± 8		97 ± 6

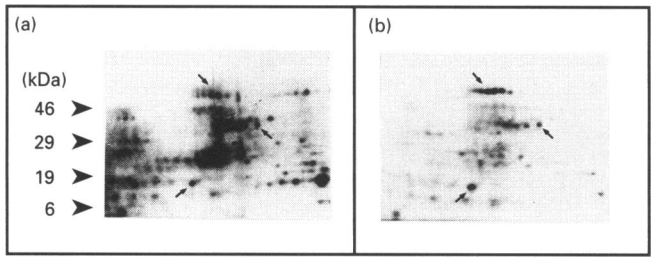

Figure 7 Effect of Itu on the ^{32}P-labelling of polypeptides in intact hepatocytes

Cells were incubated for a total period of 60 min in phosphate-free Krebs–Henseleit medium containing carrier-free radioactive P_i (1 mCi/ml). After 15 min, either 60 μM Itu (**b**) or an equivalent volume of buffer (**a**) was added. The figure shows autoradiograms after two-dimensional electrophoresis of cell homogenates, as described in the Experimental section. The pI range is 4–7 from left to right. Arrows indicate a few phosphopolypeptides that were barely affected by Itu.

phosphatase activity is derived from both type-1 and type-2 protein phosphatases [2]. We have therefore investigated whether the small effect of Itu on the phosphorylase phosphatase activity (Figure 4b) could perhaps be explained by a selective stimulation of a type-2 protein phosphatase. However, as shown in Table 2, none of the purified enzymes was significantly affected by Itu. This absence of a caffeine-like effect indicates that Itu has little affinity for the purine site in phosphorylase *a*.

Effect of Itu on the phosphorylation state of other hepatic polypeptides

In view of the broad action of Itu as inhibitor of protein kinases *in vitro*, it seemed possible that the nucleoside could affect in liver cells the phosphorylation state of proteins other than those involved in glycogen metabolism. We have therefore investigated the effect of Itu on the phosphorylation level of polypeptides in ^{32}P-prelabelled hepatocytes, as measured by autoradiography after two-dimensional electrophoresis (Figure 7). Itu indeed decreased the amount of radiolabel in most phosphopolypeptides, with some clear exceptions indicated by arrows in Figure 7.

We can exclude the possibility that the decreased labelling of phosphopolypeptides in the presence of Itu might result from a decreased specific radioactivity of the γ-phosphate of ATP. If anything, the specific radioactivity of the γ-phosphate of ATP was slightly increased after preincubation with Itu in conditions identical with those in Figure 7 (results not shown).

DISCUSSION

Mechanism of the glycogenic action of Itu

Previous work has shown that the effects of Itu on the activities of glycogen synthase and phosphorylase are not mediated by cell swelling or by an inhibition of adenosine kinase [12]. The glycogenic action of Itu could not be explained either by an effect on the concentration of cyclic AMP [11,12] or Ca^{2+} (the present study). However, the ability of Itu to antagonize both the Ca^{2+}-mediated and the cyclic AMP-mediated effects on glycogen synthase and phosphorylase (Figure 3) suggested that the nucleoside affects the common terminal step of both signalling pathways, i.e. the protein kinases and/or the protein phosphatases that act on glycogen synthase and phosphorylase. Although Itu stimulated slightly the phosphorylase phosphatase activity in a liver extract (Figure 4b), this effect is small and cannot explain the activation of glycogen synthase (see below). On the other hand, Itu emerged as a potent inhibitor of protein kinases, including phosphorylase kinase and the catalytic subunit of protein kinase A (Figure 4a), which operate sequentially in the activation of phosphorylase. The activation of glycogen synthase in hepatocytes occurred progressively over a much wider range of Itu concentrations (Figure 2). This is in keeping with the fact that multiple protein kinases are able to phosphorylate various sites in liver glycogen synthase [1]; several of these glycogen-synthase kinases were tested (protein kinase A, phosphorylase kinase, both casein kinases and protein kinase C) and found to differ widely in their sensitivity to Itu (Figures 4a and 5). Inhibition of protein kinases appears therefore to account for the Itu-induced inactivation of phosphorylase and activation of glycogen synthase in hepatocytes.

Itu as a general inhibitor of protein kinases

Like most protein kinase inhibitors [33,34], Itu competes with ATP (Figure 6, Table 1). The kinetic behaviour with respect to the protein substrate is unusual and indicates that Itu binds with higher affinity to the enzyme–substrate complex than to free enzyme [35]. In accordance with its broad inhibitory action on protein kinases *in vitro*, Itu decreased the extent of phosphorylation of many phosphopolypeptides in hepatocytes (Figure 7). The fact that the phosphorylation level of some hepatic polypeptides was not affected by Itu may indicate that some protein kinases are not inhibited by Itu in the physiological intracellular milieu. However, some of these phosphopolypeptides may represent phospho-enzyme intermediates that are formed during the catalytic action of, for example, various phosphotransferases that are insensitive to Itu (Table 2). The massive effect of Itu to promote the dephosphorylation of so many polypeptides strongly suggests that several biological effects of Itu are linked to an

inhibition of protein kinases. For example, an inhibition of myosin light chain kinase by Itu could explain previous observations that Itu causes relaxation of smooth muscles when injected into mice [10,11]. In this respect, it is also significant that an inhibition of protein phosphatase(s) by okadaic acid causes muscle contraction [36]. An additional argument comes from the observations that tubercidin (a very weak protein kinase inhibitor) did not affect the activities of glycogen synthase and phosphorylase (the present study) and did not cause muscle relaxation *in vivo* [11]. We propose that Itu can serve as a general protein kinase inhibitor in intact cells. Combined with the use of specific protein phosphatase inhibitors, Itu could thus be helpful in delineating the role of protein kinases and protein phosphatases in intracellular signalling. The large difference in Itu-sensitivity of various protein kinases (Figure 5) may be used to identify the protein kinases involved in the regulation of particular cell functions.

The role of phosphorylase *a* in the activation of glycogen synthase

Our initial hypothesis was that Itu would bind to phosphorylase, promote its dephosphorylation, and hence allow the activation of glycogen synthase by removing phosphorylase *a* which inhibits the glycogen-bound synthase phosphatase. However, Itu failed to promote the dephosphorylation of phosphorylase by various purified protein phosphatases (Table 2), and inspection of Figures 1 and 2 reveals that the activation of glycogen synthase could not be explained merely by the inactivation of phosphorylase. Similar conclusions have recently been reached by Carabaza et al. [37] in a study on the effects of glucose analogues in liver cells: all of the compounds were found to promote the inactivation of phosphorylase, but only those that could be phosphorylated on carbon-6 were able to activate glycogen synthase. This was taken as evidence that the generation of hexose 6-phosphate was essential for the activation of glycogen synthase. Combined with previous observations that the glucose-induced activation of glycogen synthase *in vivo* and in crude liver fractions requires prior inactivation of phosphorylase [2,4], these results [37] and our present observations seem to imply that the inactivation of phosphorylase is necessary but not sufficient for the activation of glycogen synthase by the glycogen-bound protein phosphatase.

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