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Acute changes in *myo*-inositol uptake and $^{22}Na^+$ flux in murine neuroblastoma cells (N1E-115) following insulin

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myo-Inositol uptake was investigated in a murine neuroblastoma clone (N1E-115) to determine the effect of altered Na^+,K^+-ATPase activity. The Na^+ ionophone monensin, and veratridine, an alkaloid affecting voltage-dependent Na^+ entry, increased acute $^{22}Na^+$ uptake and $^{22}Na^+$ efflux from pre-loaded cells, concomitant with enhanced *myo*-inositol uptake. This effect was also seen following insulin. Insulin-stimulated *myo*-inositol uptake was inhibited by amiloride, ouabain and pyrithiamine. Amiloride inhibition suggests that activation of Na^+/H^+ exchange preceding Na^+,K^+-ATPase activation is involved in insulin stimulation of *myo*-inositol uptake. Pyrithiamine inhibition is an indication of prior activation of the Na^+,K^+-ATPase α^+ catalytic subunit by insulin. The results provide evidence that insulin contributes to the maintenance of Na^+,K^+-ATPase in neuronal tissue.

myo-Inositol uptake; Na^+; ($Na^+ + K^+$)-ATPase; Insulin; Pyrithiamine; (Neuroblastoma clone)

1. INTRODUCTION

The cellular uptake of *myo*-inositol is dependent on Na^+ flux maintained by Na^+- and K^+-dependent adenosine triphosphatase (Na^+,K^+-ATPase [1]. This enzyme is the basis of active cellular transport of Na^+ and K^+ and specific subunits of the Na^+- and K^+-sensitive complex alter reactivity in response to ion concentration, phosphorylation state and plasma membrane environment [2–4]. In diabetic neuropathy, there is considerable evidence to indicate that a defect in Na^+,K^+-ATPase in peripheral nerve, and autonomic ganglia, reflects an underlying alteration in cellular *myo*-inositol uptake [5,6]. The neuronal system therefore represents a very tightly coupled system, as the Na^+ gradient generated by the action of Na^+,K^+-ATPase is required for the uptake of *myo*-inositol and subsequent formation of phosphoinositides. The intracellular actions of phosphoinositide-derived, 1,2-diacylglycerol and inositol phosphates are thus limited by the availability of *myo*-inositol. To investigate possible changes to Na^+,K^+-ATPase activity which may be involved in neuronal responses in diabetes, a murine neuroblastoma clone, N1E-115, was used. Recently, we have shown that cells of this clone respond in a very similar manner to peripheral motor nerve when Na^+-dependent *myo*-inositol uptake is measured after a number of metabolic manipulations [7]. Altered Na^+ fluxes were induced in these cells to determine conditions under which Na^+,K^+-ATPase may be modified and the effect of this on *myo*-inositol uptake. In particular, evidence was sought for an insulin-sensitive activation contributing to *myo*-inositol uptake. An α^+-subunit of Na^+,K^+-ATPase has been described in a number of tissues including brain [8,9]. The complex containing this α^+-subunit is exclusively the insulin-sensitive form of Na^+,K^+-ATPase activity [10]. Na^+-pump activity was stimulated by two means, rapidly by addi-

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tion of the Na^+ ionophore, monensin, and more gradually by prolonged incubation with the alkaloid veratridine, stabilizing an open form of voltage-sensitive Na^+ channels [11], which will recruit Na^+,K^+-ATPase activity to the cell membrane [12]. Inhibition of Na^+-pump activity was investigated using ouabain and with pyrithiamine, an anti-metabolite of thiamine with considerable specificity for the α^+-subunit contained in the Na^+,K^+-ATPase complex [13]. Cellular *myo*-[2-^{3}H]inositol uptake was determined concomitant with $^{22}Na^+$ content.

2. MATERIALS AND METHODS

2.1. *Culture of murine neuroblastoma cells (N1E-115)*

N1E-115 neuroblastoma cells, used at passage 16–25, were a gift from Dr E. Richelson (Department of Psychiatry and Pharmacology, Mayo Clinic, Rochester, MN). The cells were grown at 37°C in humidified air containing 5% CO_2 in Dulbecco's modification of minimum essential medium/fetal bovine serum (90:10, v/v). Prior to experimentation, cells were subcultured and plated at a density of 1×10^6 cells in Dulbecco's medium/fetal bovine serum (90:10, v/v) containing glucose (5.6 or 25 mmol/l).

2.2. myo-*Inositol uptake*

myo-Inositol uptake was determined as in [7]. Briefly, cells were incubated in a modified buffer containing 135 mmol/l NaCl, 5.4 mmol/l KCl, 1.2 mmol/l $CaCl_2$, 1 mmol/l NaH_2PO_4, 1.3 mmol/l $MgSO_4$ and 5 mmol/l Hepes, pH 7 (330 mosmol/l) containing *myo*-[2-^{3}H]inositol (4×10^{-5} mol/l, 10 μCi/ml) for 20 min.

At the end of this time, cells were removed, washed with three changes of medium or buffer without added radiolabelled *myo*-inositol, blotted and solubilized in 0.3 ml of 0.75 N NaOH at 80°C. *myo*-[2-^{3}H]Inositol content was quantitated in a gel formed by the addition of 4 ml H_2O and 10 ml pseudocumene scintillant.

In separate experiments labelled *myo*-inositol was replaced with 5 μCi L-[1-^{3}H(N)]glucose (final L-glucose concentration 5×10^7 mol/l) for determination of non-specific uptake and entrapped extracellular medium.

2.3. $^{22}Na^+$ *content and efflux from N1E-115 cells*

Cells were incubated for 4 h in Dulbecco's medium containing $^{23}Na^+$ (149 mmol/l) and $^{22}Na^+$ ($^{22}Na^+$ at final spec. act. 1 μCi/ml). The incubations were either continued for a further 20 min in the presence of added agents or the medium removed and replaced with medium containing $^{23}Na^+$ only for a further 20 min period. Cells were washed and solubilized as for *myo*-inositol determinations.

2.4. *Addition of agents*

When present, insulin, pyrithiamine, ouabain, monesin or amiloride, dissolved appropriately, were added either singly or in combination, for the last 20 min of incubation in Dulbecco's medium or for 20 min of buffer incubation with either *myo*-[2-^{3}H]inositol, or to cells pre-loaded for 4 h with $^{22}Na^+$. Veratridine was included for 40 h in culture. The solutions used for suspending the agents, H_2O, ethanol or dimethyl sulphoxide were included in control incubations.

2.5. *Cellular protein determinations*

Protein was determined on solubilized cell preparations using Coomassie brilliant blue dye [14]. *myo*-[^{3}H]Inositol and $^{22}Na^+$ content were corrected for extracellular content and expressed as pmol/100 μg protein and nmol/100 μg protein, respectively.

2.6. *Statistical analysis*

Data were analysed using the Student's *t*-test for paired data, significance being taken at the 5% level ($p < 0.05$) unless indicated otherwise.

3. RESULTS

3.1. myo-*Inositol uptake*

The results for *myo*-inositol uptake are summarized in table 1. When N1E-115 cells were cultured at two glucose concentrations, the results confirmed previous observations of inhibition of *myo*-inositol uptake in the presence of elevated glucose (4.01 ± 0.17 vs 2.14 ± 0.06 pmol/100 μg protein at 5.6 and 25 mmol/l glucose, respectively). No effect of insulin was seen at the lower glucose concentration but a significant increase was seen with insulin in cells incubated at

Table 1

myo-Inositol uptake in N1E-115 neuroblastoma cells

Additions	5.6 mmol/l glucose		25 mmol/l glucose	
	No added insulin	Insulin (0.7 nmol/l)	No added insulin	Insulin (0.7 nmol/l)
No pre-incubation				
Nil	4.01 ± 0.17	4.12 ± 0.27	2.14 ± 0.06[a]	3.71 ± 0.04[b]
Ouabain (10^{-4} M)	3.32 ± 0.12[a]	3.41 ± 0.12[a]	1.31 ± 0.04[b]	1.42 ± 0.03[c]
Pyrithiamine (10^{-5} M)	3.78 ± 0.09	3.87 ± 0.08	1.89 ± 0.05[b]	1.91 ± 0.04[c]
Amiloride (10^{-5} M)	3.71 ± 0.09	3.67 ± 0.08	1.88 ± 0.07[b]	1.89 ± 0.02[c]
Monensin (10^{-6} M)	4.79 ± 0.14[a]	–	2.78 ± 0.08[b]	–
Veratridine (10^{-5} M)	4.51 ± 0.16[a]	–	2.89 ± 0.08[b]	–
Pre-incubation; veratridine (10^{-5} M)				
Nil			6.61 ± 0.27[b]	–
Ouabain (10^{-4} M)			3.90 ± 0.24[b]	–
Pyrithiamine (10^{-5} M)			4.89 ± 0.24[b]	–

[a] Significantly different from incubation in the presence of 5.6 mmol/l glucose without additions
[b] Significantly different from incubation in the presence of 25 mmol/l glucose without additions
[c] Significantly different from incubation in the presence of 25 mmol/l glucose with added insulin

Values shown are means ± SE (8 experimental observations) and are expressed as pmol/100 μg protein

25 mmol/l glucose. A dose-response curve for this effect is shown in fig.1, indicating the effect to be near maximal within the range of physiological insulin concentrations. Incubations were continued at 0.7 nmol/l insulin. The increase in *myo*-inositol uptake was significantly reduced by concurrent incubation with ouabain, pyrithiamine or amiloride. Ouabain, but not pyrithiamine or amiloride, also significantly reduced *myo*-inositol uptake in non-insulin-stimulated cells. A significant increase in *myo*-inositol uptake was seen when the Na^+ ionophores monensin and veratridine were present during the uptake experiment. The effect was most marked, however, followed pre-treatment with veratridine. This significant increase in *myo*-inositol uptake was partially inhibited by ouabain or pyrithiamine present during the uptake period.

3.2. *Cellular $^{22}Na^+$ uptake*

In cells which had been incubated with $^{22}Na^+$ for 4 h in the presence of 5.6 or 25 mmol/l glucose, the net cellular $^{22}Na^+$ was 5.77 ± 0.7 and 5.42 ± 0.2 nmol/100 μg protein, respectively (no significant difference). At 25 mmol/l glucose, when 0.7 nmol/l insulin was present for a final 20 min,

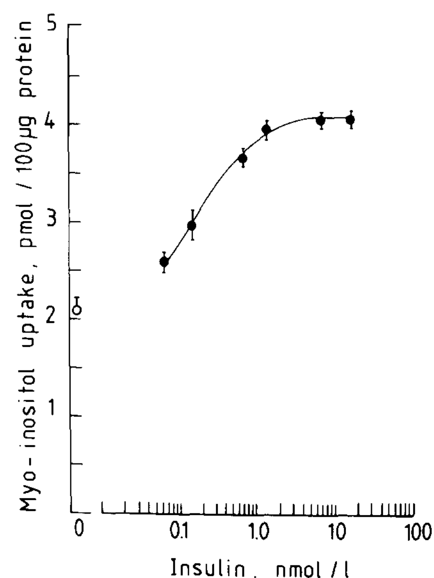

Fig.1. Effect of insulin on *myo*-inositol uptake in N1E-115 cells. *myo*-Inositol uptake was determined over 20 min in the presence of 0.07–70 nmol/l insulin. Values shown are means ± SE for 6 determinations at each point.

Table 2

$^{22}Na^+$ content of N1E-115 neuroblastoma cells

Addition	$^{22}Na^+$ content (nmol/100 μg protein	
	No added insulin	Insulin (0.7 nmol/l)
Nil	5.42 ± 0.21	7.25 ± 0.51[a]
Pyrithiamine (10^{-5} M)	6.42 ± 1.07	8.74 ± 0.37[b]
Amiloride (10^{-5} M)	5.41 ± 1.71	5.34 ± 0.56[b]
Ouabain (10^{-4} M)	13.46 ± 1.38[a]	15.42 ± 1.76[b]
Monensin (10^{-6} M)	19.76 ± 1.51[a]	
Veratridine (10^{-5} M)	12.06 ± 0.87[a]	
Veratridine (10^{-5} M); pre-incubation	5.92 ± 1.2	

[a] Significantly different from cells incubated without added insulin

[b] Significantly different from cells incubated with added insulin

in the presence of external $^{22}Na^+$, cellular $^{22}Na^+$ rose significantly to 7.25 ± 0.5 nmol/100 μg protein. This effect was blocked by the concurrent presence of amiloride. Pyrithiamine, together with insulin, caused a significant increase in $^{22}Na^+$ content of cells loaded and incubated in the presence of $^{22}Na^+$. Similarly, ouabain and monensin or veratridine added acutely raised cellular $^{22}Na^+$ content significantly (table 2).

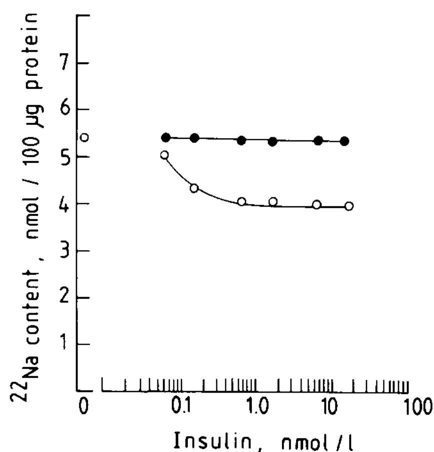

Fig.2. Effect of insulin on $^{22}Na^+$ content of N1E-115 cells. After incubation with $^{22}Na^+$ as described in section 2, cells were incubated in $^{22}Na^+$-free medium for 20 min in the presence of 0.07–70 nmol/l insulin and in the presence (●) or absence (○) of 10^{-5} M pyrithiamine. Results are means for triplicate estimations within an experiment.

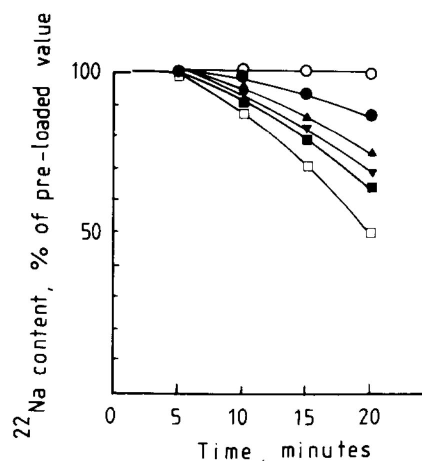

Fig.3. Time course of $^{22}Na^+$ efflux from N1E-115 cells pre-loaded with $^{22}Na^+$. Cells were incubated in $^{22}Na^+$-free medium, following loading with $^{22}Na^+$. $^{22}Na^+$ content was determined over a 20 min period in cells which had been pre-incubated without additions (●) or pre-incubated in the presence of 10^{-5} M veratridine (□). The figure indicates the effect of 10^{-4} M ouabain (○), 0.7 nmol/l insulin (▲), 10^{-6} M monensin (▼), or 10^{-5} M veratridine (■) added acutely during the efflux period.

3.3. *$^{22}Na^+$ efflux from pre-loaded cells*

$^{22}Na^+$ efflux from pre-loaded cells was determined by monitoring $^{22}Na^+$ content over 20 min in cells removed to $^{22}Na^+$-free buffer. Insulin promoted $^{22}Na^+$ loss in a dose-dependent manner, which was blocked by the concurrent presence of pyrithiamine (fig.2). Similarly, $^{22}Na^+$ efflux was greater in cells to which monensin or veratridine had been added for 20 min or to cells pre-incubated with veratridine. The loss of cellular $^{22}Na^+$ could be blocked by the concurrent presence of ouabain (fig.3).

4. DISCUSSION

Our results indicate that *myo*-inositol uptake may be altered in neuroblastoma cells by manipulations which alter cellular Na^+ flux. A comparison of uptake under different experimen-

tal conditions permits inferences to be drawn about the activity of Na^+,K^+-ATPase. Insulin, in particular, altered *myo*-inositol uptake associated with a pyrithiamine-inhibitable rise in $^{22}Na^+$ efflux, a strong indication of Na^+,K^+-ATPase activation. It is possible that the activation of insulin-stimulated Na^+,K^+-ATPase follows amiloride-sensitive Na^+/H^+ exchange in the neuroblastoma cell. This possibility is supported by experiments showing an amiloride-sensitive increase in $^{22}Na^+$ content induced by insulin. Na^+/H^+ exchange has been found in a number of cell types and proposed to be a ubiquitous component of mammalian cell plasma membranes including neuronal cells [15–17]. There is considerable evidence that growth factors stimulate the activity of Na^+,K^+-ATPase at the cell membrane secondarily to this increased Na^+ entry into cells.

Whether the Na^+,K^+-ATPase activation is insulin-specific in all cells remains to be determined. Here, the insulin activation of Na^+,K^+-ATPase appears to be specific for a pyrithiamine-inhibitable form. Both the α and insulin-sensitive, α^+ catalytic subunits of Na^+,K^+-ATPase have been described in neurones [8]. The inhibition by pyrithiamine of $^{22}Na^+$ efflux and *myo*-inositol uptake in insulin-stimulated cells suggests the presence of the α^+ form in the neuroblastoma line.

Similarly, increased Na^+,K^+-ATPase activity following Na^+ influx due to veratridine would indicate that, as described for cultured skeletal muscle [12], veratridine can increase Na^+,K^+-ATPase activity by stimulated biosynthesis of Na^+,K^+-ATPase subunits. In the neuroblastoma, pyrithiamine inhibition of Na^+,K^+-ATPase and *myo*-inositol uptake after veratridine would confirm the presence and activation of the α^+ catalytic subunit.

Reduction of Na^+,K^+-ATPase activity in diabetic nerve is associated with reduced *myo*-inositol uptake [18–20]. This can be prevented and normal Na^+ flux and nerve conduction maintained by *myo*-inositol supplementation [20] or the administration of an aldose reductase inhibitor, correcting the metabolic consequences of hyperglycaemia [6,22]. The present study indicates that insulin may also contribute to improved *myo*-inositol uptake directly by increasing Na^+,K^+-ATPase activity.

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