ADP modifies the function of the glucose transporter: studies with reconstituted liposomes

Mitsuhiro SOFUE, Yoshiyuki YOSHIMURA, Mikio NISHIDA and Jun KAWADA*
Faculty of Pharmaceutical Sciences, University of Tokushima, Tokushima 770, Japan

Modification of function of the glucose transporter by nucleotides was studied by using liposomes reconstituted with the human erythrocyte glucose transporter. ADP enclosed in the liposomes inhibited the uptake of D-glucose and nicotinamide in a dose-dependent manner, but other enclosed nucleotides (ATP, AMP, CDP, GDP, UDP) showed no effect on the uptake of both. Only intraliposomal ADP was effective, and extra-liposomal ADP was not, under our experimental conditions. Intraliposomal ADP did not change K_m, but decreased $V_{max.}$ to approximately one-third of control for uptake of both D-glucose and nicotinamide. However, the binding and the affinity of cytochalasin B to the reconstituted liposomes were not affected by intraliposomal ADP. The uptake of uridine was not changed in the presence of ADP, indicating that the nucleoside transporter co-existing in the liposomal membranes is not regulated by ADP. Human erythrocytes whose intracellular ATP was decreased by Ca^{2+} ionophore A23187 also showed decreased uptake of 2-deoxy-D-glucose and nicotinamide. This phenomenon was very similar to that found in the liposomes. These findings suggest the possibility that the function of the glucose transporter is directly and negatively modified by an increased concentration of intracellular ADP.

INTRODUCTION

Glucose transporter proteins are widely distributed in the cell membranes of most tissues, and their basic function has been thought to be the translocation of D-glucose from outside to inside of cells, although they are classified into several types, depending on differences in their modes of expression in tissues (Bell et al., 1990). However, it is still uncertain whether there are any direct intra- or extra-cellular factor(s) regulating the function of this transporter. In human erythrocytes, the uptake of 3-*O*-methyl-D-glucose was decreased when the concentration of intracellular ATP was decreased by Ca^{2+} ionophore A23187 (May, 1988). This phenomenon has been confirmed in detail in our laboratory, as described below. The uptake of 2-deoxy-D-glucose in cultured rat pancreatic β-cells was inhibited by decreased intracellular ATP concentration caused by streptozotocin and also by oligomycin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), known as a potent uncoupler (Sofue et al., 1991). In a more recent paper, we demonstrated the possible uptake of nicotinamide through the glucose transporter, using liposomes reconstituted with the human erythrocyte glucose transporter (Sofue et al., 1992). Inhibition of nicotinamide transport in pancreatic β-cells treated with streptozotocin, oligomycin and CCCP was also observed (Sofue et al., 1991). These findings seemed to suggest that the concentration of intracellular ATP is an important regulatory factor of the glucose transporter for uptake of D-glucose and nicotinamide. However, contrary to expectation, ATP had no influence on the function of the glucose transporter, as described in the present paper. Therefore, we examined the effect of ADP, because the concentration of intracellular ADP is relatively increased under conditions where ATP generation is inhibited or ATP is forced to be consumed. The present paper reports these studies using liposomes reconstituted with human erythrocyte glucose transporter and human erythrocytes treated with Ca^{2+} ionophore A23187.

EXPERIMENTAL

Materials

[2,8-^{3}H]ADP (0.98 TBq/mmol), D-[U-^{14}C]glucose (10.6 GBq/mmol) and [4-^{3}H]cytochalasin B (555.0 GBq/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). [2,8-^{3}H]ATP (740 GBq/mmol) and L-[1-^{14}C]glucose (2.035 GBq/mmol) were from American Radiolabelled Chemicals (St. Louis, MO, U.S.A.). [2-^{3}H]AMP (444.0 GBq/mmol), [*carbonyl*-^{14}C]nicotinamide (2.07 GBq/mmol) and [2-^{14}C]uridine (2.04 GBq/mmol) were from Amersham International (Amersham, Bucks., U.K.). 2-Deoxy-D-[1,2-^{3}H]glucose (1.48 TBq/mmol) was from ICN Radiochemicals (Irvine, CA, U.S.A.). Soybean phospholipids (Type II-S), ATP, ADP, AMP, CDP, GDP and UDP were from Sigma (St. Louis, MO, U.S.A.). Adenosine and uridine were from Nacalai Tesque (Kyoto, Japan). All other reagents used were of analytical grade. Fresh human erythrocytes were obtained from the Red Cross Blood Center (Tokushima, Japan).

Preparation of glucose transporter from human erythrocytes

Preparation of erythrocyte ghosts (Steck and Kant, 1974) and alkali-treated vesicles (Baldwin et al., 1980) was as reported. The glucose transporter was solubilized with Triton X-100 and purified by DEAE-cellulose column chromatography (Kasahara and Hinkle, 1977).

Preparation of reconstituted liposomes enclosing nucleotides

Three different concentrations (2.5, 5 and 10 μM) each of radiolabelled ATP, ADP and AMP (each 37 KBq) were added when soybean phospholipid dispersions were prepared by sonication, and then reconstituted liposomes were made by the freeze–thaw method (Kasahara and Hinkle, 1977; Sase et al., 1982). Reconstituted liposomes were separated from unenclosed

Abbreviation used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.
* To whom correspondence should be addressed.

nucleotides and unreconstituted transporter proteins by centrifugation in 0.4 M sucrose (Wheeler and Hinkle, 1981). The cloudy layer obtained by centrifugation was collected, and sucrose was removed on a Sephadex G-50 column. Samples (185 μl) of the cloudy eluate were used for uptake and binding experiments. The amounts of nucleotides enclosed were calculated from the specific radioactivity of tracers used. For preparing reconstituted liposomes enclosing CDP, GDP and UDP, 10 μM each of these unlabelled nucleotides was used, and other procedures were the same as described above. Protein was determined by the method of Lowry et al. (1951) with BSA as a standard.

Uptake experiments with liposomes

The reconstituted liposomes were incubated with D-[^{14}C]glucose (14.8 KBq, final concn. 0.2 mM), [^{14}C]nicotinamide (7.4 KBq, final concn. 0.2 mM), and [^{14}C]uridine (7.4 KBq, final concn. 0.2 mM) at 25 °C for 30 s. Then the uptake reaction was stopped by adding 5 ml of cold stopping solution (Sofue et al., 1992). The mixture was filtered on a 0.2 μm-pore membrane filter (Advantec) and washed with 2 × 5 ml of the same solution. The washed filters were solubilized in Bray's (1960) solution and radioactivity was determined in a liquid-scintillation counter (Aloka). The amounts of transported substrates were calculated from the specific radioactivity of each tracer.

For kinetic studies, the ADP-enclosed liposomes prepared with 10 μM ADP were incubated with D-[^{14}C]glucose and [^{14}C]nicotinamide in the presence of 0–32 mM unlabelled D-glucose or unlabelled nicotinamide, respectively, at 25 °C for 30 s. The values of K_m and $V_{max.}$ were determined from an Eadie–Hofstee plot.

Cytochalasin B binding experiments

The reconstituted liposomes containing various concentrations of ADP described above were incubated with three different concentrations of [^{3}H]cytochalasin B (20 KBq; final concns. 4 μM, 0.4 μM and 40 nM) and cytochalasin E (final concentrations were the same as those of cytochalasin B) for 5 min at 25 °C. The reaction was stopped by adding 5 ml of cold stopping solution. The mixture was filtered on the membrane filter and washed with 3 × 5 ml of the same solution. This number of washings did not cause dissociation of the bound cytochalasin B, as shown previously (Sofue et al., 1992). The washed mixture was treated in the same way as described above for radioactivity counting.

Uptake experiments with human erythrocytes

Samples (100 μl) of fresh human erythrocytes (6 × 10^8 cells) were washed with 2 × 1 ml of balanced salt solution (May, 1988). The resulting pellet was suspended in 1 ml of 100 mM phosphate buffer (pH 7.4) and incubated with various concentrations of A23187 for 1 h at 37 °C. After incubation, 10 μl of the suspension was transferred to a microtube and the cells were washed twice with the same buffer. The resulting pellets were used for uptake experiments. 2-Deoxy-D-[^{3}H]glucose (37 KBq/sample) and [^{14}C]-nicotinamide (7.4 KBq/sample) were incubated for 30 min and for 2 h, respectively, at 37 °C. The cell pellets were then washed and decolorized by treatment with 500 μl of H_2O_2 and 500 μl of propan-2-ol containing 2 M KOH for 12 h at 37 °C. The solution was neutralized by addition of 1 ml of 10 % (v/v) acetic acid, and counted for radioactivity in 10 ml of Aquasol 2 (New England Nuclear). Intracellular ATP concentrations of erythrocytes were determined with luciferin–luciferase (Sigma) (Strehler and Totter, 1952) in a Lumac/3M Bio-counter.

RESULTS

Reconstituted liposomes enclosing ADP

The volumes of reconstituted liposomes enclosing various concentrations of ADP were determined by measuring 24 h uptake of L-[^{14}C]glucose (Sase et al., 1982). The results are summarized in Table 1. The volumes of liposomes and also the amounts of transporter proteins incorporated into liposomal membranes were not affected by ADP. These results suggest that the reconstitution process does not change the function of liposomes in our experimental conditions, unlike the results reported by Wheeler (1989) for ATP. Therefore, the results described below are thought to be due to the activity of reconstituted glucose transporters.

Uptake of D-glucose and nicotinamide

Intraliposomal adenosine 5′-phosphates were increased almost linearly depending on the concentration of each phosphate added when the liposomes were prepared. However, the uptake

Table 1 Effect of enclosing ADP on volumes of reconstituted liposomes and the amounts of incorporated glucose transporter proteins

Values are means ± S.E.M. for three samples.

ADP added for preparation of liposomes (μmol)	Volume (μl/mg of protein)*	Protein (μg)
0	438 ± 14	5.18
2.5	450 ± 11	5.31
5	444 ± 16	5.24
10	419 ± 3	5.29

Table 2 Effects of adenosine 5′-phosphates on the uptake of D-glucose and nicotinamide in reconstituted liposomes

Values are means ± S.E.M.: *$P < 0.01$ versus control (Student's t test).

Adenosine phosphate added for preparation of liposomes (μmol)		Adenosine phosphate enclosed into liposomes (mM) ($n = 6$)	Uptake (nmol/min per mg of protein)	
			D-Glucose ($n = 3$)	Nicotinamide ($n = 3$)
ATP	0	0	18.9 ± 1.2	21.8 ± 0.8
	2.5	1.5 ± 0.19	17.8 ± 0.8	20.7 ± 0.9
	5	3.5 ± 0.28	18.6 ± 0.7	26.4 ± 0.4
	10	6.5 ± 0.30	18.4 ± 0.2	21.7 ± 1.5
AMP	0	0	21.8 ± 1.2	26.2 ± 1.0
	2.5	3.2 ± 0.18	24.0 ± 0.8	26.9 ± 2.0
	5	6.0 ± 0.20	18.1 ± 0.8	26.5 ± 0.3
	10	10.3 ± 0.3	19.4 ± 1.1	26.3 ± 1.5
ADP	0	0	26.8 ± 0.8	32.8 ± 1.3
	2.5	2.1 ± 0.12	14.7 ± 0.4*	23.1 ± 0.6*
	5	4.8 ± 0.32	13.2 ± 0.8*	17.7 ± 1.5*
	10	8.6 ± 0.67	8.4 ± 1.3*	12.0 ± 1.8*

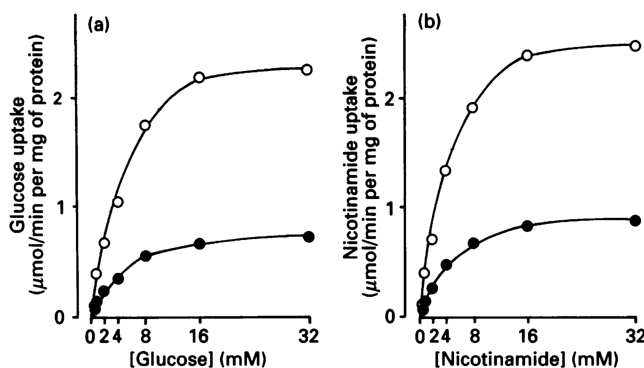


Figure 1 Concentration-dependence of uptake of D-glucose and nicotinamide by reconstituted liposomes

(**a**) Reconstituted liposomes were incubated with D-[^{14}C]glucose in the presence of various concentrations of unlabelled D-glucose without (○) or with (●) enclosed ADP. (**b**) Reconstituted liposomes were incubated with [^{14}C]nicotinamide in the presence of various concentrations of unlabelled nicotinamide without (○) or with (●) enclosed ADP. ADP concentration was 10 μM when the liposomes were prepared for both (**a**) and (**b**).

Table 3 Kinetic constants of D-glucose and nicotinamide uptake in the presence or absence of ADP

Values are means ± S.E.M. for three samples.

	D-Glucose uptake		Nicotinamide uptake	
	−ADP	+ADP	−ADP	+ADP
K_m (mM)	6.9 ± 0.37	7.2 ± 0.45	6.6 ± 0.36	5.9 ± 0.33
$V_{max.}$ (μmol/min per mg of protein)	3.4 ± 0.27	1.2 ± 0.13	3.6 ± 0.19	1.3 ± 0.06

Table 4 Effect of ADP on the uptake of uridine in reconstituted liposomes

Values are means ± S.E.M. for three samples.

ADP concn. used for preparation of liposomes (μM)	ADP enclosed into liposomes (mM)	Uridine uptake (nmol/min per mg of protein)
0	0	39.0 ± 1.2
2.5	2.5 ± 0.08	42.2 ± 1.8
5	5.5 ± 0.06	45.7 ± 1.7
10	7.2 ± 0.08	49.0 ± 1.0

of D-glucose and nicotinamide was not affected by either ATP or AMP, regardless of their intraliposomal concentrations. These findings indicate that both ATP and AMP molecules do not modify the function of the glucose transporter. On the contrary, the uptake of D-glucose and nicotinamide was decreased, depending on the increased concentration of enclosed ADP. At the highest concentration of ADP, their uptake was inhibited to about 30% of those of controls (Table 2). Such inhibition was not observed in reconstituted liposomes containing no ADP

Table 5 Effect of ADP on cytochalasin B binding to reconstituted liposomes

Units of binding: nmol/5 min per mg of protein for 4 μM; pmol/5 min per mg of protein for 0.4 μM and 40 nM. Values are means ± S.E.M. for three samples.

[Cytochalasin B]	ADP concn. (μM)	Binding
4 μM	0	4.0 ± 0.50
	2.5	4.2 ± 0.23
	5	3.9 ± 0.57
	10	4.1 ± 0.28
0.4 μM	0	405 ± 4
	2.5	413 ± 24
	5	383 ± 28
	10	401 ± 21
40 nM	0	45.6 ± 4.0
	2.5	44.7 ± 2.7
	5	41.1 ± 2.7
	10	40.5 ± 0.4

when ADP was present in the medium during uptake experiments, showing that extra-liposomal ADP was ineffective (results not shown). Furthermore, other nucleotides (CDP, GDP and UDP) enclosed in liposomes did not change the rates of uptake of D-glucose and nicotinamide (results not shown).

Kinetic studies were carried out for inhibition of the uptake of D-glucose and nicotinamide (Figures 1a and 1b). An Eadie–Hofstee plot (v versus v/s) of each Figure gave kinetic data as summarized in Table 3. In the presence of intraliposomal ADP the K_m values were not changed, but $V_{max.}$ values were decreased to approximately one-third of controls for either D-glucose or nicotinamide uptake. These results suggest the possibility that only ADP among nucleoside diphosphates interacts with the glucose transporter from the inside of liposomes and negatively modifies its transport activity.

Uptake of uridine

It is reported that the nucleoside transporter protein is co-purified with that of the glucose transporter and is detected together on band 4.5 in SDS/PAGE (Jarvis and Young, 1981; Wu et al., 1983; Young and Jarvis, 1983). Therefore reconstituted liposomes used in our experiments contain nucleoside transporters, as shown in our previous paper (Sofue et al., 1992). Then we examined whether or not the function of the nucleoside transporter is modified by ADP like the glucose transporter. The same experiments were carried out for uridine uptake as for uptake of D-glucose and nicotinamide. However, enclosed ADP did not affect the uptake of uridine in reconstituted liposomes, showing the distinct difference in these two transporters for ADP (Table 4).

Cytochalasin B binding

The values of $V_{max.}$ for the uptake of both D-glucose and nicotinamide were decreased to one-third of controls by the presence of intraliposomal ADP. Therefore we examined whether the binding of cytochalasin B to reconstituted liposomes is affected by ADP. The binding of cytochalasin B was not changed in the range of ADP concentrations where the uptake of D-glucose and nicotinamide was significantly decreased (Table 5). Furthermore, intraliposomal ADP did not modify the affinity of cytochalasin B to the glucose transporter, even when the con-

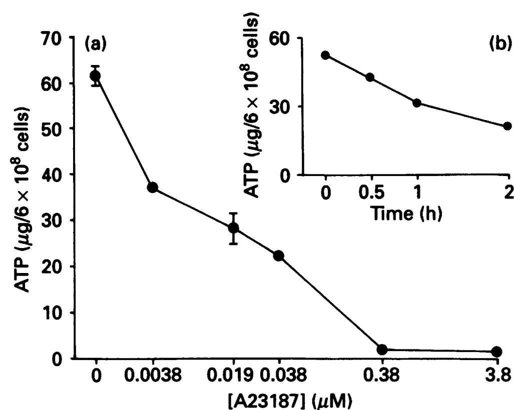


Figure 2 Change in intracellular ATP concentration of human erythrocytes treated with Ca^{2+} ionophore A23187

(**a**) Dose-dependence curve. Values are means ± S.E.M. for three samples. The bar is omitted for values with an S.E.M. of less than 5% of the mean. (**b**) Change in ATP concentration with time of treatment with 0.038 μM A23187. Values are means for six samples, but all S.E.M. values are less than 5% of the mean.

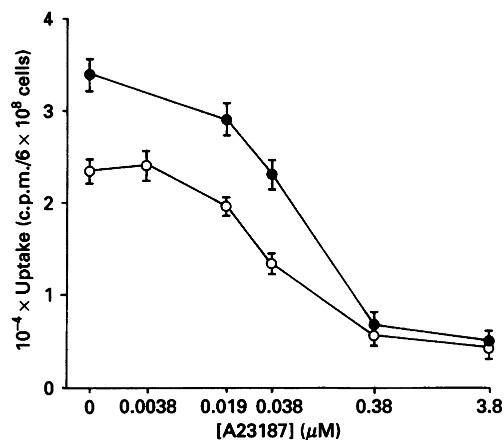


Figure 3 Uptake of 2-deoxy-D-[^{3}H]glucose (○) and [^{14}C]nicotinamide (●) by human erythrocytes treated with various concentrations of Ca^{2+} ionophore A23187

Values are means ± S.E.M. for eight samples.

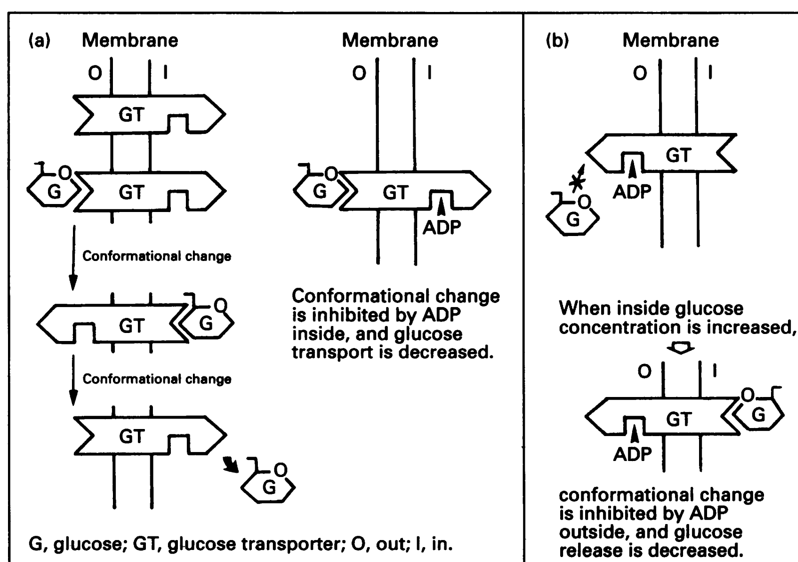
centration of cytochalasin B was changed widely. These results suggest that the site of molecular interaction between ADP and the glucose transporter is not in the close vicinity of its cytochalasin B binding site.

Uptake of 2-deoxy-D-glucose and nicotinamide by A23187-treated human erythrocytes

The intracellular ATP concentration of human erythrocytes was decreased by Ca^{2+} ionophore A23187 in a dose-dependent manner (Figure 2a) and also time-dependently (Figure 2b). Thus the effect of A23187 on ATP concentration was quantitative under our experimental conditions. In such erythrocytes, the uptake of 2-deoxy-D-[^{3}H]glucose and [^{14}C]nicotinamide was decreased by increasing concentrations of A23187 (Figure 3). This phenomenon was very similar to that found in the reconstituted liposomes enclosing ADP described above.

DISCUSSION

There are some reports showing regulation of the glucose transporter by nucleotides. Direct activation of the glucose transporter by physiological concentrations of ATP was demonstrated in human erythrocytes (Jaquez, 1983; Hebert and Carruthers, 1986; Carruthers, 1986a,b). On the contrary, we demonstrated that ADP, not ATP, directly inhibited the function of glucose transporter in our model system using reconstituted liposomes.



Scheme 1 Possible effect of internal and external ADP in reconstituted liposomes

Our results seem to give a reasonable explanation of the previously reported findings that hexose uptake was inhibited in human erythrocytes treated with Ca^{2+} ionophore A23187 (May, 1988), and also our findings with the same material, reported here, and that the uptake of D-glucose and nicotinamide was decreased in pancreatic β-cells exposed to streptozotocin, oligomycin and CCCP (Sofue et al., 1991), because in all of these cases the concentration of intracellular ATP was decreased by each chemical, and thus ADP concentration was thought to be relatively increased.

Another fact that we presented here, that only intraliposomal ADP, but not extra-liposomal ADP, was effective suggests that the molecular interaction of ADP and the glucose transporter protein to cause regulation is localized on the hydrophilic region of the transporter peptide facing the inner side of the liposomal or probably cellular membranes. However, the site of cytochalasin B binding in the peptide molecule is excluded, as demonstrated in our binding experiments. Considering the facts that ADP does not change K_m, but decreases $V_{max.}$ and does not affect the binding of cytochalasin B, the inhibitory action of ADP on D-glucose and nicotinamide uptake might be attributable to its hindering effect on the conformational change of the glucose transporter.

Further discussion is needed of the ineffectiveness of extra-liposomal ADP. In the membranes of reconstituted liposomes, the orientation of the glucose transporters is probably 50% inward/50% outward (Wheeler, 1989). However, the outside concentration of glucose is much higher than the inside, and macroscopically no reverse transport (i.e. from inside to outside) of glucose is observed in an early phase of the transport process. In such conditions, the regulation by internal ADP is to be expected if the ADP-binding site is assumed to be located as shown in Scheme 1(a). When the transporter is reversely oriented, external glucose is not transported, because no glucose binding site is exposed to the outside. In such conditions, external ADP has no effect (Scheme 1b). However, in the actual reconstituted liposomes incorporating transporters oriented in a 50% inward/50% outward ratio, external ADP will become effective when the glucose concentration is approaching equilibrium. However, the apparent uptake of glucose or nicotinamide seems to be unchanged, because the release caused by reversely oriented transporters is inhibited.

Our reconstituted liposomes contain nucleoside transporters (Sofue et al., 1992). However, uridine uptake was not affected by ADP, as shown in our present experiments. Young and Jarvis (1983) reported that glucose and nucleoside transporters are functionally different from each other, and we also reached the same conclusion, from the results obtained by using reconstituted liposomes, that cytochalasin B did not affect the uptake of adenosine, but inhibited D-glucose uptake, and nitrobenzylthioinosine decreased the uptake of adenosine, but not of D-glucose (Sofue et al., 1992). The present results provide further evidence that these two transporters are different also in respect of their regulation mechanisms.

Beutler (1990) reported that the concentrations of ATP and ADP in normal human erythrocytes were around 1.5 mM and 0.2 mM respectively. This value of ADP concentration is one order of magnitude lower than that of intraliposomal ADP in our experiments. Therefore the exact physiological significance of our findings is uncertain at present. However, a possible regulatory role of low concentrations of ADP on the glucose transporter would be expected in native cells, considering that the function of the glucose transporter in native cells is probably more efficient than that in artificial reconstituted liposomes.

Irrespective of the physiological significance, the present findings, showing the inhibition by ADP of the uptakes of nicotinamide and D-glucose, are also further evidence supporting a possible multi-function of the glucose transporter proposed by us (Sofue et al., 1992).

REFERENCES

Baldwin, J. M., Lienhard, G. E. and Baldwin, S. A. (1980) Biochim. Biophys. Acta. **599**, 699–714

Bell, G. I., Kayano, T., Buse, J. B., Burant, C. F., Takeda, J., Lin, D., Fukumoto, H. and Seino, S. (1990) Diabetes Care **13**, 198–207

Beutler, E. (1990) in Hematology, 4th Edn. (Williams, W. J., Beutler, E., Erslev, A. J. and Lichtman, M. A., eds.), pp. 317–332, McGraw-Hill Publishing Co., New York

Bray, G. A. (1960) Anal. Biochem. **1**, 279–285

Carruthers, A. (1986a) Biochemistry **25**, 3592–3602

Carruthers, A. (1986b) J. Biol. Chem. **261**, 11028–11037

Hebert, D. N. and Carruthers, A. (1986) J. Biol. Chem. **261**, 10093–10099

Jaquez, J. A. (1983) Biochim. Biophys. Acta **727**, 367–378

Jarvis, S. M. and Young, J. D. (1981) Biochem. J. **194**, 331–339

Kasahara, M. and Hinkle, P. C. (1977) J. Biol. Chem. **252**, 7384–7390

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275

May, J. M. (1988) FEBS Lett. **241**, 188–190

Sase, S., Anraku, Y., Nagano, M., Osumi, M. and Kasahara, M. (1982) J. Biol. Chem. **257**, 11100–11105

Sofue, M., Yoshimura, Y., Nishida, M. and Kawada, J. (1991) J. Endocrinol. **131**, 135–138

Sofue, M., Yoshimura, Y., Nishida, M. and Kawada, J. (1992) Biochem. J. **288**, 669–674

Steck, T. L. and Kant, J. A. (1974) Methods Enzymol. **31**, 172–180

Strehler, B. L. and Totter, J. R. (1952) Arch. Biochem. Biophys. **40**, 28–41

Wheeler, T. J. (1989) Biochemistry **28**, 3413–3420

Wheeler, T. J. and Hinkle, P. C. (1981) J. Biol. Chem. **256**, 8907–8914

Wu, J.-S., Jarvis, S. M. and Young, J. D. (1983) Biochem. J. **214**, 995–997

Young, J. D. and Jarvis, S. M. (1983) Biosci. Rep. **3**, 309–322

Received 24 August 1992/4 January 1993; accepted 22 January 1993