Volume 281, number 1,2, 30–32 FEBS 09542 April 1991

ADONIS 001457939100252D

Sodium dependent inositol transport in HL60 cells is not related to Na^+/K^+ ATPase activity

M.A. Baxter[1], G. Grafton[1], C.M. Bunce[2], M.C. Sheppard[1] and G. Brown[2]

Departments of [1]Medicine and [2]Immunology, University of Birmingham, Birmingham, B15 2TT, UK

Received 30 January 1991

In HL60 cells, inositol transport is sodium-dependent but functionally independent of Na^+/K^+ ATPase activity. This observation has implications for the currently proposed theory for the development of diabetic complications.

Inositol transport; Na^+/K^+ ATPase activity; Diabetic complication

1. INTRODUCTION

Inositol has been shown to enter many mammalian cell types via a specific, saturable, active and sodium-dependent transport mechanism [1–3]. The rate of inositol transport appears to regulate intracellular inositol concentrations [4,5]. The mechanisms regulating the transporter's activity are unknown but some reports have suggested that inositol transport is intimately linked to cell membrane $Na^+/^+$ ATPase activity. Indeed it has been proposed that the interaction of Na^+/K^+ ATPase and inositol transport may underly the pathological decline in the intracellular inositol concentrations which is thought to be important in the development of diabetic complications [6–8]. Intracellular inositol is required for the structural integrity and enzyme activity of the Na^+/K^+ ATPase complex [9]. Intracellular inositol depletion may also cause inhibition of Na^+/K^+ ATPase activity via a protein kinase C-mediated protein phosphorylation [10,11].

It is well established that inositol transport is sodium-dependent and it has been proposed that the sodium gradient created by the Na^+/K^+ ATPase enzyme may actually drive the inositol transporter [6–8]. The studies suggesting a link between inositol transport and Na^+/K^+ ATPase activity have relied on the observation that ouabain, a powerful inhibitor of Na^+/K^+ ATPase, can also inhibit inositol transport [6,10,12]. These studies have not however determined the relative sensitivities of these processes to ouabain and do not therefore provide conclusive evidence of a mechanistic link. We have addressed this issue by studying the dose response of Na^+/K^+ ATPase activity and inositol transport to ouabain inhibition in the human promyeloid cell line HL60.

Correspondence address: M.A. Baxter, Dept. of Medicine, University of Birmingham, B15 2TT, UK

2. MATERIALS AND METHODS

Cell culture and assay of inositol uptake was as described previously [5]. In brief:

2.1. *Cell culture*

HL60 cells were maintained in long term exponential growth in RPMI 1640 medium (Northumbria Biologicals Ltd) supplemented with 10% fetal calf serum, L-glutamate (Gibco) and antibiotics (100 U/ml penicillin and 50 μg/ml streptomycin, Gibco). Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

2.2. *Inositol transport assay*

HL60 cells were harvested from the culture medium (10 min, 1000 rpm, MSE3000) and washed (3 times) in either phosphate buffered saline (PBS) or 20 mM HEPES pH 7.4 containing 5 mM potassium chloride, 2.0 mM magnesium chloride, 2 mM calcium chloride and either sodium or caesium chloride (140 mM).

Cells were resuspended in buffer at a concentration of 1–2 × 10^7 cells/ml and a 500 μl aliquot was mixed with 1 μCi [^{14}C]inulin (9.24 mCi/mmol, Amersham International, UK), glucose (0–20 mM) and ouabain (0–200 μM). In a separate tube, 500 μl of buffer containing inositol (15 μM), ouabain (0–200 μM), glucose (0–20 mM) and [^{3}H]inositol (Amersham International) at a specific activity of 0.2 μCi/nmol inositol was simultaneously incubated for 10 min at 37°C. Inositol uptake was initiated by the addition of 500 μl of the cell mixture to this tube. 200–600 μl aliquots were taken at time 0 and after 10 min incubation. The reaction was terminated by dilution in cold buffer (400 μl) and the immediate recovery of the cells by centrifugation (30 s, microfuge, Beckman, High Wycome, UK) through dibutylpthalate oil (Sigma, Poole, UK). The tubes were snap-frozen in liquid nitrogen and the tip containing the cell pellet removed and placed in scintillation vials containing 400 μl of distilled water and 50 μl of Optisolve (FSA laboratory supplies, Loughbrough, UK). After the cell pellet was dispersed 4 ml of scintillation fluid was added and [^{14}C] and [^{3}H] radioactivity measured using a dual label programme (LKB Mk3, LKB-Pharmacia, Milton Keyenes, UK).

2.3. *Na^+/K^+ ATPase activity assay*

The assay was in essence identical to that described for inositol transport except that [^{14}C]inulin was excluded and ^{86}Rb (0.2 μCi per assay) was substituted for both inositol and [^{3}H]inositol in the assay mixture. ^{86}Rb-radioactivity was measured using standard liquid scintillation counting.

 Published by Elsevier Science Publishers B.V.

3. RESULTS

A description and validation of the inositol-transport assay in HL60 cells has been described previously [5].

A plot of the rate of inositol uptake in HL60 cells against inositol concentration is shown in Fig. 1. The curve is characteristic of a process with saturation kinetics. This is supported by the linearity of the Hanes plot (S/V vs. S; insert to Fig. 1). Inositol uptake into HL60 cells is active and saturable and under the assay conditions described uptake represents inositol transport [5]. Analysis of the data shown in the insert to Fig. 1 shows that the plot has a linear regression of 0.98 allowing accurate estimation of the inositol transporter's kinetics. The K_m value under the assay conditions was 27.0 ± 3.0 ($n=5$) μM, while the V_{max} was 4.09 ± 0.59 ($n=5$) pmol/min/10^6 cells. These values are in good agreement with published data on the HL60 cell line [3,5].

The rate of inositol uptake at a substrate concentration of 15 μM and in the presence of 140 mM sodium chloride was 1.68 ± 0.2 ($n=6$) pmol/min/10^6 cells. In contrast the inositol transport rate in the absence of sodium (the buffer containing equimolar caesium chloride) was only 0.11 ± 0.02 ($n=6$) pmol/min/10^6 cells. These data suggest that 93.2 ± 1.9% ($n=6$) of inositol transport activity is sodium dependent. The residual 6.8 ± 1.9% ($n=6$) of uptake presumably reflects inositol transport via a sodium-independent

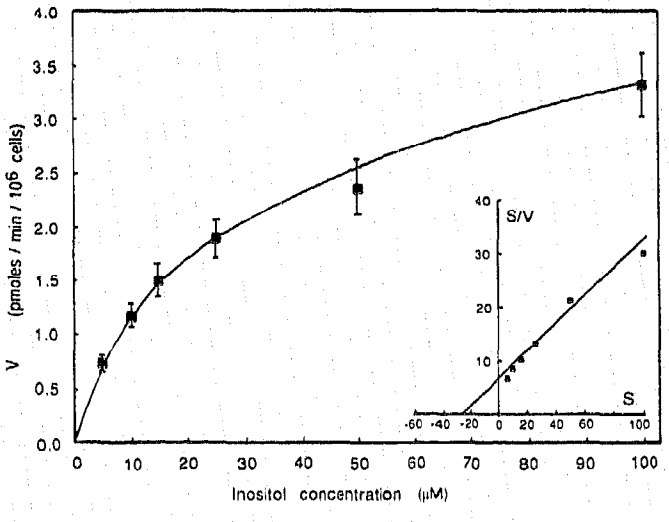

Fig. 1. Rates of inositol uptake into HL60 cells vs. inositol concentration. HL60 cells (0.1–0.8 × 10^6 cells/ml) were incubated for 10 min at 37°C in HEPES buffer, supplemented as described in section 2, and containing inositol (0–100 μM) and [^{3}H]inositol (0.2 μCi/nmol). Aliquots were taken for the estimation of inositol uptake and the cells collected by spinning through dibutyl phtalate oil. Radioactive counts present in the cell pellet were estimated using liquid scintillation spectrometry. Values shown are mean ± SEM of 5–7 determinations. Insert shows the data presented in Fig. 1 transformed using a Hanes plot (S/V vs. S). The intercept on the x-axis = K_m and on the y-axis = K_m/V_{max}.

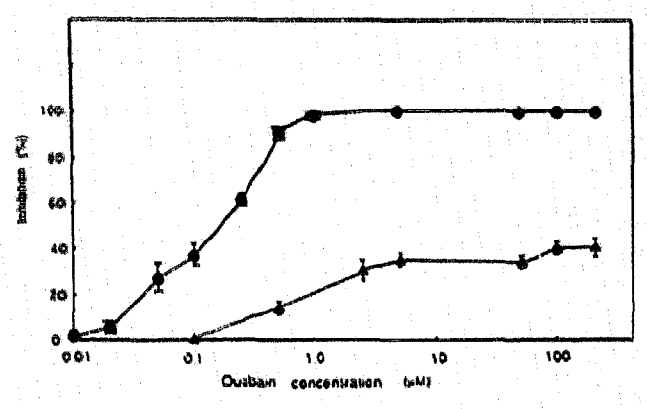

Fig. 2. Effect of ouabain on the rate of inositol transport and Na^+/K^+ ATPase activity. Inositol transport assays were performed in PBS buffer at an inositol concentration of 15 μM. Preincubation and assay tubes contained ouabain (0–200 μM). Cell viability assessed by phase-contrast microscopy and the ability of the cells to spin through the dibutyl phthalate oil (dependent on the cells volume to mass ratio) was not impaired by exposure to ouabain. Na^+/K^+ ATPase activity was assessed using ^{86}Rb uptake (see section 2). Incubations were as described for the assay of inositol transport except that inositol was omitted and ^{86}Rb (0.2 μCi/assay) substituted for [^{3}H]inositol and [^{14}C]inulin. The percentage inhibition of inositol transport (▲) and Na^+/K^+ ATPase (●) is plotted against ouabain concentration (μM). Data are the mean ± SEM of 5–7 experiments.

mechanism which is generally assumed to be physiologically unimportant [3,12].

Na^+/K^+ ATPase activity was assayed using ^{86}Rb uptake [8]. At ouabain concentrations of 5 μM or greater, 89.3 ± 1.4% ($n=6$) of ^{86}Rb uptake into HL60 cells was inhibited. This was taken to represent maximal Na^+/K^+ ATPase activity (100%) and was determined

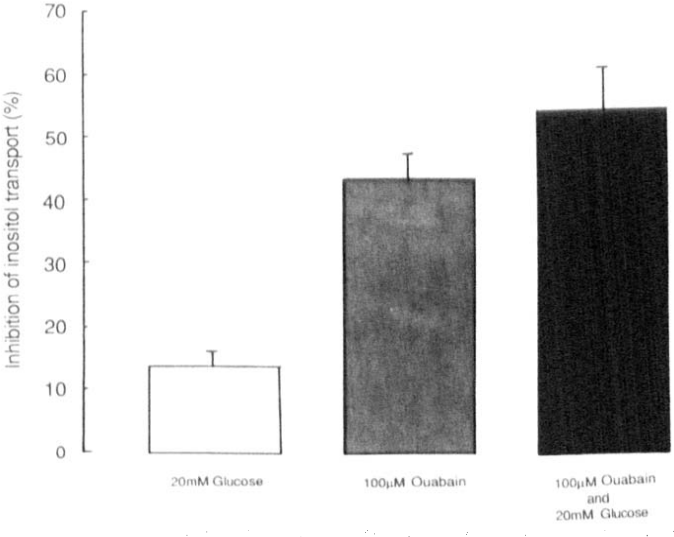

Fig. 3. The effect of ouabain and glucose on the rate of inositol transport. The assay of inositol transport was as described in the legend to Fig. 2. All assays were performed at an inositol concentration of 15 μM. Glucose (20 mM) and/or ouabain (100 μM) were added as indicated. Initial transport rates were 1.1 ± 0.9 pmol/min/10^6 cells ($n=16$) and the data shown represent the percentage inhibition in transport rates. Data are mean ± SEM of 4–6 experiments.

for each individual experiment. The effect of lower concentrations of ouabain on ^{86}Rb uptake was assessed in relation to the maximal Na^+/K^+ ATPase activity and fractional inhibitions were determined accordingly. Data obtained for the effects of ouabain on both Na^+/K^+ ATPase activity and inositol transport in HL60 cells are shown in Fig. 2. A significant inhibition of Na^+/K^+ ATPase activity (28 ± 6.8% ($n = 4$)) was observed at a ouabain concentration of 0.05 μM. A 10-fold increase in ouabain concentration (0.5 μM) produced a 90.5 ± 2.1% ($n = 8$) inhibition of Na^+/K^+ ATPase activity. Complete inhibition of enzyme activity (100%) was seen at an ouabain concentration of 5 μM. In contrast, inositol transport was relatively insensitive to ouabain, since only 15 ± 2.7% ($n = 6$) of transport activity was inhibited at an ouabain concentration of 5 μM. Greater levels of inhibition (41.6 ± 4.0% ($n = 7$)) required an ouabain concentration of 100 μM and concentrations of ouabain up to 10 mM produced no more than a 50% inhibition of inositol transport. The duration of exposure of the cells to ouabain (10 min–2 h) had no effect on the final level of inhibition achieved. If the concentrations of ouabain required to achieve 50% of the maximal inhibition of Na^+/K^+ ATPase and inositol transport are compared, it is clear that Na^+/K^+ ATPase is 10-fold more sensitive to ouabain than inositol transport (0.13 μM vs. 1.3 μM).

The fact that a large, if not major, proportion of inositol transport is ouabain-insensitive is supported by the observation that the inhibition of inositol transport seen with high concentrations of glucose (20 mM) is additive to the maximal effects of ouabain (Fig. 3).

4. DISCUSSION

Inositol transport is a potentially important regulator of intracellular inositol concentration. A recent report has shown that the changes in the intracellular inositol concentration which occur in HL60 cells during differentiation towards neutrophils are mediated by activation of inositol transport [5]. The mechanisms regulating the inositol transporter remain unknown. In many tissues, including HL60 cells, inositol transport is sodium-dependent [3,6] and it seems probable that the transport rate will, at least in part, be governed by the prevailing transmembrane sodium gradient. In most cells Na^+/K^+ ATPase activity is a major determinant of transcellular sodium flux and it might therefore be predicted that inositol transport and Na^+/K^+ ATPase activity are linked. Indeed these arguments have formed the basis of a hypothesis which attempts to explain the development of diabetic complications [7]. The metabolic or polyol theory suggests that in diabetes a decline in the rate of inositol transport leads to intracellular inositol depletion and a decline in Na^+/K^+ ATPase activity [9–11]. This results in a further reduction in inositol transport rate perpetuating the pathophysiological abnormalities and promoting the development of diabetic neuro- and microangiopathy [6–8]. Studies have shown that ouabain, a Na^+/K^+ ATPase inhibitor, reduces the rate of inositol transport and it has been argued that this demonstrates that inositol transport is dependent on the activity of Na^+/K^+ ATPase [6,7,12]. However, this assertion demands that both processes exhibit a similar sensitivity to ouabain inhibition and to date no studies have attempted to show the dose response of inositol transport and Na^+/K^+ ATPase activity to ouabain inhibition.

In this study we have confirmed the sodium dependence of inositol transport in HL60 cells. Na^+/K^+ ATPase activity was extremely sensitive to ouabain inhibition and this is in keeping with previous studies of the enzyme derived from human tissues [13]. In contrast, inositol transport was relatively insensitive to ouabain inhibition and only 15.0 ± 2.7% ($n = 6$) of inositol transport was inhibited at an ouabain concentration of 0.5 μM. Despite the fact that both inositol transport and Na^+/K^+ ATPase activity are inhibited by ouabain, the 10-fold difference in sensitivity of these two processes to inhibition make it improbable that they are mechanistically linked. This assertion is supported by the observation that no more than 50% of inositol transport can be inhibited by ouabain.

The data presented in this paper show that although inositol transport is sodium-dependent it is functionally independent of Na^+/K^+ ATPase activity. This observation suggests that an interaction between inositol transport and Na^+/K^+ ATPase does not underly the development of diabetic complications.

REFERENCES

[1] Hammerman, M.R., Sacktor, B. and Daughday, W.H. (1980) J. Physiol. 239, F113-F120.
[2] Greene, D.A. and Lattimer, S.A. (1982) J. Clin. Invest. 70, 1009-1018.
[3] Yorek, M.A., Dunlap, J.A. and Ginsberg, B.H. (1986) Arch. Biochem. Biophys. 246, 801-807.
[4] Gillon, K.R. and Hawthorne, J.N. (1983) Biochem. J. 210, 775-781.
[5] Baxter, M.A., Bunce, C.M., Lord, J.M., French, P.J., Michell, R.H. and Brown, G. (1991) Biochim. Biophys. Acta, in press.
[6] Greene, D.A. and Lattimer, S.A. (1984) Am. J. Physiol. 246, E311-E318.
[7] Greene, D.A., Lattimer, S.A. and Sima, A.A. (1987) N. Eng. J. Med. 316, 599-605.
[8] Simmonds, D.A. and Winegrad, A.I. (1989) Diabetologia 32, 402-408.
[9] Mandersloot, J.G., Roelofson, B. and DeGlen, J. (1978) Biochim. Biophys. Acta 508, 478-485.
[10] Greene, D.A. and Lattimer, S.A. (1986) Diabetes 35, 242-245.
[11] Kowluru, R., Bitensky, M.W., Kowluru, A., Demb, M., Keaton, P.A. and Burcain, T. (1989) Proc. Natl. Acad. Sci. USA 86, 3327-3351.
[12] Simmonds, D., Bomford, J. and Ng, L.L. (1990) Clin. Sci. 78, 335-341.
[13] Skou, J.C. (1990) FEBS Lett. 268, 314-324.