Evidence that activation of 2-deoxy-D-glucose transport in rat thymocyte suspensions results from enhanced coupling between transport and hexokinase activity

Richard J. NAFTALIN and Robert J. RIST

Department of Physiology, King's College London, Strand, London WC2R 2LS, U.K.

1. Suspensions of rat thymocytes accumulate free 2-deoxy-D-glucose (2-dGlc) within the cytosol to a concentration approx. 25-fold above the external concentration. This active accumulation was enhanced by 40 nM-phorbol 12-myristate 13-acetate (phorbol). 2. The K_m for zero-*trans* uptake in control cells was 2.3 ± 0.14 mM and V_{max} was 0.41 ± 0.08 μ mol/min per 10^{10} cells ($n = 6$). In cells treated with phorbol (40 nM) the K_m for zero-*trans* uptake was 1.2 ± 0.13 mM and V_{max} 0.46 ± 0.03 μ mol/min per 10^{10} cells ($n = 6$). The K_m was decreased significantly by phorbol ($P < 0.01$). 3. Phorbol-dependent activation of thymocytes delayed exit of free 2-dGlc into sugar-free solution and prevented exchange exit. Activation had no effect on 3-*O*-methyl D-glucoside (3-OMG) exit. 4. Coupling of 2-dGlc transport to hexokinase activity was determined by observing the effects of various concentrations of unlabelled cytosolic 2-dGlc on influx of labelled 2-dGlc into the hexose phosphate pool. In control cells this coupling was 0.81 ± 0.02 and in phorbol-activated cells it was 0.92 ± 0.01 ($P < 0.01$). 5. The high-affinity inhibitor of hexokinase, mannoheptulose, inhibited uptake of 2-dGlc in both control and phorbol-treated cells. These data are consistent with a model for activation of sugar transport in which hexokinase activity is integrated with the sugar transporter at the endofacial surface. The results suggest that phorbol increases the degree of coupling transport with hexokinase activity, thereby leading to an increase in the rate of uptake of 2-dGlc, a decrease in exit of free 2-dGlc from the cytosol and an increase in free 2-dGlc accumulation.

INTRODUCTION

2-Deoxy-D-glucose (2-dGlc) influx into rat thymocytes is increased by a number of agents, which include phorbol 12-myristate 13-acetate (phorbol), the lectin concanavalin A, the calcium ionophore A23187, raised cyclic AMP and reducing agents, e.g. dithiothreitol (Whitesell & Regen, 1978; Nordenberg *et al.*, 1983; Klip *et al.*, 1984, 1986; Segal, 1987). However, thymocyte sugar transport is insensitive to insulin, is independent of ion gradients and can be inhibited with phloretin, $HgCl_2$ or cytochalasin B.

The mechanism of activation of 2-dGlc transport in thymocytes is still unclear. A model for control of sugar transport by insulin and corticosteroids in adipocytes and fibroblasts, which is considered to be an analogue of activation of sugar transport in thymocytes, is that transporters are transferred from the endoplasmic reticulum to the plasma membrane (Carter-Su & Okamoto, 1985; Haspel *et al.*, 1986). After activation an increased V_{max} , without change in the K_m of the transport process is predicted (Scheme 1). However, there can be a divergence between the number of transporters within the plasma membrane and the activity of the transport system (Matthaei *et al.*, 1987). It is also reported that insulin-dependent activation of rat adipocytes leads to a decrease in the K_m for uptake of the non-metabolized sugar 3-*O*-methyl D-glucoside (3-OMG) (Whitesell & Abumrad, 1985). [Sugar transport and metabolism in mammalian cells are conventionally considered as a

tandem process where sugar is transported across the membrane and enters the cytosol before phosphorylation via the hexokinase reaction (Graff *et al.*, 1978).]

Variants of the tandem model have been described; it has been suggested that there are two populations of thymocytes, active and quiescent; in active cells the V_{max} for 3-OMG transport is higher, and K_m is lower, than in quiescent cells (Whitesell & Regen, 1978). Another view is that there are cells which have two parallel sugar-transport systems of high and low affinity. The high-affinity system is increased during activation (Christopher *et al.*, 1976; D'Amore & Lo, 1986).

A result which is inconsistent with general applicability of the tandem model is that activation of rat adipocytes by insulin leads to a 50-fold accumulation of unphosphorylated 2-dGlc from an external concentration of 7 μ M (Foley & Gliemann, 1981). There is no evidence that the sugar is sequestered within the cytosol. This accumulation of free sugar has been confirmed by Wieringa *et al.* (1984). They showed that the transport is in part related to intracellular concentrations of ATP.

Thus an alternative model is required to explain free-sugar accumulation which is stimulated by insulin or other activators.

An alternative model proposed to explain accumulation of free sugar is that hexokinase transforms the permeant sugar ligand to impermeant hexose phosphate on the cytosolic surface of the transporter (Naftalin & Smith, 1987). Sugar is transported across the membrane via the transporter. At the endofacial surface the emerg-

Scheme 1. Differences between the kinetic predictions of tandem transport and sugar phosphorylation with transport and sugar phosphorylation on the cytosolic surface of the transporter

ent sugar can either be released into the cytosol by dissociation from the transporter without phosphorylation via the transport cycle, or can move back to the external surface in a 'futile cycle', or can be transformed by hexokinase action at the endofacial surface into 2-dGlc 6-phosphate and then released into the cytosol via the combined transport and phosphorylation cycle. The chemical reaction at the inner membrane surface prevents recycling of transported ligand and thereby reduces the proportion of endofacial transporter sites liganded to permeant sugar, thus reducing the number of futile cycles. This increases the rate of net sugar uptake. The model predicts that a decrease in the apparent K_m for zero-trans net uptake and an apparent increase in V_{max} for zero-trans net uptake follows upon activation of the hexokinase reaction at the endofacial surface. Additionally, endofacial surface activity of hexokinase whereby sugar from the cytosolic source is bound to the transporter sites and transformed to hexose phosphate via the phosphorylation cycle, also reduces the rate of exit of free sugar from the cytosol by recapturing the sugar residue before it escapes to the exterior (Scheme 1).

The combined effect of hexokinase in increasing uptake and decreasing exit transforms the passive membrane transporter into an active rectifier of free sugar flow. Regeneration of free sugar within the cytosol from hexose phosphate completes the active-transport process and results in free sugar accumulation. The maximal extent of free sugar accumulation is dependent on the relative rates of sugar phosphorylation on the cytosolic surface of the transporter and the rate of sugar dissociation from the transporter into the cytosol, i.e.:

$$[G_{in}]/[G_{out}] = E \cdot s / (w + 1)$$

where $[G_{in}]$ and $[G_{out}]$ are the intra- and extra-cellular concentrations of free sugar, $E = \exp(-dG_{hex}/RT)$; dG_{hex} is the free energy of the hexokinase reaction, R is the gas constant and T the absolute temperature; s is the

forward rate of the endofacial hexokinase reaction and w is the rate of dissociation of free sugar from the endofacial transporter surface into the cytosol. Hence, a high thermodynamic affinity of the hexokinase reaction, i.e. high $[ATP]$, low $[ADP]$ and low $[hexose\ phosphate]$, also a high surface activity of hexokinase and a high binding affinity of the sugar for the transporter, i.e. a low dissociation rate and the dissociation constant, $K_{s(sugar)}$, all promote accumulation of free sugar. It should be noted that this formula applies only to the ideal distribution ratio. The real distribution ratio will be modified by the relative rates of cytosolic hexokinase and phosphatase activity.

The model is an example of transport loosely coupled to a phosphorylation reaction, as the uptake of sugar across the membrane can be independent of hexokinase activity or can be incorporated in the hexokinase reaction. The ratio between the transport cycle and the phosphorylation reaction is not fixed. The relative rates of sugar flux via these independent cycles depend on the thermodynamic driving forces generating flow of free sugar and the hexokinase reaction and mainly on the ratio s/w . Active sugar influx and uphill accumulation of free sugar depend on the endofacial hexokinase reaction with sugar at an activity different from that within the cytosol; hence the endofacial sugar is in an intermediate compartment in series with both the exofacial surface and cytosol.

A good test of the degree of coupling is to determine the extent to which labelled sugar from the external solution is incorporated into the cytosolic hexose phosphate pool in preference to free sugar from the cytosolic pool. The model predicts that the higher the degree of coupling, the less will be the inhibitory effect of intracellular free sugar on incorporation of extrinsic labelled sugar into hexose phosphates.

In contrast, the tandem model requires all the free sugar should enter the cytosolic pool before it is

incorporated into hexose phosphate; hence uptake of labelled sugar into the hexose phosphate pool will be inversely related to the concentration of unlabelled cytosolic free sugar.

Thus a prediction of the model is that the higher the degree of coupling or the tighter the coupling, the less the degree of slippage of free sugar past the phosphorylation cycle, the more will sugar influx be activated, the less will be the rate of free sugar exit from the cytosol and the higher will be the free sugar accumulation within the cytosol. Thus, an agent such as phorbol ester may affect free sugar exit and accumulation in the way predicted by the loose-coupling model.

The model predicts that sugars which are not substrates of the hexokinase reaction, e.g. 3-OMG, will be relatively unaffected by activation of the coupling between transport and metabolism, except where they are in competition with residual free sugar in the cytosol. Integration of hexokinase with the transporter has also been suggested as a possible means of activation of transport (Wieringa *et al.*, 1984).

Previous objections to models in which transport is directly linked to phosphorylation in yeasts (Jaspers & Van Steveninck, 1975) renal cortex (Kleinzeller & McAvoy, 1976) and intestinal epithelia (Elsas & MacDonnell, 1972) have been that the sugar influx is apparently unrelated to the rate of phosphorylation, as free sugar enters the cytosol more rapidly than it is phosphorylated; also, when extracellular sugar concentration is reduced, the subsequent loss of cytosolic free sugar occurs independently of any alteration in intracellular hexose phosphate. This evidence of 'slippage' is hard to reconcile with transport tightly coupled to phosphorylation. However, some degree of slippage is intrinsic to any loose-coupling model (Naftalin & Smith 1987). In the present paper these predictions are tested using the rat thymocyte sugar-transport system and its activation by phorbol ester.

The possibility of a parallel system of active transport by Na^+ -ion-linked transport has been eliminated by using 2-dGlc as a substrate. As this sugar does not have a hydroxy residue at the 2-position, it cannot be used as a transported ligand by the Na^+ /glucose co-transporter (Bihler *et al.*, 1962). Furthermore, we show that 3-OMG, a sugar which is a normal substrate of the Na^+ /glucose co-transport system, is not accumulated by thymocytes; additionally phloretin is an ineffective inhibitor of Na^+ /glucose co-transport, and yet is very effective at inhibiting active 2-dGlc transport and accumulation in thymocytes.

EXPERIMENTAL

Cell preparation

The method of cell preparation was adapted from Klip *et al.* (1986). Immature Wistar rats weighing between 120 and 175 g were killed by cervical dislocation after chloroform anaesthesia. The thymuses were removed by blunt dissection and immersed in Hepes buffered saline (pH 7.3) at 20 °C. The tissue was chopped into small pieces and disaggregated in about 50 ml of Hepes/saline by repeated suction into and extrusion from the nozzle of a 10 ml plastic syringe (18–20 cycles). Any floating debris was removed. The cell suspensions were lightly centrifuged at 400 *g* for 1 min to precipitate any heavy aggregates and the supernatants decanted and re-

centrifuged for 3 min at 2000 *g* in a bench centrifuge to concentrate the cells.

Microscopy and cell counting

This method of disaggregation does not cause discernible damage to the appearance or integrity of the cells. There is very little cell debris in the preparations, and it is hard to detect any dead cells by using Evan's Blue uptake.

All sugar-uptake rates and estimates of intracellular concentrations of free sugar and hexose phosphates are normalized to 10^{10} cells. As the mean cell volume is approx. 100 fl, 10^{10} cells are equivalent to approx. 1 ml of cell water. The cell numbers were estimated using a Coulter counter model B. The concentration of cells in the suspension used for influx was approx. 5×10^6 cells/ml and for efflux $(1-2) \times 10^7$ cells/ml.

Activation with phorbol

Before measuring uptake, the cell suspensions were preincubated for a period of 30 min in solutions containing phorbol 12-myristate 13-acetate (40 nM) or in control solutions. This concentration of phorbol ester was found to give optimal activation over a prolonged incubation.

Radioactive-sugar-uptake studies

Cell suspensions were incubated in standard 5 mM-Hepes/140 mM-NaCl/5 mM-KCl/1.2 mM-MgCl₂, buffered to pH 7.3, containing various concentrations of sugars as stated. It was found that uptakes were higher in phosphate-free buffer than in buffers containing 1–10 mM phosphate. The presence or absence of Ca^{2+} had no significant effect on 2-dGlc uptake as was found previously by Segal & Ingbar (1981); removal of Ca^{2+} has no effect on phorbol-activated sugar uptake. Time courses of uptake were measured over periods varying from 2 min to 3 h. Uptake of sugars into the cells was measured by using 2-D-[1-³H]Glc (Amersham International), initial specific radioactivity 11.5 Ci/mmol at a final isotope concentration of 0.2 $\mu\text{Ci/ml}$, or ¹⁴C-labelled 3-O-[U-¹⁴C]methylglucose (Amersham International), initial specific radioactivity 74.2 mCi/mmol at a final isotope concentration of 0.05 $\mu\text{Ci/ml}$.

After incubation, sugar uptakes were halted by adding iso-osmotic ice-cold stopping solution to the cell suspensions. This contained enough phloretin and HgCl₂ to give a final concentration of 100 μM and 1 M respectively. The cell suspensions were centrifuged in a bench centrifuge for 2 min at 3000 *g*. The supernatants were removed and a sample retained for estimation of extracellular radioactivity. Then the cells were washed twice more by suspension and re-centrifugation in 4 ml of stopping solution to remove extracellular isotope.

Finally, the cell pellets were lysed in 4 ml of ice-cold distilled water. This lysate was used to determine the total uptake, free sugar and hexose phosphate concentrations within the cell pellet.

The possibility of breakdown of hexose phosphate to free sugar in the lysate was tested by determining the rate of breakdown for hexose phosphate to free sugar at 0 °C and 37 °C. It can be seen from Fig. 1 that there is no significant breakdown of hexose phosphate in the lysate from controls or phorbol-treated cells at 0 °C over a 3 h incubation period, although raising the temperature to 37 °C produced considerable dephosphorylation.

Fig. 1. Effect of temperature on the ratio of free sugar to hexose phosphate in cell lysates from control (circles) and phorbol-treated (squares) tissue at 0 °C (closed symbols) and at 37 °C (open symbols)

Each point is the mean value of two determinations.

Studies of radioactive sugar exit from preloaded cell suspensions

Suspensions containing approx. 2×10^7 cells/ml were used in the preincubation solutions. Cells were incubated with either 0.1 mM- or 1 mM-labelled 2-dGlc or 10 mM-labelled 3-OMG for 2 h in control conditions and for the next hour with or without phorbol (40 nM). At the end of the incubation period the cells were loosely packed by centrifugation at 1500 *g* for 3 min and the supernatant removed. The cell pellet was suspended in buffered saline at 20 °C and the cells were immediately re-centrifuged at room temperature for 30 s. This washed cell pellet was diluted approx. 50-fold by addition to fresh solution containing nominally zero sugar at 37 °C, (2-dGlc < 0.02 mM when the loading concentration was with 1 mM-2-dGlc).

Zero-*trans* net exit was measured by observing the time-dependent loss of radioactivity from cell sugar and hexose phosphate pools.

Exchange exit was measured by adding the cell pellet to solutions containing unlabelled 2-dGlc (5 mM). Higher concentrations of unlabelled 2-dGlc were found to decrease the rate of loss of labelled sugar from the cells.

The initial concentration of labelled sugar within the cells was measured by adding a volume of indiluted cell suspension to stopping solution. Samples of suspension were taken at intervals and added to stopping solution and centrifuged, the supernatants were removed and the pellets lysed in distilled water before analysis of the total radioisotope, free sugar and hexose phosphate in the lysates, as described below. The cell concentration in each suspension was determined separately using the Coulter counter. The same procedures were used to determine 2-dGlc and 3-OMG exits from cells, except that with 3-OMG only the total radioisotope was estimated.

Estimation of unphosphorylated and phosphorylated sugar

A Whatman DE81 2.3 cm filter was washed with 2 ml of ice-cold glass distilled water by suction via a Buchner filtration tube; then 1 ml of ice-cold cell lysate from approx. $(1-2) \times 10^6$ cells were filtered and the filtrate retained; the filter was washed with a further 2 ml of distilled water, and this secondary wash filtrate was added to the primary filtrate. The combined filtrate was sampled for free sugar and the remainder rejected. The hexose phosphate retained on the filter was then eluted by washing with 2 ml of 0.5 M-HCl, and a sample of this filtrate was counted to estimate hexose phosphates. Estimates of total, free sugar and hexose phosphates/ 10^{10} cells were obtained after correction for quench and dilution. The samples were counted in scintillation fluid with the following composition: 500 ml of toluene; 500 ml of Synperonic N (Durham Chemicals Distributors, Birtley, Chester-le-Street, Co. Durham, U.K.; 2.5 g of 2,5-diphenyloxazole (Sigma Chemical Co., Poole, Dorset, U.K.).

The efficiency of the separation process was continuously checked by determining whether the sum of the recoveries of free sugar and sugar phosphates was equal to the separately determined total sugar. This method was also checked by using a cell-free system in which D-[3 H]glucose 6-phosphate was generated from labelled D-glucose and ATP catalysed by Baker's yeast hexokinase obtained from Sigma.

T.l.c.

Lysates obtained from control and phorbol-treated cells which had been incubated for 2 h with 1 mM-2-dGlc ($2 \mu\text{Ci}$ of ^3H label/ml) then freeze-dried and resuspended in 0.1 ml of water. Portions (5 μl) of the extracts were spotted on to thin-layer silica chromatography plates (0.25 mm thickness) and eluted with a mixture of ethyl acetate/methanol/water (9:2:1, by vol.) for approx. 20 min. After drying, the sugars were located on the plates with aniline/diphenylamine spray reagent (Sigma). The silica from parallel unstained tracks was scraped from the plate, extracted in 0.5 ml of water and counted for radioactivity.

Two radioactive spots were obtained: one associated with hexose phosphate, which does not migrate, and the other with free sugar with the same R_f as 2-dGlc. The proportion of label in each spot was the same $\pm 5\%$ (four estimates) as that determined by the ion-exchange-chromatographic separation described above in both control and phorbol-treated cells. Hence we conclude that the quick method we routinely used for determination of free sugar and hexose phosphates is reliable.

Preloading using hypo-osmotic shock

It has been reported that mannoheptulose, the high-affinity competitive inhibitor of hexokinase, inhibits 2-dGlc uptake into rat adipocytes stimulated with insulin (T. Weiringa, personal communication).

The following method was used to load cells with high concentrations of 2-dGlc or mannoheptulose. Cells were exposed to hypo-osmotic media, 200 mosM/kg, containing the inhibitor mannoheptulose or labelled 2-dGlc (10 mM) at 20 °C. A large increase in labelled-sugar uptake was observed from hypo-osmotic medium containing 10 mM-2-dGlc compared with cells incubated

in iso-osmotic media (approx. 250%). After 30 min exposure, the cells were transferred to media at 37 °C. There was no significant change in either sugar uptake or loss of internal label from cells incubated at this osmoticity compared with controls pre-incubated in iso-osmotic solutions, although preincubation in lower osmoticity solution, i.e. 150 mosM·kg⁻¹ reduced subsequent 2-dGlc influx, suggesting a loss of cell ATP.

Statistics

Parameters to Michaelis–Menten equations were fitted by using Enzfitter non-linear regression with proportional weighting of each of the points. The statistical significance of the changes was determined by using Student's *t* test.

RESULTS

Uptake of 2-dGlc into thymocytes

(a) **Time course of uptake.** The time course of uptake of 0.1 mM-2-dGlc is shown in Fig. 2. After approx. 15 min incubation at 37 °C, free sugar was present within the cytosol of both control and phorbol-treated cells at higher concentrations than in the extracellular solution. The initial rate of accumulation of free sugar and hexose phosphates during the first 15 min was higher in phorbol-treated cells than in control cells. In phorbol-treated cells accumulation of both free sugar and hexose phosphate slowed after 2 h, whereas in control cells sugar accumulation was linear during the first 3 h. The ratio of

free sugar to hexose phosphate remained constant during the entire time course in both control and phorbol-treated suspensions.

Accumulation after 3 h incubation

The accumulation of total sugar, free sugar and hexose phosphates after 3 h incubation is shown in Table 1.

Phorbol increased free sugar accumulation by 153% and hexose phosphates by 208%.

The effect of varying the concentration of 2-dGlc on the combined total accumulation of free sugar and hexose phosphate is shown in Fig. 3. Also shown is the accumulation of 3-OMG incubated for the same time with the same external sugar concentration range as with 2-dGlc. The accumulations are plotted as distribution ratios between cell water and extracellular fluid, assuming 10¹⁰ cells have a cytosolic water space of 1 ml.

Raising the concentration of extracellular 2-dGlc decreased the sugar distribution ratio, but did not affect the relative proportion of intracellular free sugar and hexose phosphates. However, when extracellular [2-dGlc] was raised to 7.5 mM or above, there was no longer any significant phorbol-dependent increase in either total, free or hexose phosphate accumulation.

There was no effect of changing the extracellular concentration of 3-OMG in the range 0.1 mM–20 mM on the distribution ratio of 3-OMG, which was approximately equal to 1 at all concentrations. This is a useful corroboration of estimates of cell water space determined from cell number and volume with the Coulter Counter. There was no effect of phorbol (40 nM) on 3-OMG distribution in the concentration range 0.1–20 mM. These findings indicate that there is no Na⁺-dependent co-transport of sugar in thymocytes (Bihler *et al.*, 1962).

Fig. 2. Time course of uptake of 2-dGlc from an external concentration of 0.1 mM into 10¹⁰ cells (data from a single experiment)

The total uptake is the sum of radioactivity in the free-sugar and hexose phosphate pools. Closed symbols, total uptakes; squares, phorbol (40 nM)-treated cells; open symbols, hexose phosphate (squares, phorbol treated; circles, controls).

Effects of phorbol on zero-trans influx kinetics of 2-dGlc

Total uptake of 2-dGlc into both free sugar and hexose phosphate pools was initially determined over a period varying from 30 s to 15 min. As the uptake was linear within experimental error over the first 10 min, uptake rates were measured at either 5 or 10 min and normalized to uptake/min per 10¹⁰ cells. Uptakes were corrected for phloretin/HgCl₂-insensitive fluxes, which varied between 5% of total uptake at low concentrations to approx. 30–40% at high concentrations. There was no significant effect of phorbol on this inhibitor-insensitive uptake.

The *K_m* for zero-trans uptake in control cells was 2.3 ± 0.14 mM and *V_{max}* was 0.41 ± 0.08 μmol/min per 10¹⁰ cells (*n* = 6). In cells treated with phorbol (40 nM), the *K_m* for zero-trans uptake was 1.2 ± 0.13 mM and *V_{max}* 0.46 ± 0.03 μmol/min per 10¹⁰ cells (*n* = 6). It follows that the *K_m* was reduced significantly by phorbol (*P* < 0.01), whereas *V_{max}* was unaffected by phorbol (Fig. 4).

Effects of equilibrated concentrations of 3-OMG on 2-dGlc influx

Thymocytes were equilibrated with various concentrations of unlabelled 3-OMG in the range 0–25 mM for 60 min. After this equilibration period, uptake of various concentrations of ³H-labelled 2-dGlc (0.1–5 mM) were measured over a 10 min interval. The *K_i* i.e. the

Table 1. Uptake of 2-dGlc after 3 h incubation with an external concentration of 0.1 mM

Phorbol (40 nM) causes a significant increase in total, free sugar and hexose phosphate accumulation by rat thymocytes (** $P < 0.01$).

	Uptake ($\mu\text{mol}/10^{10}$ cells)		
	Total	Free sugar	Hexose phosphate
Control	2.3 ± 0.1 (5)	1.5 ± 0.1 (5)	0.5 ± 0.05 (5)
Phorbol (40 nM)	$5.2 \pm 0.7^{**}$ (6)	$3.8 \pm 0.5^{**}$ (6)	$1.6 \pm 0.4^{**}$ (6)

Fig. 3. Effect of varying the concentration of 2-dGlc and 3-OMG on total accumulation of sugar into 10^{10} cells after 3 h incubation

Squares, phorbol (40 nM)-treated cells (mean $\pm$ S.E.M.; $n = 6$ for 2-dGlc, $n = 5$ for 3-OMG). Circles, control, $n = 6$ for 2-dGlc; $n = 5$ for 3-OMG (n = number of separate experiments). The distribution ratio is estimated as:

$$\frac{\text{Total amount of labelled sugar (hexose phosphate + free sugar) } (\mu\text{mol}/10^{10} \text{ cells})}{\text{Radioactive sugar } (\mu\text{mol}/\text{ml of incubation fluid})}$$

estimated concentration of 3-OMG required to reduce influx of 2-dGlc from the following equation:

$$K_i = i / [(K_p / K_m) - 1]$$

where K_p is the effective K_m in the presence of inhibitor at concentration i (Dixon & Webb, 1964) was determined from the non-linear least-squares linear regression line of the data using the Enzfitter program. The K_i for inhibition of control influx was 10.45 ± 1.6 mM ($n = 5$) and for phorbol-treated cells the K_i was 7.4 ± 1.4 mM ($n = 5$).

As 3-OMG is not a substrate for hexokinase, the absence of any significant change in the K_i of 3-OMG-

Fig. 4. Phloretin-sensitive rates of uptake of 2-dGlc into 10^{10} cells in control (a) (squares, $n = 6$) or after exposure to phorbol (40 nM) (b) (open squares, $n = 6$) and also the effect of 25 mM 3-OMG on phloretin-sensitive uptake of 2-dGlc into the control (filled circles, a, $n = 5$) and phorbol (40 nM)-treated (b, $n = 5$) cells

Lines were fitted by non-linear least-squares regression using the Enzfitter program of the Michaelis-Menten equation with proportional weighting of the uptakes at each concentration. The apparent K_p values at 25 mM-3-OMG and the K_m in control and phorbol-treated tissues were used to derive the K_i of 3-OMG-dependent inhibition of 2-dGlc uptake. In control tissue the K_p was 7.81 ± 1.27 mM and $V_{\max}$ was 0.53 ± 0.045 $\mu\text{mol}/\text{min}$ per 10^{10} cells in phorbol-treated cells, K_p was 5.7 ± 0.33 mM and $V_{\max}$ was 0.57 ± 0.033 $\mu\text{mol}/\text{min}$ per 10^{10} cells.

dependent inhibition of 2-dGlc influx by phorbol indicates that phorbol does not affect the intrinsic affinity of the transporter for sugars. It may be deduced that the phorbol-dependent reduction in the K_m of 2-dGlc influx is likely to be due to a metabolic reaction of 2-dGlc which alters the apparent affinity of the transport process.

Effects of phorbol on sugar exit from preloaded cells: zero-trans exit of 3-OMG

Cells preloaded with ^{14}C -labelled 10 mM-3-OMG for 2 h were concentrated by centrifugation and resuspended in medium which was nominally free of 3-OMG (final concn. 0.1 mM).

Intracellular sugar decreased mono-exponentially ($t_{1/2} \approx 10$ min) in both control and phorbol-treated cells (see Fig. 5). There was no evidence of a two-phase exit of 3-OMG, as was reported in rat thymocytes (Whitesell &

Fig. 5. Time-dependent decrease of ¹⁴C-labelled (3-OMG) (μmol/10¹⁰ cells) from cells preloaded with 10 mM from control cells (closed circles, *n* = 4) and phorbol-treated cells (40 nM) (open circles, *n* = 4)

There was no significant effect of phorbol on exit.

Regen, 1978) and no evidence of any phorbol-dependent increase in 3-OMG exit.

Effects of phorbol on zero-trans net exit of 2-dGlc

Cells were preincubated with 0.1 mM- or 1 mM-³H-labelled 2-dGlc for 3 h in the presence or absence of 40 nM-phorbol. The loss of radioactivity from the cells was determined as described in the Experimental section.

In Fig. 6 the decrease in total amount of sugar/10¹⁰ cells is plotted as a function of time. The closed symbols represent fluxes into solutions which were nominally sugar-free. There was no significant decrease in radioactivity from phorbol-treated cells during the first 20 min of incubation, whereas there was a rapid initial rate of exit from control cells during the first 10 min, followed by a slow decline. These data indicate that phorbol-dependent activation of sugar transport showed the net rate of 2-dGlc loss from the cells, as predicted for a sugar-transport system loosely coupled to hexokinase activity (Naftalin & Smith, 1987).

Effect of phorbol on exchange exit of 2-dGlc

The intracellular free sugar concentration was in the range 5–12 mM after 3 h incubation with 1 mM-2-dGlc. Unlabelled 2-dGlc at 5 mM was added to the external solution to give near-optimal conditions for equilibrium exchange.

Exit of labelled sugar from control cells into saline containing 5 mM-2-dGlc was significantly increased during the initial 20 min period compared with net flux (*P* < 0.01); there was a small, but statistically insignificant, increase in the loss of label from phorbol-treated cells. In control cells, exit of label slowed after 20 min. Exchange exit from phorbol-treated cells was not significantly increased above net exit.

Estimation of the coupling coefficient between transport and hexokinase

(a) **Theory.** Coupling between 2-dGlc transport and phosphorylation can only occur if the sugar is phosphorylated before it equilibrates with the cytosolic pool. A quantitative test for coupling between transport and hexokinase action at the cytosolic surface of the membrane is to determine the effect of a decrease in the

Fig. 6. Time-dependent decrease of total amount ($\pm$ S.E.M.) of [³H]2-dGlc/10¹⁰ cells from cells preloaded for 3 h with 1 mM-2-dGlc

Circles, control cells; squares, phorbol (40 nM)-treated cells; closed symbols, zero-trans exit into nominally sugar-free solution. Open symbols, equilibrium exchange exit into 2-dGlc (*n* = 6 for all conditions). Phorbol decreases both net and exchange ('ex') exit of sugar from the cells (*P* < 0.001).

specific radioactivity of free sugar in the cytosolic pool on incorporation of label into the hexose phosphate pool.

If 2-dGlc is phosphorylated entirely within the cytosol, a decrease in the specific radioactivity of cytosolic free sugar will lower the rate of incorporation of label into the hexose phosphate pool, providing the concentration of cytosolic free sugar exceeds the *K_m* of the hexokinase reaction by a factor < 3–5-fold. However, if phosphorylation of labelled 2-dGlc takes place on the endofacial cytosolic surface of the cell membrane before equilibration within the cytosol and access of cytosolic sugar to the active site of the membrane-bound hexokinase is hindered, then decreasing the specific radioactivity of the cytosolic free-sugar pool will have little effect on the rate of label incorporation into the hexose phosphate pool.

Hence if:

$$\text{Uptake rate} = k \times \text{sp. radioactivity}$$

where *k* is the coefficient relating uptake of 2-dGlc into cytosolic hexose phosphates to the specific radioactivity of the cytosolic free-sugar pool, then:

$$\frac{\text{Uptake rate}_{\text{ref.}}}{\text{Uptake}_{\text{inhibited}}} = \frac{\text{Sp. radioactivity}_{\text{ref.}}}{\text{Sp. radioactivity}_{\text{inhibited}}}$$

i.e.:

$$\frac{1}{\text{Fractional inhibition of uptake}} = \frac{1}{\text{Fractional decrease in sp. radioactivity}}$$

If the control_{ref.} influx of 2-dGlc (0.1 mM) into hexose phosphate when the cells are preincubated with zero sugar is taken as the reference data for influx, and intracellular labelled 2-dGlc under the same conditions is taken as the reference intracellular concentration, then the dependence of inhibition of influx on the decrease in specific radioactivity with pre-loading with various concentrations of unlabelled 2-dGlc will be a measure of the extent to which transport is independent of the hexokinase reaction.

As the reference point of the plot of 1/(fractional inhibition of uptake) = 1 and the reference point of 1/(fractional decrease in specific radioactivity) is zero, the slope of the regression line between 1/(fractional inhibition of influx) and 1/(fractional decrease in specific radioactivity) when influx is completely uncoupled from hexokinase will be 0.9.

Hence the degree of coupling between transport and hexokinase activity can be obtained from the following relationship:

$$\text{Degree of coupling} = 1 - (\text{Regression coefficient}/0.9).$$

Observing the effect of preloading the cells with unlabelled 2-dGlc on the initial rate of incorporation of labelled 2-dGlc from external solution therefore provides

a quantitative index of coupling between transport and hexokinase activity. When this value is 1, the coupling ratio or transfer coefficient is unity, i.e. dilution of the cytosolic free-sugar specific radioactivity is without effect on incorporation of labelled sugar into hexose phosphate. When the value is zero then the coupling ratio is zero.

The specific-radioactivity decrease of the sugar is estimated as follows:

$$\text{Sp. activity decrease} =$$

$$\frac{\text{Total uptake of sugar in 10 min}}{\text{Total uptake of sugar in 10 min + free sugar accumulated within the cell during 2 h preincubation}}$$

Scattergrams of individual estimates of fractional inhibition of uptake into hexose phosphate were plotted against the appropriate fractional decrease in intracellular specific radioactivity.

(b) Protocol. Preincubation. Cells were preloaded, usually for 2 h, with labelled or unlabelled 2-dGlc concentrations in the range 0–1 mM in the presence or absence of 40 nM-phorbol. The cells incubated with label at this stage were used to estimate the free-sugar concentration of those cells incubated with identical concentrations of unlabelled sugar (Fig. 7a). In cells incubated with 1 mM-2-dGlc and phorbol (40 nM), accumulation of free sugar increased to approx. 2.5 mol/10¹⁰ cells (□, *n* = 6) for each condition. In control cells (■) the accumulation of free sugar was less.

Influx. Total uptake of ³H-labelled 2-dGlc (0.1 mM)

Fig. 7. Estimation of the coupling coefficient between 2-dGlc transport and hexokinase activity

(a) Amount of free 2-dGlc in cells [closed squares, control; open squares, phorbol (40 nM)-treated] and total uptake (circles) (mol/10¹⁰ cells, mean ± S.E.M.) (closed circles, control; open circles, phorbol-treated cells (*n* = 6) after incubation for 2 h in solutions containing 2-dGlc at the concentrations stated. Lines are the Michaelis-Menten least-squares non-linear fits to the data. (b) Effect of preloading cells with various concentrations of unlabelled 2-dGlc for 2 h in the presence or absence of phorbol, on total uptake of 0.1 mM-³H-labelled 2-dGlc into 10¹⁰ cells for 10 min (*n* = 6) (closed circles, control; open circles, phorbol-treated cells). Also plotted is the uptake of 0.1 mM-³H-labelled 2-dGlc into the hexose phosphate pool (closed squares, control; open squares, phorbol-treated cells). Lines are two-parameter least-squares non-linear fits to the data. (c) Graph of 1/(fractional decrease in the rate of 2-dGlc uptake) into 10¹⁰ cells in 10 min plotted against 1/(fractional decrease in specific radioactivity of the 2-dGlc label). Closed circles, control; open circles; phorbol-treated. Lines are the least-squares linear-regression lines to the data points.

for a 10 min period expressed as mol of labelled sugar/10 min per 10^{10} cells after preincubation with 2dGlc at the concentrations shown in Fig. 7b [○, phorbol (40 nM); ●, controls, $n = 6$]. Uptake of labelled sugar into the hexose phosphate pool is also shown in Fig. 7b [□, with phorbol (40 nM); ■, controls]. Increased influx was observed into both hexose phosphate and total sugar pools with cells incubated with phorbol. The effect of preloading with increasing concentrations of unlabelled 2-dGlc is to decrease total uptake of labelled 2-dGlc and also uptake into hexose phosphate.

The slopes of the least-squares linear fit of the reciprocal of the fractional decrease in external 2-dGlc influx into hexose phosphate versus the reciprocal of the estimated decrease in the specific radioactivity of cytosolic free sugar indicate that, in control cells, the coupling coefficient was 0.81 ± 0.02 , whereas in phorbol-treated cells the coefficient increased to 0.92 ± 0.01 (Fig. 7c). This phorbol-dependent increase in the coefficient is statistically significant ($P < 0.001$). Both lines have slopes significantly different either from zero or 1 ($P < 0.001$), indicating that the coupling process between hexokinase and sugar transport is loose in both control and phorbol-activated thymocytes.

Effects of preloading with varying concentrations of mannoheptulose

Mannoheptulose is known to be a high-affinity inhibitor of hexokinase (Coore & Randle 1964; Ashcroft & Randle, 1970). Preliminary experiments were carried out with cells loaded with various concentrations of mannoheptulose by exposure to hypo-osmotic solutions containing either mannoheptulose or sedoheptulose or sucrose. It was found that mannoheptulose had a significant effect on subsequent 2-dGlc uptake when it was present in the hypo-osmotic loading solution at 10 mM or 20 mM whereas neither sedoheptulose nor sucrose had any effect. In both control and phorbol-treated cells there was a significant mannoheptulose-dependent decrease in uptake of 2-dGlc, which suggests that the initial rate of uptake of sugar is linked to hexokinase activity. The proportional inhibition of mannoheptulose is approximately the same in controls and phorbol-treated

cells, i.e. 25 and 20 %. These results partially confirm unpublished data on 2-dGlc uptake into rat adipocytes, kindly made available by Dr. T. Wieringa, Academisch Ziekenhuis, Leiden, The Netherlands.

Mannoheptulose was without significant effect on uptake of 10 mM-3-OMG into control and phorbol-treated cells.

DISCUSSION

The following new results have been described.

(1) When thymocytes were incubated with 0.1 mM-2-dGlc for 3 h, free sugar accumulates within the cytosol of rat thymocytes approx. 25-fold above the concentration present in the external solution. Accumulation is stimulated by phorbol ester (Table 1 and Figs. 2 and 3).

(2) The accumulation arises because of the large asymmetry between influx and efflux of free sugar. Influx of sugar maximally exceeds efflux in phorbol-treated cells by approx. 200-fold. In control cells this flux ratio is approx. 50. Activation by phorbol both stimulates influx and retards exit.

(3) The K_m for 2-dGlc uptake is decreased by phorbol activation (Fig. 4); however, the K_i for 3-OMG-dependent inhibition of 2-dGlc influx is unaffected by phorbol (Fig. 4). The former effect is consistent with depletion of permeant 2-dGlc ligand from the inner surface of the transporter. The loose-coupling model predicts that increased hexokinase activity at the inner surface of the transporter will reduce the proportion of internal sites liganded to permeant 2-dGlc. This will increase influx of 2-dGlc and hence also reduce the proportion of sites on the external surface liganded to 2-dGlc. With zero-*trans* net transport, this depletion of liganded 2-dGlc reduces the apparent K_m of the transport process. The absence of any effect of phorbol on the K_i of 3-OMG-dependent inhibition of 2-dGlc uptake is consistent with the view that the only alteration of the transport system by phorbol is due to interaction of hexokinase with 2-dGlc.

(4) Increased exchange exit of 2-dGlc from control cells is observed, but this is not evident in phorbol-treated cells (Fig. 6).

Table 2. Effect of preloading with mannoheptulose (20 mM) on 2-dGlc (0.1 mM) uptake into total, free-sugar and hexose phosphate pools in 10 min

Mannoheptulose has a significant inhibitory effect on 2-dGlc uptake into the total, free-sugar and hexose phosphate pool in both control and phorbol-treated cells. Phorbol induces a significant increase in 2-dGlc uptake (statistical significance of phorbol versus control in same column ($^{pp}P < 0.01$, two tailed *t*-test, paired difference); the statistical significance of the mannoheptulose effect versus control or phorbol-treated cells is shown ($^mP < 0.05$; $^{mm}P < 0.01$); n is the number of separate experiments. Mannoheptulose inhibits the same proportion of influx into control cells as in phorbol-treated cells; for this reason the mannoheptulose-dependent decrease in influx is greater in phorbol-treated cells than in controls.

Conditions	<i>n</i>	Uptake ($\mu\text{mol}/10^{10}$ cells)		
		Total sugar	Free sugar	Hexose phosphate
Control	7	0.30 ± 0.03	0.16 ± 0.04	0.16 ± 0.02
Control + 20 mM-mannoheptulose	7	0.22 ± 0.01^{mm}	0.11 ± 0.01^{mm}	0.10 ± 0.01^{mm}
Phorbol	6	0.81 ± 0.06^{pp}	0.37 ± 0.03^{pp}	0.35 ± 0.02^{pp}
Phorbol + 20 mM-mannoheptulose	6	0.65 ± 0.04^{mm}	0.26 ± 0.01^{mm}	0.26 ± 0.02^m

(5) Mannoheptulose inhibits uptake of 2-dGlc into both control and phorbol-treated cells (Table 2).

The mannoheptulose-dependent percentage decrease in influx is the same in both control and phorbol-treated cells, i.e. there is a greater net effect of mannoheptulose in phorbol-treated cells.

The results indicate that 2-dGlc uptake and accumulation is related to hexokinase activity and that phorbol affects some function of the transport system related to hexokinase.

The following data are inconsistent with either a change in the number or in the intrinsic activity of the sugar transporters within the thymocyte membrane. (i) No significant phorbol-dependent increase is observed in the $V_{\max}$ for 2-dGlc influx (Fig. 4); (ii) there is evidence that 3-OMG and 2-dGlc share the same transporter because (a) influx and exit of both sugars are inhibited by phloretin and HgCl_2 , and (c) equilibrium distributions of 3-OMG inhibit 2-dGlc uptake competitively (Fig. 4).

Although 3-OMG and 2-dGlc share the same transporter, phorbol does not affect the 3-OMG exit (Fig. 5), whereas it lowers the rate of 2-dGlc exit. This is evidence that phorbol-dependent activation of sugar transport in rat thymocytes is related to phosphorylation of the transported sugar rather than to an effect on either the intrinsic activity of the transporter or the number of transporters. Other evidence in support of this view is as follows: (1) the absence of any effect of phorbol on the apparent affinity of 3-OMG for the transporter as the K_t of 3-OMG-dependent inhibition of 2-dGlc influx is unaffected by phorbol (Fig. 4); (2) the phorbol-dependent increase in the coupling coefficient between 2-dGlc influx and its incorporation into cytosolic hexose phosphatase (Fig. 7c); (3) the inhibition of 2-dGlc influx by mannoheptulose in both control and phorbol-treated tissue without any effect of mannoheptulose on 3-OMG influx.

A possible explanation for the higher coupling coefficient between transport and hexokinase activity in phorbol-treated cells is that there is a stimulation of hexokinase adhesion to the endofacial surface of the transporter. This mechanism could be useful in explaining the activation of sugar transport in other systems, in particular the insulin-dependent activation of 2-dGlc transport in adipocytes.

We are grateful to the Medical Research Council for a project grant which supported this work.

REFERENCES

- Ashcroft, S. J. H. & Randle, P. J. (1970) *Biochem. J.* **119**, 5–15
- Bihler, I., Hawkins, K. A. & Crane, R. K. (1962) *Biochim. Biophys. Acta* **59**, 94–102
- Carter-Su, C. & Okamoto, K. (1985) *J. Biol. Chem.* **260**, 11091–11098
- Christopher, C. W., Kohlbacher, M. S. & Amos, H. (1976) *Biochem. J.* **158**, 439–450
- Coore, H. G. & Randle, P. J. (1964) *Biochem. J.* **91**, 56–59
- D'Amore, T. & Lo, T. C. Y. (1986) *J. Cell. Physiol.* **127**, 95–105
- Dixon, M. & Webb, E. C. (1964) *Enzymes* 2nd Edn., Longmans, Green & Co., London
- Elsas, L. J. & MacDonnell, R. C. (1972) *Biochim. Biophys. Acta* **255**, 948–959
- Foley, J. J. & Gliemann, J. (1981) *Biochim. Biophys. Acta* **648**, 100–106
- Graff, J. C., Wohlheuter, R. M. & Plagemann, P. G. (1978) *J. Cell Physiol.* **96**, 171–178
- Haspel, H. C., Wilk, E. W., Birnbaum, M. J., Cushman, S. W. & Rosen, O. M. (1986) *J. Biol. Chem.* **261**, 6778–6789
- Jaspers, H. T. A. & Van Stevininck, J. (1975) *Biochim. Biophys. Acta* **406**, 370–385
- Kleinzeller, A. & McAvoy, E. M. (1976) *Biochim. Biophys. Acta* **45**, 126–143
- Klip, A., Rothstein, A. & Mack, E. (1984) *Biochem. Biophys. Res. Commun.* **124**, 14–22
- Klip, A., Mack, E., Cragoe, E. J. & Grinstein, S. (1986) *J. Cell Physiol.* **127**, 244–252
- Matthaei, S., Olefsky, J. M. & Karnielli, E. (1987) *Biochem. J.* **251**, 491–497
- Naftalin, R. J. & Smith, P. M. (1987) *Biochim. Biophys. Acta* **897**, 93–111
- Nordenberg, J., Stenzel, K. H. & Novogrodsky, A. (1983) *J. Cell Physiol.* **117**, 183–188
- Segal, J. (1987) *Biochem. J.* **246**, 561–566
- Segal, J. & Ingbar, S. H. (1981) *J. Clin. Invest.* **68**, 103–110
- Whitesell, R. T. & Abumrad, N. A. (1984) *J. Biol. Chem.* **260**, 2894–2899
- Whitesell, R. R. & Regen, D. M. (1978) *J. Biol. Chem.* **253**, 7289–7294
- Wieringa, T., Van Dam, K., Bos, M. P., Van Putte, J. P. M. & Krans, H. M. J. (1984) *Biochim. Biophys. Acta* **803**, 123–128