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Resting membrane potential in 41A3 mouse neuroblastoma cells. Effect of increased glucose and galactose concentrations

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Neuroblastoma cells were used to examine the effect of high concentrations of glucose or galactose and accumulation of polyols on the resting membrane potential. Polyol levels are increased and *myo*-inositol content decreased when neuroblastoma cells are chronically exposed to media containing 30 mM glucose or 30 mM galactose compared to cells grown in media containing 30 mM fructose. Furthermore, the 6 h accumulation and incorporation into phospholipid of extracellular *myo*-inositol is decreased in cells exposed to media containing 30 mM glucose or 30 mM galactose compared to cells grown in media containing 30 mM fructose. The resting membrane potential was determined by examining the steady-state accumulation of the lipophilic cation tetra[^{3}H]phenylphosphonium bromide (TPP^+). The resting membrane potential of cells grown in media containing 30 mM fructose is about −70 mV which is very similar to the resting membrane potential of cells grown in unsupplemented media. The resting membrane potential is significantly decreased in cells grown in media containing 30 mM glucose or 30 mM galactose. *myo*-Inositol metabolism and content and polyol levels are maintained at near normal values and the resting membrane potential is improved when media containing 30 mM glucose or 30 mM galactose are supplemented with 0.4 mM sorbinil. Acute exposure of neuroblastoma cells to 2 mM ouabain had no significant effect on [^{3}H]TPP^+ accumulation. This suggests that acute inhibition of Na^+/K^+ pump activity does not decrease the resting membrane potential of neuroblastoma cells. The decrease in resting membrane potential may be induced by the metabolic abnormalities and/or chronic decrease in Na^+/K^+ pump activity which occur when neuroblastoma cells are chronically exposed to increased glucose or galactose concentrations.

Introduction

A major pathological disorder in diabetes is a neuropathy which is characterized by a reduction in nerve conduction velocity. The changes which occur in the diabetic nerve which are responsible for the pathology of diabetic neuropathy are not known. Presently metabolic abnormalities such as high concentrations of circulating glucose are considered to be major contributing factors to the development of diabetic neuropathy [1–7]. Investigations with diabetic animals have led to a theory linking high glucose levels and the intracellular accumulation of sorbitol and depletion of *myo*-inositol to the progressive development of diabetic neuropathy. Greene et al. [8,9] have proposed a mechanism suggesting that decreased Na^+/K^+-ATPase activity, a primary defect in diabetic neuropathy, may be responsible for the development of this disorder. Decreased Na^+/K^+-ATPase activity in peripheral nerve can be restored in diabetic animals by treatment with aldose reductase inhibitors or *myo*-inositol supplementation to the diet [2,9,10]. Other defects in diabetic neuropathy including reduced axon transport and nerve conduction velocities are also improved by aldose reductase inhibitor treatment or *myo*-inositol supplementation [11,12]. This suggests that alterations in polyol levels and *myo*-inositol metabolism are partially responsible for the development of disorders which occur in diabetic neuropathy. To further examine the relationship between increased glucose concentration, intracellular polyol accumulation and decreased *myo*-inositol metabolism and content we have been examining cultured neuroblastoma cells chronically exposed to increased glucose levels. Our previous studies have shown that high concentrations of

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glucose are responsible for decreasing *myo*-inositol metabolism and content and phosphatidylinositol level in neuroblastoma cells [13]. These changes were accompanied by a decrease in Na^+/K^+-ATPase transport activity [14,15]. The decrease in *myo*-inositol metabolism and content and Na^+/K^+-ATPase transport activity were significantly improved by sorbinil treatment of the glucose supplemented media [14,16]. These data support the theory that increased glucose concentration is responsible for altering *myo*-inositol metabolism, increasing sorbitol accumulation and inducing functional defects at the cellular level. To determine if other defects may occur in neural cells exposed to high glucose concentrations we have examined the resting membrane potential of cultured neuroblastoma cells exposed to media containing 30 mM glucose, 30 mM galactose or 30 mM fructose. Many properties of neural cells rely on the electrical potential across the plasma membrane and an alteration in this property could impair many functional processes. Many neurophysiological properties have been examined and described using neuroblastoma cell lines making them an appropriate model system for studies of the nervous system. Neuroblastoma cells express many of the properties of normal neurons and after differentiation will form synapses in culture and are electrically responsive (for reviews, see Refs. 17–19). Our studies have shown that cultured neuroblastoma cells may also be a good model system to examine the effect of high glucose concentrations on neural cell metabolism and function [13–16].

Materials and Methods

Cell culture

Mouse neuroblastoma cells NB41A3 were cultured in Ham's F10 medium, which contains 5.6 mM glucose and supplemented with 15% horse serum, 2.5% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 294 μg/ml glutamine. The final inositol concentration of the media was determined to be 43.3 μM with the serum contributing greater than 90% of the content. The cells were grown in Corning 75 cm^2 tissue culture flasks in an incubator maintained at 37°C, with 5% CO_2 in humidified air as the gas phase. For experimental purposes, cells were cultured in this media supplemented with 30 mM D-glucose, 30 mM galactose or 30 mM D-fructose in the absence or presence of 0.4 mM sorbinil. Sorbinil was a gift from Dr. Nancy J. Hutson of Pfizer Inc. Cells grown in media containing 30 mM fructose were used as an osmotic control for cells exposed to media containing 30 mM glucose or 30 mM galactose [13–16]. Cells were maintained in normal media for a minimum of four passages (four weeks) prior to experimental manipulation. Cultures were used for a maximum of eighteen weeks. Prior to experimental use, cells to be used for *myo*-inositol metabolism investigations were seeded into Lux 4-well plates and assays conducted in quadruplicate when cells were near confluency. For resting potential studies cells were seeded in Corning 150 cm^2 tissue culture flasks and prior to experimental evaluation were harvested using 0.25% Trypsin/0.1% EDTA.

Inositol metabolism

Uptake. Accumulation of extracellular *myo*-inositol was measured following a wash of the cells with media at 37°C. The cells were incubated in supplemented media (37°C) containing tracer amounts of *myo*-[2-^{3}H]inositol, 20 Ci/mmol purchased from Amersham/Searle, Arlington Heights, IL. After the 6-h incubation, the cells were washed two times with ice-cold buffer and collected in a total volume of 1.5 ml. The cell suspension was then sonicated for 5 s to obtain a uniform mixture, and samples taken to determine protein content and extracellular *myo*-[2-^{3}H]inositol accumulation and incorporation into inositol phospholipids. For net uptake analysis, duplicate 0.25 ml aliquots of the cell suspension were taken, 5.0 ml of scintillation fluid added, and the radioactivity measured with a liquid scintillation spectrometer. Quenching was monitored with a ^{226}Ra external standard. Protein content was determined with 50-μl aliquots using a modification of the Lowry method [21]. To determine the effect of the experimental conditions on extracellular *myo*-[2-^{3}H]inositol incorporation into phospholipid, a 0.50-ml aliquot of the 1.5 ml suspension was extracted with 20 ml of chloroform/ methanol/ HCl (2 : 1 : 0.015, v/v/v) [13,14,16]. The lipid fraction was then isolated following phase separation produced by adding 4 ml of 154 mM NaCl containing 0.5 M HCl. The entire lipid fraction was then collected in a 20 ml scintillation vial, dried under N_2 and analyzed for radioactivity. The incorporation of myo-inositol into phospholipid is based solely upon the percent of radioactivity recovered in lipid compared to the total radioactivity taken up by the cells following the 6-h incubation. The amount of *myo*-inositol incorporated into phospholipid was determined by multiplying this percentage by the total uptake. All of these calculations were based upon mg of cell protein and do not take into account the content of the intracellular inositol pools, one of which may be specific for phospholipid precursors. *myo*-Inositol taken up from the medium by neuroblastoma cells does not equilibrate with the entire intracellular inositol pool, but is preferentially incorporated into phospholipids [14,16]. The *myo*-inositol not incorporated into phospholipid is presumably available to maintain the cellular inositol pool.

Intracellular inositol and sorbitol

Intracellular levels of *myo*-inositol and sorbitol were analyzed by gas-liquid chromatography as described by

Clements and Reynertson [22] and Tomlinson et al. [12]. Cells were collected after trypsinization and washed with glucose free Hepes buffer. Methyl α-mannoside (100 μg/ml) was added as an internal standard and cell samples sonicated. The samples were collected after centrifugation (25000 × g), lyophilized, and silylated with a 1.0-ml mixture of pyridine, hexamethyldisilazane, and trimethylchlorosilane (10:2:1, v/v/v) and constant mixing for 16 h. The samples were then extracted with cyclohexane and analyzed by gas-liquid chromatography. The samples were injected into a Hewlett Packard 5890A GLC equipped with a glass column packed with 3% OV3 on chromosorb W and a flame ionization detector. Thermal programming of the gas chromatograph was used to maximize the separation. The column was held at 180°C for 2 min after injection, then the temperature increased at a rate of 4 C°/min to 225°C and maintained at this temperature for 5 min. Elution times of sorbitol and *myo*-inositol were determined by comparison of the peaks to a standard, and recovery and quantitation of the sample by comparison to methyl α-mannoside.

Resting membrane potential

Neuroblastoma cells were harvested, washed with buffer, counted and resuspended in a 'low K^+ buffer', containing 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.5 mM glucose and 50 mM Tris (pH 7.4) or 'high K^+ buffer', containing the same components as the low K^+ buffer except that 130 mM KCl was substituted for NaCl [20]. Following a 10-min equilibration period at 37°C reactions were initiated by adding 100 μl of washed cells (10^6) to a 900 μl solution containing either the low or high K^+ buffer and 1–50 μM tetra[^{3}H]phenylphosphonium bromide (TPP^+) (purchased from Amersham/Searle, 26 Ci/mmol). The reactions were terminated after an appropriate period of time by centrifugation in an Eppendorf microfuge for 1 min. The supernatants were aspirated and the pellets resuspended in 0.5 ml of NCS tissue solubilizer (Amersham/Searle) and incubated overnight at room temperature. Afterwards 5 ml of Neutralizer cocktail (Research Products International, Elk Grove Village, IL) was added and radioactivity determined by liquid scintillation spectrometry. All assays were conducted in triplicate. The resting membrane potential was also examined in neuroblastoma cells differentiated by exposing the cells for 8 days to 10^{-7} M phorbol 12-myristate 13-acetate (phorbol ester), 10^{-3} M dibutyryl cyclic AMP (DcAMP) or 10^{-5} M retinoic acid. The medium was changed every two days. The procedures used to differentiate the cells were similar to those described by Yu et al. [23] and Prashad et al. [24]. The neuroblastoma cells used in these studies have previously been shown to differentiate with exposure to 10^{-3} M DcAMP [19]. Upon examination by light microscopy of the differentiated cells following 8 days of exposure to the differentiating agents the cells appeared to have extended neurite growth compared to control cells (data not shown).

Intracellular space

Cells were harvested, washed with buffer, counted and resuspended in the low or high K^+ buffer, containing [^{3}H]H_2O and [^{14}C]inulin. After a 30 min incubation at 4°C the cells were pelleted by centrifugation. The intracellular space was determined from the [^{3}H]H_2O content of the cell pellet which was corrected for extracellular trapping by determining the [^{14}C]inulin content.

Data analysis

All values were calculated per milligram of cell protein or per 10^6 cells. Resting membrane potentials were determined as described by Lichtshtein et al. [20] using the following equations.

$$[TPP^+]_{(intra,\ low\ K^+)} - [TPP^+]_{(intra,\ high\ K^+)} = [TPP^+]_{(intra,\ corrected)}$$

Inserting this value into the Nernst equation results in the resting membrane potential.

$$\psi = -61 \log([TPP^+]_{(intra,\ corrected)}/[TPP^+]_{(extra)})$$

All comparisons for significance were made using Student's *t*-test.

Results

TPP^+ accumulation

Neuroblastoma cells suspended in a buffer containing physiological concentrations of Na^+ and K^+ took up [^{3}H]TPP^+ rapidly for 15 min and achieved a steady-state level of accumulation after 20 min (Fig. 1). When the cells were resuspended in a buffer containing a high K^+ concentration the steady-state level of [^{3}H]TPP^+ accumulation was decreased by about 70%. Fig. 2 shows that steady-state accumulation of [^{3}H]TPP^+ by cells resuspended in either normal or high K^+ buffer was linear for [^{3}H]TPP^+ concentrations ranging from 1 to 25 μM. [^{3}H]TPP^+ accumulation (2 μM) was approximately linear with respect to cell number ranging from 0.25 to $1 \cdot 10^6$ cells (Fig. 3). The accumulation of [^{3}H]TPP^+ by neuroblastoma cells at a concentration greater than $1 \cdot 10^6$ decreased with respect to cell number. This may be due to the depletion of substrate.

Additional studies have shown that [^{3}H]TPP^+ does not bind to the cells. Intracellular [^{3}H]TPP^+ is rapidly lost following a prelabeling incubation with [^{3}H]TPP^+ and a subsequent incubation in buffer free of [^{3}H]TPP^+ (data not shown). These data support the conclusion

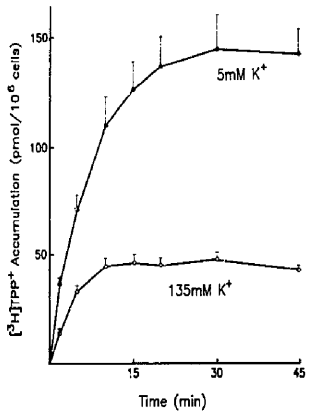

Fig. 1. Time course of $[^3H]TPP^+$ accumulation in high and low K^+ buffer. Neuroblastoma cells, 10^6, were incubated in high or low K^+ buffer containing 2 μM $[^3H]TPP^+$. Each value is the mean of six separate determinations. The standard error is indicated by the vertical line.

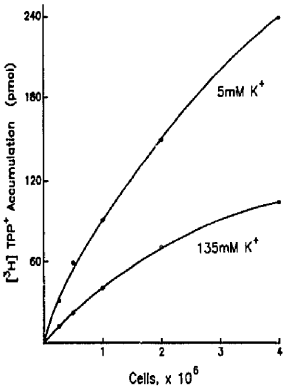

Fig. 3. Effect of cell number on $[^3H]TPP^+$ accumulation. Neuroblastoma cells, $(0.25–4)\cdot10^6$, were incubated for 20 min in high or low K^+ buffer containing 2 μM $[^3H]TPP^+$. Each value is the mean of six separate determinations. The standard error was less than 10% of the mean value for each determination.

that $[^3H]TPP^+$ accumulation by neuroblastoma cells is mediated by a nonsaturable process and that the plateau of TPP^+ accumulation represents a steady state condition (i.e., $[^3H]TPP^+$ influx = $[^3H]TPP^+$ efflux) [20]. These results are similar to those reported by Lichtshtein et al. [20] for the determination of the resting membrane potential in a neuroblastoma-glioma hybrid cell line. Based upon these results the determination of the resting membrane potential was done by incubating $1\cdot10^6$ cells at 37°C for 20 min with 2 μM $[^3H]TPP^+$. The

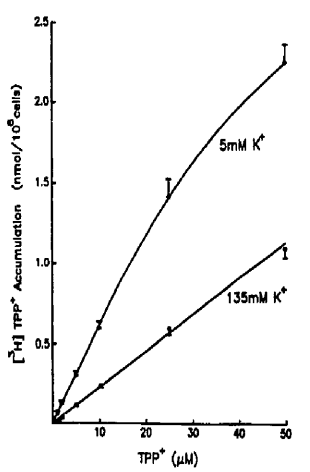

Fig. 2. Effect of $[^3H]TPP^+$ concentration on accumulation. Neuroblastoma cells, 10^6, were incubated for 20 min in high or low K^+ buffer containing 2–50 μM $[^3H]TPP^+$. Each value is the mean of six separate determinations. The standard error was less than 10% of the mean value for each determination.

accumulation of $[^3H]TPP^+$ in these conditions is reduced by 60–70% when the K^+ concentration is increased from 5 mM to 135 mM.

To calculate TPP^+ concentration gradients the intracellular volume of cells from each of the experimental conditions was determined. Cells resuspended in normal or high K^+ buffer had an intracellular volume of 3.24 and 2.74 $\mu l/10^6$ cells, respectively. These values did not significantly change for cells grown in the experimental culture conditions. The resting membrane potential in isolated neurons and neuroblastoma cells is mostly due to the K^+ diffusion potential [20]. To determine the extent that the K^+ gradient influences $[^3H]TPP^+$ accumulation the effect of external K^+ concentration on $[^3H]TPP^+$ accumulation by neuroblastoma cells was examined. Increasing the external K^+ concentration from 5 mM to 135 mM caused a decrease in $[^3H]TPP^+$ accumulation (Fig. 4). At 25, 50, 75, 100 and 135 mM K^+ $[^3H]TPP^+$ accumulation was decreased by 27%, 42%, 49%, 59% and 64%, respectively. Therefore, about 35% of the $[^3H]TPP^+$ accumulated by the cells at steady state is insensitive to an external K^+ concentration that abolishes the resting membrane potential across the plasma membrane [20]. This is likely due to the accumulation of $[^3H]TPP^+$ by internal organelles, such as the mitochondria, which have a negative potential that is not sensitive to the extracellular K^+ concentration.

Resting membrane potential

Steady-state $[^3H]TPP^+$ accumulation was used to determine the resting membrane potential of neuroblastoma cells exposed to media containing 30 mM glucose, 30 mM galactose or 30 mM fructose ± 0.4 mM sorbinil (Table I). $[^3H]TPP^+$ accumulated in the presence of high K^+ buffer and corrected for the intra-

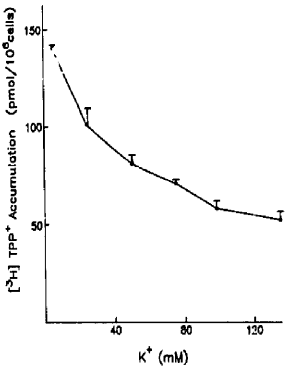

Fig. 4. Effect of increasing potassium concentration on [^{3}H]TPP$^+$ accumulation. Neuroblastoma cells, 10^6 were incubated in buffer containing 5, 25, 50, 75, 100 and 135 mM K^+ and 2 μM [^{3}H]TPP$^+$. After 20 min the amount of [^{3}H]TPP$^+$ accumulated by the cells was determined. Each value is the mean of four separate determinations. The standard error is indicated by the vertical line.

cellular space was subtracted from [^{3}H]TPP$^+$ accumulated in normal buffer. This value was then inserted into the Nernst equation to obtain the resting membrane potential. The resting membrane potential was significantly decreased in cells exposed for a minimum of 2 weeks to media containing 30 mM glucose or 30 mM galactose compared to cells grown in media containing 30 mM fructose. The resting membrane potential of cells grown in unsupplemented media, -69.0 ± 1.3 mV, is similar to the value obtained for cells grown in media containing 30 mM fructose. We have previously used cells grown in media containing 30 mM fructose as an osmotic control for cells grown in media containing 30 mM glucose or 30 mM galactose [13-16]. The addition of sorbinil, an aldose reductase inhibitor, to the glucose or galactose supplemented media partially protected the cells from the decrease in resting membrane potential. The resting membrane potential of neuroblastoma cells cultured in media containing 30 mM glucose was significantly improved when sorbinil was added to the glucose supplemented media. A similar improvement was observed when sorbinil was added to media containing 30 mM galactose, however, the difference did not attain statistical significance. Similar results were obtained from a study of [^{3}H]TPP$^+$ accumulation conducted using cells that remained attached to tissue culture plates. In this study which was conducted in triplicate the average [^{3}H]TPP$^+$ accumulation by cells cultured in media containing 30 mM glucose, 30 mM fructose, 30 mM glucose and 0.4 mM sorbinil or 30 mM fructose and 0.4 mM sorbinil was 20800, 29400, 25200 and 29100 cpm/mg protein, respectively. These values were corrected for extracellular trapping by using [^{14}C]inulin and for [^{3}H]TPP$^+$ accumulation in high-K^+ buffer which did not differ among the different growth conditions. In a similar study neuroblastoma cells cultured in unsupplemented media or media containing 30 mM galactose, 30 mM fructose or 30 mM galactose and 0.4 mM sorbinil accumulated 28600, 20500, 27800 and 25800 cpm/mg protein [^{3}H]TPP$^+$, respectively. The resting membrane potential was also determined in neuroblastoma cells differentiated by DcAMP, retinoic acid or phorbol ester. These studies were also conducted with cells attached to tissue culture plates and the amount of [^{3}H]TPP$^+$ accumulated in cells incubated in low-K^+ and high-K^+ buffer was determined. The resting membrane potential of undifferentiated cells analyzed by this method was -76.9 mV ($n = 2$, conducted in triplicate). The resting membrane potential of cells differentiated by DcAMP, retinoic acid or phorbol ester was -83.2, -61.2 and -74.7 mV, respectively.

TABLE I

Resting membrane potential of neuroblastoma cells cultured in 30 mM fructose, glucose or galactose ± 0.4 mM sorbinil

Neuroblastoma cells were grown for 2–4 weeks in the supplemented media. Resting membrane potential was determined by incubating 10^6 cells for 20 min in high- or low-K^+ buffer containing 2 μM [^{3}H]TPP$^+$. [^{3}H]TPP$^+$ accumulation was determined and the resting potential calculated as described in Materials and Methods. Each value is the mean of 17 separate determinations ± S.E.

Culture conditions	Resting membrane potential (mV)
Fructose	-70.6 ± 2.1
Glucose	-59.9 ± 1.9 [a,b]
Galactose	-62.3 ± 2.1 [a]
Fructose + sorbinil	-70.3 ± 1.6
Glucose + sorbinil	-66.4 ± 1.7
Galactose + sorbinil	-66.8 ± 2.0

[a] $P < 0.05$, compared to fructose supplemented cells.
[b] $P < 0.05$, compared to the glucose + sorbinil supplemented cells.

Effect of veratridine and ouabain on TPP$^+$ accumulation

Na^+/K^+-ATPase transport activity is decreased in cells chronically exposed to media containing 30 mM glucose or 30 mM galactose [14,15]. To determine if decreased Na^+/K^+-ATPase activity could contribute to the decrease in the resting membrane potential the effect of ouabain was examined. Fig. 5 presents data on the [^{3}H]TPP$^+$ content of cells prelabeled with [^{3}H]TPP$^+$ for 20 min then transferred to buffer containing 5 mM K^+ with or without 2 mM ouabain, a concentration which we have previously shown blocks Na^+/K^+-ATPase activity by 90%. After 1 h ouabain had no significant effect on the efflux of [^{3}H]TPP$^+$. In contrast, the addition of 2 mM veratrine caused a rapid decrease in [^{3}H]TPP$^+$ content similar to the effect observed when the cells were transferred to a buffer containing 135 mM K^+. The effect of veratridine was blocked by the prior addition of 5 μM tetrodotoxin (data not shown). Furthermore, ouabain has no significant effect

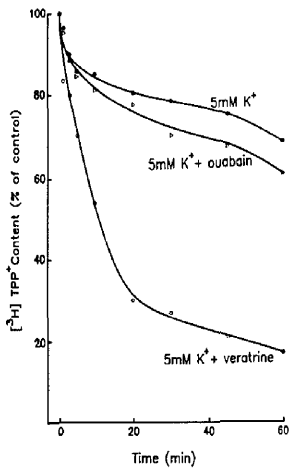

Fig. 5. Effect of ouabain and veratrine on [^{3}H]TPP$^+$ accumulation. Neuroblastoma cells were incubated with [^{3}H]TPP$^+$ for 20 min. Afterwards 2 mM ouabain or 2 mM veratrine was added to the cell suspension and [^{3}H]TPP$^+$ content determined during a 60 min incubation. The data are the mean of three separate determinations and is presented as a percentage of the [^{3}H]TPP$^+$ remaining in the cells following the initial 20 min incubation. The standard error was less than 15% of the mean value for each determination.

on [^{3}H]TPP$^+$ accumulation by the cells. Cells pretreated with buffer containing 5 mM K^+ with or without 2 mM ouabain for 30 min followed by the addition of [^{3}H]TPP$^+$ accumulated 26 500 and 23 650 cpm/10^6 cells, respectively.

myo-Inositol metabolism

To correlate changes in *myo*-inositol metabolism with the resting membrane potential cells were analyzed for *myo*-inositol and polyol content and extracellular *myo*-[2-^{3}H]inositol accumulation and incorporation into inositol phospholipids (Table II). The sorbitol and galactitol content is increased about 5-fold in cells exposed to media containing 30 mM glucose and about 30-fold in cells exposed to media containing 30 mM galactose, respectively, compared to cells grown in media containing 30 mM fructose. Supplementing media containing 30 mM glucose or 30 mM galactose with 0.4 mM sorbinil blocks polyol accumulation. The *myo*-inositol content of cells grown in media containing 30 mM glucose or 30 mM galactose is decreased about 60%, but is maintained at near control levels when the glucose or galactose supplemented media contains 0.4 mM sorbinil. Extracellular *myo*-[2-^{3}H]inositol accumulation and incorporation into inositol phospholipids, primarily phosphatidylinositol, is decreased 20–25% in cells exposed to media containing 30 mM glucose or 30 mM galactose compared to cells grown in media containing 30 mM fructose. *myo*-Inositol metabolism is maintained at near control levels when the glucose or galactose supplemented media contains 0.4 mM sorbinil.

Discussion

Decreases in nerve conduction velocity, axon transport and Na^+/K^+-ATPase activity have been documented in the diabetic nerve [1,10–12,25–29]. Furthermore, a decrease in Na^+/K^+-ATPase activity has been proposed to be a contributing factor to the development of diabetic neuropathy [8,9]. In diabetic animal models these disorders are improved by treatment with aldose reductase inhibitors or supplementing the diet with *myo*-inositol [1,11,12,25,26]. This suggests that an in-

TABLE II

myo-Inositol metabolism and myo-inositol and polyol content in neuroblastoma cells cultured in 30 mM glucose, 30 mM galactose or 30 mM fructose ± 0.4 mM sorbinil

Neurobalstoma cells were grown for 2–4 weeks in the supplemented medium. *myo*-Inositol accumulation and incorporation into phospholipid was determined following a 6-h incubation with *myo*-[2-^{3}H]inositol. Cells were also analyzed for the intracellular content of *myo*-inositol and polyols, sorbitol or galactitol following exposure of the cells to 30 mM glucose or 30 mM galactose, respectively. Each value is the mean ± S.E. of 17 separate determinations.

Determination	Media supplementation					
	fructose	glucose	galactose	fructose + sorbinil	glucose + sorbinil	galactose + sorbinil
myo-Inositol metabolism[a]						
Accumulation	232.0 ± 11.9	171.4 ± 15.2[c,d]	165.2 ± 6.1[c,e]	247.2 ± 19.7	221.0 ± 16.8	210.8 ± 8.2
Incorporation	165.5 ± 7.3	122.1 ± 11.9[c,d]	132.8 ± 5.0[c,e]	160.5 ± 10.9	173.1 ± 13.5	158.6 ± 6.0
Content[b]						
myo-Inositol	2.7 ± 0.3	1.0 ± 0.1[c,d]	1.1 ± 0.1[c,e]	3.0 ± 0.5	2.4 ± 0.4	2.2 ± 0.3
Polyol	2.5 ± 0.2	13.0 ± 1.7[c,d]	75.6 ± 13.6[c,e]	2.0 ± 0.2	3.1 ± 0.3	5.8 ± 0.4

[a] pmol/mg of protein.
[b] nmol/mg of protein.
[c] $P < 0.05$, compared to fructose supplemented medium.
[d] $P < 0.05$, compared to glucose + sorbinil supplemented medium.
[e] $P < 0.05$, compared to galactose + sorbinil supplemented medium.

crease in polyol levels and/or a decrease in *myo*-inositol content, metabolic derangements which occur in the diabetic peripheral nerve, may have a role in the development of these disorders. The hypothesis being examined in our studies is that high concentrations of glucose causes an increase in the sorbitol content and by yet an unexplained mechanism(s) is responsible for the decrease in *myo*-inositol levels [13,16]. Because the decrease in *myo*-inositol content is blocked by treatment with aldose reductase inhibitors we propose that sorbitol accumulation may be partially responsible for *myo*-inositol depletion. Our studies with neuroblastoma cells have indicated that accumulation of polyols by the cells is responsible for a non-competitive inhibition of *myo*-inositol transport and we have proposed that this causes a decrease in *myo*-inositol accumulation and content [13,15,16]. These results are supported by studies of Li et al. [30] using cultured bovine retinal capillary pericytes. However, it has also been shown that the presence of increased glucose concentration acutely decreases *myo*-inositol uptake by a competitive inhibition [2].

Exposing neuroblastoma cells to increased glucose or galactose concentrations for a minimum of two weeks causes a decrease in ouabain-sensitive Na^+/K^+-ATPase transport activity [14,15]. The decrease in Na^+/K^+ pump activity was accompanied by a decrease in *myo*-inositol metabolism and content and an increase in polyol levels. These effects were blocked by the addition of sorbinil to the supplemented media and is similar to results obtained from studies of Na^+/K^+-ATPase activity in diabetic peripheral nerves [26]. To further determine the effect metabolic derangements or decreased Na^+/K^+ pump activity may have on neural cell properties the resting membrane potential was examined. A decrease in the resting membrane potential would likely cause an alteration in the ability of a nerve cell to propagate a signal which could be partially responsible for the decrease in nerve conduction velocity. Exposing neuroblastoma cells to increased glucose or galactose concentrations caused a decrease in the resting membrane potential. This was accompanied by a decrease in *myo*-inositol metabolism and content and an increase in polyol levels. The addition of sorbinil to the glucose- or galactose-enriched media at a concentration that restored *myo*-inositol metabolism and content and blocked polyol accumulation improved the resting membrane potential. The resting membrane potential in neuroblastoma cells differentiated by DcAMP or phorbol ester was similar to the resting membrane potential of undifferentiated cells. Neurobiastoma cells differentiated by retinoic acid demonstrated a resting membrane potential which was less than the undifferentiated cells. The reason for the decrease in the resting membrane potential in neuroblastoma cells differentiated in retinoic acid is not known, however, these data indicate that the resting membrane potential of differentiated and undifferentiated cells is similar. These studies suggest that cultured neuroblastoma cells may be a useful cellular model system to evaluate changes which may occur in neural cells exposed to high ambient glucose concentrations.

The decrease in the resting membrane potential in cells cultured in the presence of media containing 30 mM glucose or 30 mM galactose could be partially due to the decrease in Na^+/K^+-ATPase transport activity [14,15]. To examine this we analyzed the effect of ouabain treatment on $[^3H]TPP^+$ accumulation by the cells. The addition of ouabain at a concentration which blocks Na^+/K^+-ATPase activity had little effect on the steady-state levels or accumulation of $[^3H]TPP^+$ by neuroblastoma cells. This suggests that the acute inhibition of Na^+/K^+ pump activity does not cause a decrease in the resting membrane potential in neuroblastoma cells. Therefore, the K^+ diffusion gradient which is almost totally responsible for the resting membrane potential is apparently maintained in the cells even when the Na^+/K^+ pump is acutely inactivated [20]. However, it is possible that chronic inhibition of Na^+/K^+-ATPase transport activity may contribute to the decrease in the resting membrane potential. Potential clamp analysis of myelinated nerve fibers isolated from the diabetic BB-Wistar rat showed that the fibers had a decreased Na^+ equilibrium potential, decreased Na^+ currents and an increase in K^+ permeability [31]. It was concluded from these studies that changes in Na^+ homeostasis precede changes related to the pathology of diabetic neuropathy. Furthermore, at physiological potassium concentrations the resting membrane potential in rat skeletal myotubules depends on the activity of the Na^+/K^+ pump [32].

Other changes besides a decrease in Na^+/K^+-ATPase activity may also contribute to decreasing the resting membrane potential. Because the resting membrane potential is maintained in cells exposed to media containing 30 mM glucose or 30 mM galactose when 0.4 mM sorbinil is added to the media it is possible that the metabolic derangements which occur in these cells are responsible for the decrease in the resting membrane potential. Perturbations of the plasma membrane could contribute to a decrease in the membrane resting potential. It has been shown that changes in the lipid content of plasma membranes of cultured neurons alters electrophysiology properties of the neuron [33,34]. In this regard inositol phospholipid content and metabolism is altered in diabetic peripheral nerves and neuroblastoma cells cultured in media containing 30 mM glucose or 30 mM galactose [13,14,16,35–39]. It is also possible that ion channel properties are altered in neural cells exposed to increased glucose or galactose levels. Ca^{2+} and K^+ channels in neuroblastoma cells are regulated by the phosphatidylinositol (PI) cycle and Greene et al. [8]

have postulated that phosphoinositide turnover is altered in the diabetic peripheral nerve [40–42]. This could cause an alteration in the production of the second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) which could alter Ca^{2+} homeostasis and the activity of protein kinase C (PKC). Ca^{2+} channels are regulated by PKC activity and Na^+ channels may also be regulated by phosphorylation [41,43]. Therefore, alterations in PI cycle activity could affect the regulation of ionic channels. In addition, Williamson et al. [44] have proposed that the NAD^+/NADH ratio is altered by high glucose concentrations. This could effect the homeostasis of the glycolytic pathway which could result in the alteration of Na^+ channels which have been shown to be regulated by metabolites of the glycolytic pathway [45]. Future studies will focus on determining the mechanism responsible for the decrease in the resting membrane potential. Currently whole cell patch clamp studies of the Na^+ and K^+ channels are being conducted with neuroblastoma cells chronically exposed to high glucose or galactose concentrations.

These studies show that the resting membrane potential of neuroblastoma cells is altered by chronic exposure to conditions which mimic the diabetic milieu. This alteration may be related to the metabolic derangement or chronic decrease in Na^+/K^+ pump activity which occurs when the cells are chronically exposed to media containing increased glucose or galactose concentrations.

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