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Trehalose Uptake through P2X₇ Purinergic Channels

Provides Dehydration Protection

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ABSTRACT

The tetra-anionic form of ATP (ATP^{4-}) is known to induce monovalent and divalent ion fluxes in cells that express purinergic P2X_7 receptors (Steinberg *et al.*, 1987; Sung *et al.*, 1985), and with sustained application of ATP it has been shown that dyes as large as 831 daltons can permeate the cell membrane (Steinberg *et al.*, 1987). The current study explores the kinetics of loading α,α -trehalose (342 daltons) into ATP stimulated J774.A1 cells, which are known to express the purinergic P2X_7 receptor (Steinberg *et al.*, 1987). Cells that were incubated at 37°C in a 50 mM phosphate buffer (pH 7.0) containing 225 mM trehalose and 5 mM ATP, were shown to load trehalose linearly over time. Concentrations of ~ 50 mM were reached within 90 min of incubation. Cells incubated in the same solution at 4°C loaded minimally, consistent with the inactivity of the receptor at low temperatures. However, extended incubation at 37°C (>60 min) resulted in zero next-day survival, with adverse effects appearing even with incubation periods as short as 30 min. By using a two-step protocol with a short time period at 37°C to allow pore formation, followed by an extended loading period on ice, cells could be loaded with up to 50 mM trehalose while maintaining good next day recovery ($49\% \pm 12\%$ by Trypan Blue exclusion, $56 \pm 20\%$ by Alamar BlueTM assay). Cells porated by this method and allowed an overnight recovery period exhibited improved dehydration tolerance suggesting a role for ATP poration in the anhydrous preservation of cells.

INTRODUCTION

A variety of organisms, such as encysted embryos of the brine shrimp *Artemia franciscana*, tardigrades, many seeds, bacteria and yeast cope with freezing and desiccation by producing disaccharides such as trehalose and sucrose (Crowe *et al.*, 1992; Crowe and Crowe, 2000). In naturally desiccation-tolerant organisms trehalose is thought to offer protection by several means. Because of the unique orientation of the hydroxyl groups on the trehalose molecule, structurally it can effectively replace water molecules that are fundamental to maintaining proper folding of proteins during dehydration and storage in the dry state (Crowe *et al.*, 1992). In a similar manner the trehalose hydroxyl groups can bind to phospholipid headgroups of the lipid bilayer, lowering the liquid-to-gel transition temperature and thereby preventing cytoplasmic leakage during rehydration (for review see Oliver *et al.*, 1998). This protection mode is referred to as the ‘water replacement hypothesis’ (Crowe, 1987). Additionally trehalose is known to form a glass at low water contents. The formation of a glass is thought to reduce the mobility of molecules and consequently the rates of deleterious chemical and physical interactions, thereby allowing cells to withstand sustained storage in the dry state (Sun and Leopold, 1997; Oliver *et al.*, 1998).

Recently there has been an increased interest in engineering mammalian cells to achieve desiccation tolerance to facilitate biopreservation in the dry state. Trehalose has been shown to effectively improve the survival of a number of organisms and structures following drying, including liposomes (Crowe *et al.*, 1987; Sun *et al.*, 1996), mammalian cell membranes (Chen *et al.*, 2001), bacteria (Conrad *et al.*, 2000; Israeli *et al.*, 1993; Leslie *et al.*, 1995; Potts, 1994), enzymes (Carpenter *et al.* 1987ab), retroviruses (Bieganski *et al.*, 1998), platelets (Wolkers *et al.*, 2001), human mesenchymal stem cells (Gordon *et al.*, 2001), and murine fibroblasts (Acker *et*

al., 2002). Trehalose has also been shown to improve the post-thaw viability of cryopreserved cells. The utility of trehalose as a cryoprotectant has been demonstrated for pancreatic islets (Beattie *et al.*, 1997), human oocytes (Eroglu *et al.*, 2002), as well as for fibroblasts and keratinocytes (Eroglu *et al.*, 2000).

Sugars do not easily penetrate mammalian cells unless specific proteins are present in the cell membrane to facilitate transport, such as is the case for glucose. Consequently a number of methods have been explored to introduce non-native sugars such as trehalose into mammalian cells. Beattie *et al.* (1997) exploited the thermotropic lipid phase transition event of the cell membrane to load trehalose intracellularly. Guo *et al.* (2000) transfected human embryonic kidney epithelial cells with genes that encode for trehalose synthesis. Puhlev *et al.* (2001) compared both of these methods to osmotic shock as a permeabilization method and determined that the cytotoxic effect of osmotic shock outweighed the benefits of using it as a delivery method. Toner and colleagues used a switchable engineered hemolysin-H5 pore to deliver trehalose intracellularly to a number of cell types including 3T3 fibroblasts and human keratinocytes (Russo *et al.*, 1997; Eroglu *et al.* 2000). Using the H5 technology it was possible to generate intracellular concentrations as high as 0.5 M within 60-min of exposure to an extracellular solution of the same trehalose molarity (Acker *et al.*, 2003). Eroglu *et al.* (2002, 2003) successfully used microinjection to infuse oocytes with trehalose. Various levels of endocytotic uptake of trehalose were reported by Hubel *et al.* (2002) depending on the cell type and incubation temperature. The maximum intracellular concentration was determined to be 100 mM in hepatocytes. Oliver *et al.* (2004) have reported that human mesenchymal stem cells can be loaded with trehalose by fluid phase endocytosis. In that study the intracellular localization of endocytosed trehalose was studied by fluorescence microscopy, using endocytosed Lucifer

Yellow as a substitute for trehalose. The data indicated that the endocytosed material was initially encapsulated within endosomes but that it left the vesicle and distributed throughout the cytosol over time. Intracellular trehalose concentrations were reported to be in the range of 10 - 20 mM.

The current work explores the activation of P2X₇ purinergic receptor channels to provide a means of loading trehalose into cells. The P2X₇ receptor belongs to a family of ATP-gated ionotropic receptors (North and Benard, 1997). These ATP-sensitive receptors are multimeric membrane proteins, with two trans-membrane regions. These receptors are predominantly expressed in antigen-expressing immune cells and epithelia (Virginio *et al*, 1999, Coutinho-Silva *et al*, 1999), as well as cells of hematopoietic origin (Gudipaty *et al.*, 2001). For a review of cell types expressing purinergic receptors see Dubyak and El-Moatassim (1993) and Collo *et al.* (1997).

The tetra-anionic form of ATP (ATP⁴⁻) is known to induce monovalent and divalent ion fluxes and membrane depolarization in cells that express purinergic receptors, in a dose-dependent manner (Steinberg *et al*, 1987; Sung *et al*, 1985, Rassendren *et. al*, 1997). The membrane depolarizing effect of ATP is very specific. Other analogs of ATP such as ADP, AMP, GTP, and numerous others have been shown to have no effect on membrane potential (Sung *et al*, 1985). With sustained or repeated application of ATP the ion channel dilates and forms a large non-selective pore. It has been shown that dyes as large as 831 da can pass through these channels (Steinberg *et al*, 1987) and that membrane-impermeant metabolic effectors can be loaded in this manner as well (Menze *et al*, 2005). It was therefore hypothesized that trehalose could be effectively delivered into the intracellular space using these channels as a gateway.

The time course of trehalose uptake following ATP stimulation of J774.A1 mouse macrophages was explored in this study. Intracellular trehalose concentrations of ~50 mM could be achieved with acceptable survival levels, without compromising long-term survival and growth potential. Cells that had been porated and loaded with trehalose were also tested for desiccation tolerance over a range of final moisture contents. Next day survival of cells was better for trehalose loaded cells at all levels of desiccation.

MATERIALS AND METHODS

Cell Culture

J774.A1 mouse macrophage cells were obtained from American Type Culture Collection (Manassas, VA). An ATP-resistant subclone of mouse macrophage cells (ATP-R) derived from J774.A1 cells were a generous gift from Thomas Steinberg (Washington University, St. Louis, MO); these cells express greatly reduced levels of the functional P2X₇ receptor channel. All cells were maintained as a suspension in spinner flasks with Dulbecco's Minimum Essential Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT) and penicillin (100U/mL)–streptomycin(100ug/mL) (Invitrogen). Cells were maintained at a density of less than 1×10^6 cells / mL.

Trehalose Loading by ATP Dependent Channel

Cells were collected from spinner flasks, washed once with phosphate buffered saline (PBS) (Invitrogen), and counted using a Z1 Cell and Particle Counter (Beckman-Coulter, Miami, FL). Cells were pelleted and resuspended in an intracellular-like poration buffer solution containing 250 mM trehalose, 1 mM MgSO₄, 5 mM glucose, 1X MEM Vitamin Solution (Invitrogen,

Carlsbad, CA), and 1 X MEM Non-Essential Amino Acids Solution (Invitrogen, Carlsbad, CA) buffered to pH 7.0 in 50 mM potassium phosphate buffer ($\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$) (Menze et al, 2005). To each sample the appropriate amount of stock 50 mM ATP (Sigma, St. Louis, MO) in PBS pH 7.0 was added to yield a final concentration of 5 mM ATP. This resulted in a final extracellular trehalose concentration of 225 mM. In control non-porated samples, the equivalent amount of PBS pH 7.0 was added to each sample instead of ATP solution. Cells were porated at a final density of 4×10^6 cells/mL. Two basic poration and loading schemes were utilized. In the isothermal scheme, cells were suspended in poration buffer supplemented with ATP (trehalose concentration: 225 mM) and then incubated at 37 °C in a water bath or on ice, for the prescribed amount of time. For studies involving 500 mM extracellular trehalose, the appropriate amount of 1 M trehalose stock solution was added together with ATP to bring the final concentration to 500 mM. In the two-step protocol, the samples were incubated with ATP supplemented poration buffer (trehalose concentration: 225 mM) in a 37 °C water bath for 5, 10, or 15 min and then moved to ice for the duration of loading. For two-step studies involving 500 mM extracellular trehalose, the appropriate aliquot of 1 M trehalose solution in poration buffer containing 5 mM ATP was added to each sample at the end of the poration period to adjust the concentration up to 500 mM, prior to incubation on ice.

HPLC quantification of Trehalose Uptake

The time course of trehalose uptake was followed by sampling from a 6-8 mL volume of cell suspension in ATP-containing poration buffer. Aliquots of 1 mL were removed from the primary volume and diluted in 10 mL of DMEM supplemented with 1 mM MgCl_2 at each time of interest. The samples were incubated at room temperature for 5 min to achieve pore closure

and then washed three times with PBS (10 mL volumes). The cells were centrifuged after every wash. This aggressive washing regimen was intended to remove extracellular trehalose as well as the cellular debris resulting from any cell lysis and damage that occurred during poration. Because cells are very fragile after poration and loading, this washing procedure was not used during routine trehalose loading. To investigate whether the trehalose would persist after an overnight recovery period, an extra set of samples (10 min incubation at 37°C, followed by 90 min incubation on ice) were returned to overnight culture and then analyzed exactly the same way after this recovery period. In preparation for intracellular trehalose extraction, the final cell pellet was resuspended in HPLC grade H₂O (Sigma), and transferred to a 1.5 mL Eppendorf tube for frozen storage at -20 °C. At the time of analysis the samples were heated for 20 min in a 95 °C hot block (VWR Scientific). After 10 min of heating, the caps were opened momentarily to release the gases expelled from the hot sample. The cell lysate was then centrifuged at 15,000 g for 10 min to pellet the cellular debris and denatured protein. The sugar-containing supernatant was filtered through a 13 mm 0.2 µm PTFE syringe filter (Fisherbrand) into 2 ml HPLC vials, and the remaining pellet used for total protein analysis. Carbohydrate peaks were separated using an Agilent 1100 Series HPLC with a Hamilton RCX10 column operating at 35 °C. The mobile phase was 100 mM NaOH, operating at a flow rate of 0.75 mL/min. The eluted peaks were quantified using pulsed amperometric detection (ESA Coulochem II, Cambridge, MA), with pulse characteristics as follows: E1=200 mV, t1=500 ms, E2=-2000mV, t2=10 ms, and E3=600 mV, t3=10 ms. Three-point trehalose standard curves were generated for every 8 samples injected.

The total protein in each HPLC sample was determined by Coomassie Plus Protein Assay (Pierce, Rockford, IL) per manufacturer's instructions for 96-well method. Optical density (570

nm) was measured on ThermoMax Microplate Reader (Molecular Devices, Sunnyvale, CA). Trehalose concentrations were normalized to total cell protein as determined per the Coomassie assay. A standard curve of total cellular protein as a function of cell number was determined for J774 cells, to allow a calculation of moles of trehalose per cell. Any data points falling outside of the linear range of the protein assay were discarded and re-analyzed at the appropriate dilution.

Cellular Water Content. To calculate the intracellular concentration of trehalose, it was necessary to estimate cell volume, as well as determine the free water content of the cell accessible to the loaded sugar. To determine the osmotically active fraction of cell water, cell volumes were measured as a function of extracellular osmolality. Solutions of varying tonicity (300 - 880 mOsm) were prepared by dilution of 10 x PBS solution with water. The osmolality of the final solution was measured in triplicate using a microOsmette osmometer (Precision Systems, Natick, MA). Cells were incubated for 15 min in the various PBS solutions and then the mean cell volume was obtained using the cell and particle counter described previously. Prior to each data acquisition the coulter counter system and aperture were filled with PBS solution of appropriate osmolality and the aperture re-calibrated using latex beads of 10 μm diameter. All measurements were made at room temperature.

Cell Viability Assays. After ATP poration, cells were transferred to spinner flasks for suspension culture. After culture for 0, 24, 48 and 72 hrs, aliquots of cell suspension were taken from the flask and membrane integrity was assessed by trypan blue exclusion (0.4% solution, Sigma). The number of viable cells was determined by counting with a hemocytometer. The immediate cell

survival after poration was normalized to that of the non-treated control. For 24, 48, and 72 h time periods the total number of viable cells in each sample was normalized to the corresponding original plated viable cell numbers. AlamarBlue™ (BioSource International Inc., Camarillo, CA) was also used to monitor the metabolic activity of the cells (Menze et al, 2005). Briefly, AlamarBlue™ stock solution was added to a final concentration of 10 % (v/v) in 2 ml of standard culture medium containing equal numbers ~~the same amount~~ of viable cells (between 20,000 to 40,000 cells per well) in a 12-well multidish (Fisher Scientific, Pittsburg, PA). The change in the absorbance at $\lambda = 570$ nm and $\lambda = 600$ nm was measured with a microplate spectrophotometer (Benchmark Plus™, BIO-RAD, Hercules, California) at various time intervals. The percent of reduced alamarBlue™ (RA %) was calculated as: $RA \% = [A_{570} - A_{600} \cdot Ro] \cdot 100$, where A_{570} and A_{600} are the wavelength-specific absorbances after subtracting the absorbance of standard culture medium without alamarBlue™. Ro is the ratio of A_{570}/A_{600} of standard medium containing 10 % alamarBlue™. The data was normalized to that of corresponding non-treated control cells.

Drying Studies. After trehalose was loaded via the P2X₇ receptor channel, cells were tested for improved desiccation tolerance. Prior to drying, cells were incubated at 37°C for 10 min in poration buffer containing 225 mM trehalose. Cells in suspension were then transferred to an ice bath for 90 min. Cells were then diluted into CO₂-equilibrated culture medium without centrifugation and given an 18-h recovery period in spinner culture. Control J774 cells received no poration treatment prior to drying. Immediately prior to desiccation experiments, cells were pelleted by centrifugation and re-suspended in either isotonic RPMI (1 part RPMI and 2 parts H₂O) containing 200 mM trehalose or in an intracellular-like buffer of similar composition as the

loading buffer (50 mM K_2HPO_4/KH_2PO_4 , 250 mM trehalose, 1 mM $MgSO_4$, 5 mM glucose, 1x MEM vitamin solution and essential amino acids, 0.5 mM alpha-lipoic acid, 10 mM NaCl, 0.1 mM EDTA, 10 mM pyruvate, pH 7.0, ~365 mOsm). Cells were washed one time in this buffer (10 mL aliquot) prior to re-suspension of the cells at the appropriate dilution for drying studies. Ten 15 μ L droplets of cell suspension were pipetted onto 35 mm tissue culture dishes and placed in a desiccation cabinet containing Drierite desiccant (W.A. Drierite Co., Xenia, OH) at ambient temperature (20 – 25 °C). Samples were dried to a range of final moisture contents, as determined gravimetrically. Water content was expressed as grams water per gram of dry mass. Sample dry mass was determined by drying parallel samples for 12 h at 60 °C. Upon reaching the target moisture content, samples were then immediately rehydrated with excess DMEM containing 10% FBS and penicillin/streptomycin and placed in a 10 % CO_2 incubator at 37 °C overnight. To quantify dehydration tolerance, cells were scored for Trypan Blue exclusion after the poration recovery period just prior to drying, and again after rehydration and overnight recovery. Cell counts after drying and recovery were normalized to cell counts immediately prior to drying.

Statistical Analysis. All trehalose loading experiments were repeated four to six times, using a minimum of three different batches of cells (i.e., different culture dates and passage levels). The data were analyzed using Statgraphics *Plus* 5.1 (Rockville, ML). Differences between groups were analyzed by ANOVA using the Tukey test for significant differences. When groups contained unequal numbers of observations, the Bonferroni test of significance was used. Significance was evaluated at both the 95 and 99 % level and reported accordingly. Slope analyses for the data in Figure 6 were performed following the procedures of Zar (1999).

RESULTS

Cellular Volume and Osmotically Active Water. To estimate the average volume of cells under physiologic conditions as well as determine the osmotically active component of the cell volume, the volume of J774 cells was measured in PBS solutions of various osmolalities. A classic Boyle van't Hoff plot was generated by plotting cell volume as a function of inverse osmolality, as is shown in Figure 1. The measured cell volume in each hyper- or hypotonic solution was normalized to the volume measured at 342.5 mOsm, as this was the average osmolality of the full complement DMEM solution in which the cells were routinely cultured. The volume at this osmolality was 1742 ± 358 fL (AVE \pm SD, n = 5). This value was also used in subsequent calculations of intracellular molarity to represent the normal physiological volume of the cell. A linear curve fit of the data revealed an intercept of 0.317, which corresponds to the osmotically inactive fraction of the cell volume. Conversely, 1190 fL of the total cell volume were considered osmotically active water and available in a volumetric sense for loaded trehalose.

Trehalose Quantification. HPLC sample peaks were integrated against trehalose standard concentration curves, yielding the total mass of trehalose in the cell lysate. Total protein in the same lysate was measured per the Coomassie protein assay. Using a calibration curve of cell number versus total cell protein (data not shown) the mass of trehalose measured per cell was computed. The conversion to intracellular molarity was achieved by utilizing the average volume of osmotically active water per cell, as determined in prior experiments. It should be noted that by normalizing to the cellular protein in the final extract, the trehalose signals reported are expected to represent only the healthy portion of the cell population that survived both the poration and the aggressive washing protocol. Trehalose that had loaded into cells that

subsequently lysed during the poration period or during the washing steps would have been washed out prior to preparation of the final cell extract. The final cell wash prior to suspension in water was also analysed for trehalose content to rule out sample contamination from extracellular trehalose. Typical chromatograms of the sample and wash solutions are shown in Figure 2, along with a trehalose standard chromatogram. The average trehalose concentration in the final PBS wash solution was $0.40 \pm 0.07 \mu\text{g/ml}$ (mean \pm SEM, $n = 42$), or approximately $1.2 \mu\text{M}$. Assuming a conservative final dilution in water of 1:10 (100- μl residual PBS wash solution in 1 mL final suspension volume), this procedure yielded a signal-to-noise ratio greater than 400 for all samples analyzed in this study.

Isothermal Trehalose Loading Protocol. The kinetics of trehalose loading at 37°C in porated cells and control cells is shown in Figure 3~~7~~ for both the J774 cell line and the J774.ATPR cell line. After 90 min of ATP stimulation, the intracellular trehalose concentration reached a level of $48 \pm 2 \text{ mM}$ (mean \pm SEM). This value represents a loading efficiency (intracellular versus extracellular concentration) of 21 %. The rate of uptake was linear throughout the duration of the experiment, ($R^2 = 0.996$). Conversely, untreated J774 cells and the treated and untreated ATP-resistant J774 cells loaded very slowly and non-linearly, achieving intracellular molarities of $< 25 \text{ mM}$ in the same time period. The difference between stimulated and non-stimulated J774 cells was statistically significant at the 99 % confidence level, whereas a significant difference between ATP-stimulated and non-stimulated J774.ATPR cells was not observed.

Two-Step Loading Protocol. In an effort to minimize the adverse effects of extended poration on next day survival (discussed in next section), while still promoting the loading of ~~being able~~ trehalose to levels useful for biopreservation, a two-step poration scheme was investigated.

Because pore formation is temperature-dependent and optimal at 37 °C, J774 cells in poration buffer containing 5 mM ATP and 225 mM trehalose were placed at 37 °C for a period of 5, 10 or 15-min. The samples were then placed on ice (0 °C) to investigate whether the pore would remain open and allow trehalose loading to proceed. As a control, cells in an identical solution were placed on ice, without the preliminary period at 37 °C to promote pore opening. The results obtained using this loading protocol are shown in Figure 4(A). ATP-stimulated cells that had been incubated for 15 min at 37 °C and then moved to an ice bath (2-step scheme) continued to load trehalose at a rate comparable to the loading rate at 37 °C (isothermal scheme). The difference between ATP-stimulated and non-stimulated J774 cells was significant at the 99 % confidence level for all incubation times at 37°C (5, 10, and 15 min). Trehalose levels in samples that were returned to overnight culture prior to analysis were statistically the same ($p < 0.01$) as samples that were not cultured overnight but analyzed immediately after the poration time period (data not shown). When a 5 min incubation at 37 °C was used, the trehalose-loading appeared to be slightly less efficient when compared to the 10 or 15 min pre-incubation period, perhaps due to a reduction in the total number of pores that formed within this shorter time period.

The loading results of J774 cells incubated on ice without a preliminary incubation step at 37 °C are shown in Figure 4(B). These cells loaded very slowly and nonlinearly, reaching a concentration < 15 mM at 90-min, consistent with previous negative controls without ATP for pore activation. A small but statistically significant difference was noted between stimulated and non-stimulated ATP resistant cells ($p < 0.01$). The effect of 500 mM extracellular trehalose on the intracellular accumulation of trehalose was also investigated. At the end of the 15-min pore formation step in poration buffer containing 225 mM trehalose, the trehalose concentration was

increased to 500 mM, and the samples were then moved to the ice bath. For comparison, J774 cells were also incubated at 37 °C in poration buffer containing 5 mM ATP and 500 mM trehalose, with no change in incubation temperature. As can be seen in Figure 5, the loading at 4 °C (2-step) is comparable to that seen at 37 °C (isothermal), resulting in equivalent amounts of intracellular trehalose at 60 and 90-min.

General observations on next day survival following poration revealed that cell losses were high when the poration time at 37 °C exceeded 15 min in both 225 mM and 500 mM trehalose solutions. Next day survival was generally better when the osmotic stress was reduced (i.e. 225 mM solutions were better than 500 mM solutions at equivalent incubation times). Based on these loading results and general viability observations, specific poration and loading protocols were further evaluated for poration toxicity and their ability to confer desiccation protection to J774 cells.

Poration and Cell Survivorship. The effect of poration time on next day survival was determined by monitoring cells that were returned to overnight culture following different periods of ATP exposure at 37°C and on ice. As can be seen from Table 1, the membrane integrity of cells was high immediately after poration and loading for all of the treatments investigated. Survival studies revealed that although a 15 minute poration period at 37°C yielded good next day survival (59%), when a 60 or 120 min loading period on ice was added, the next day survival of cells diminished to 24 and 11%, respectively. Increasing the poration time to 30 min at 37°C resulted in the loss of ~90% of cells, and the small proportion of cells that remained demonstrated no growth potential by 48 h. Reducing the poration time at 37°C to 5 or 10 min gave better overall survival results even with extended time on ice. For example, the membrane

integrity results revealed that a 10 min poration period at 37 °C followed by 60 or 120 min on ice resulted in survival of approximately half of the cell population, with the surviving cells retaining their proliferative potential, as evidenced by a doubling time similar to control. The consistent rate of Alamar BlueTM reduction compared to control over extended time periods (Table 2) suggested that the surviving fraction 24 h following poration retained its metabolic and proliferative potential. These survival and metabolic data are consistent with those reported in Menze *et al* (2004) for comparable loading conditions.

Dehydration Tolerance of Trehalose-Loaded Cells. The dehydration tolerance of trehalose-loaded cells was investigated using the two-step method of poration and loading (10 min of poration at 37 °C followed by 90 min of loading at 4 °C). The effect of drying on cell viability, as measured by the membrane integrity dye trypan blue, is shown in Figure 6. Untreated cells and cells that had been loaded with trehalose via the P2X₇ receptor channel were dried to a range of moisture contents in a desiccation cabinet (0.75-3.5 gH₂O/gdw). Untreated cells were dried either in an isotonic RPMI-based trehalose containing buffer or an intracellular-like high potassium buffer (described in Materials and Methods). Because the intracellular-like buffer gave better overall survival in controls, trehalose-loaded cells were dried exclusively in this buffer. Cells that were dried immediately after trehalose loading, i.e., without a recovery period, exhibited poor survivorship that was even lower than non-porated cells (data not shown). However, adding a recovery period of 18-h improved the survivorship over non-manipulated cells. The slopes of all three lines in Fig. 6 were statistically different from one another (P<0.05) ATP-poration using the 2-step poration and loading scheme, followed by an 18-h recovery phase, significantly improved dehydration tolerance as compared to controls.

DISCUSSION

The experiments performed in this work indicate that ATP stimulation of J774 cells to reversibly permeabilize the cell membrane is an effective means to load trehalose intracellularly. The poration medium used in these experiments was optimized to produce the best next-day survival of porated cells, the details of which are presented in Menze *et al.* 2004. In the present paper, the primary efforts were to maximize the overall loading efficacy and final intracellular concentration of trehalose, without sacrifice of next day cell survival. The optimized loading strategy involved reduction of the incubation or loading temperature to 4 °C after the initial pore formation period at 37 °C. This modification allowed the loading period to be extended to several hours before irreversible cell disruption occurred, approximately a 6-fold improvement over the non-toxic loading window of 15-min at 37 °C. This step was crucial to achieve enough intracellular trehalose to be practical for cell preservation.

A number of factors and assumptions go into determining an estimate of intracellular trehalose concentration. Various investigators normalize the raw sugar signal in different ways. In the current work, the osmotically inactive volume was determined for the particular cell type in question in order to understand the water volume that was available to solvate the loaded sugar. This approach was also used by Acker *et al* (2003) for an equilibrium loading case with good success, thereby suggesting overall validity for this method of normalization. An intermediate step between normalizing the HPLC signal to cell volume, is the determination of the amount of trehalose per cell. Because sampling error is inherent in any aliquot-based experiment despite efforts to homogenize samples, it was essential to determine the cell number in each extract prepared for sugar analysis. This was done by analyzing the total protein in each sample and estimating the cell number from a standard curve.

Because some of the poration protocols utilized in this study are known to induce cellular damage, the question can be raised as to whether the intracellular trehalose concentrations reported truly reflect sugar levels in reversibly porated cells or instead originate from cells that internalize trehalose because they have become leaky and damaged. A number of arguments can be put forward that rule out the latter scenario. The isothermal 37 °C loading data will be used as an example, since this protocol induced considerable cell damage very early in the trehalose loading process. Although identical volumes of cell suspension (1 ml) were removed at each poration endpoint, the total cell protein in the final extract was found to decrease with increasing poration time. The results of the total cell protein analysis for this data set were consistent with the observed pattern of cell viability following poration, i.e. a declining cell number with increasing poration time. If the membrane-compromised cells were not adequately removed from the population by the washing steps, then this population would contribute to the total cell protein signal determined for the sample and one would not expect to see any decrease in cell protein signals over time. The observation of protein loss over time, though not an independent indicator of cell death, does support the notion that only healthy cells are included in the final extract for sugar analysis.

Further evidence supporting the validity of the intracellular trehalose concentration estimates can be found in the raw HPLC signals. If trehalose was loading into an increasingly irreversibly permeated population of cells, one would expect the raw HPLC signals from the identical volume aliquots to increase with time, in a manner consistent with the observed cell degradation (i.e. exponentially). An examination of the raw HPLC signals for the isothermal loading data revealed no such pattern, and within the range of sampling error, the signals generally increased initially (consistent with loading into all cells), and then decreased over time

(consistent with loss of part of the population). When the raw signals were corrected for cell number using the measured protein data, a highly linear pattern of trehalose uptake was observed over the 90 minute time period (correlation coefficient = 0.998), consistent with progressive loading into a healthy population of cells.

Additional evidence that the extracts are populated by only healthy porated cells is demonstrated in the mathematical consistency of data from experiments involving different extracellular conditions. Cells porated in 500 mM trehalose versus 225 mM trehalose reveal a ~2.2-fold difference in intracellular concentration at all time points, consistent with simple diffusion through a uniform population of non-selective pores.

Finally, trehalose quantification was performed on samples immediately after poration and after they were returned to overnight culture. No statistical difference could be resolved between these groups. If only dead cells were loading trehalose, the overnight recovery period in trehalose-free culture media would result in leakage of trehalose from the dead cells and therefore a change in the measured intracellular trehalose levels. This was not observed.

It is both possible and likely that a small percentage of cells that have not undergone cell lysis during poration and processing but that eventually go into an apoptosis cascade, are included in the population analyzed for sugar content. It is also equally likely that a small percentage of cells that are sub-lethally damaged are also removed from the sample due to the robustness of the washing steps. Because both of these effects are expected to be minimal and to contribute oppositely to the reported trehalose concentration, it is believed that the estimated intracellular trehalose concentrations are both relatively and absolutely valid.

Activation of native P2X₇ receptors to load sugars or other compounds to protect against freezing or desiccation damage is a novel preservation methodology. Consequently, there is very

little literature relevant to the kinetics and toxicity of loading millimolar amounts of sugars into cells. Studies of various aspects of pore formation have been examined by others by studying the loading fluorescent dyes and other markers of cellular influx (Bennett et al., 1981; Steinberg, 1987, Virginio *et al.*, 1999). Many of these dyes were of similar size as glucopyranosyl-based mono- and di-saccharides (170-342 daltons). Virginio *et al.* (1999) have examined the kinetics of YOPRO fluorescence dye uptake as a function of the constitution of the external milieu in human embryonic kidney (HEK293) cells expressing the P2X₇ receptor. They found rapid dye uptake (detectable cellular fluorescence within seconds) with only a 5-s application of agonist. The uptake rate was higher in low divalent ion solution, such as was used in the current work. However, these experiments were performed with 2'3'-O-(benzoyl-4-benzoyl)-ATP (BzATP), a more potent agonist than ATP, making it difficult to extrapolate results to the current experiments. Gudipaty et al. (2001) demonstrated that P2X₇ receptor function in human monocytes is very sensitive to the ions that are present extracellularly. They found that replacement of extracellular Na⁺ and Cl⁻ with K⁺ and non-halide anions facilitated ATP-dependent poration, as well as increased the ATP affinity of the receptors. For these reasons potassium phosphate buffer was chosen as the base buffer for the poration medium used in this study (Menze, 2004).

In the current experiments uptake of trehalose was shown to be substantial in J774 cells even without the presence of ATP in the incubation medium. The ATP experiments performed at 4 °C with normal J774 cells and the experiments performed at 37 °C incubation with J774 ATP-resistant cells show very similar uptake patterns, suggesting that the baseline trehalose uptake in the absence of ATP-induced pore formation is not temperature dependent. Because functional pores are thought to be absent in both of these cases, the similarity of the response

curves is expected. However, the magnitude of uptake was somewhat unexpected. Contamination from residual extracellular trehalose was ruled out by examining the level of trehalose in the final wash solutions. Although detectable amounts of trehalose were present, the magnitude was such that it was expected to contribute less than 0.25% to the intracellular signal. It is possible that a fluid phase endocytosis mechanism is responsible for the baseline level of uptake observed in J774 cells.

The goal of the current work was to take advantage of non-selective endogenous pore formation to load trehalose intracellularly while retaining good long-term cell viability. It is well recognized that ATP can induce or potentiate apoptosis in cells expressing P2X₇ receptors and that prolonged exposure to ATP will ultimately result in cell death (Coutino-Silva *et al.*, 1999). However, there are a number of mediating factors that play a role in modulating the sensitivity of cells to ATP-induced cytolysis. Steinberg *et al.*, (1987) found that the dose dependency of poration agonists was not directly correlated with apoptosis sensitivity. It is also clear that permeability precedes the first stages of any observed apoptotic cascade (Zoetewij *et al.*, 1996, and Coutino-Silva *et al.*, 1999). Coutino-Silva *et al.* (1999) indicated that at least 10 min of exposure at 37 °C was necessary to cause the earliest stages of apoptosis. In contrast, permeabilization occurs almost immediately after exposure. This suggests that even if apoptosis is triggered by poration, there is the potential to intervene at the earliest stages to reverse the process. The time-temperature-concentration history necessary to induce apoptosis or necrosis, is not known, and the nature of the damage as well as the sensitivity to these variables is cell-type dependent (Zanovello, 1990). Data acquired in this study indicates that temperature can also play a key role in preventing damage associated with permeabilization.

The ionic environment of the cell can also play a very significant role in the transport of species across the cell membrane, as well as in mediating cytolytic events. Virginio *et al.* (1999) measured the time course of ionic currents as a function of the constitution of the external milieu in human embryonic kidney (HEK293) cells expressing the P2X₇ receptor. In patch clamp experiments with various ionic constitutions, they demonstrated that extracellular sodium promotes cell disruption, whereas intracellular sodium retards it. In this work extracellular sodium was minimized to maintain the highest level of cell integrity.

The improvement in cell viability that results from carrying out the ATP exposure at lower temperature is consistent with the current understanding of cellular metabolism and simple diffusion. The Q₁₀ value (defined as the factor by which the rate changes following a change of 10 °C in temperature) is of order 2 for most enzymatic reactions, including those involved in the degradative processes of cell injury and the functioning of ion pumps (Schmidt-Nielson, 1997). A change in temperature from 37 °C to 4 °C therefore results in ~10-fold reduction in the rates of many cellular reactions. In contrast, the temperature sensitivity of the coefficient of mass diffusion is very low, varying less than 2-fold over the same temperature range. A temperature drop from 37 °C to 4 °C would therefore result in a minimal loss of trehalose loading ability, as was observed in this study, while potentially preventing some cellular degradative processes from proceeding, a result that was also observed in the current study.

Although the physiologic role of the P2X₇ receptor has not been fully characterized in any cell type or species, this receptor is thought to play a role in the inflammatory response. The levels of ATP required to activate receptors (mM range) are not normal physiologic levels, but can be generated at the site of injury where cytoplasmic ATP can leak into the extracellular space. Some have hypothesized that the P2X₇ receptor acts as cytolytic pore with the primary

purpose being to elicit cell death in the activated molecule, however later studies have suggested its role was much more elaborate, facilitating cell-cell communication (Falzoni, 1995).

The current work has provided evidence that ATP-induced cell permeabilization of P2X₇ expressing cell types can load high levels of intracellular trehalose (up to 130mM) in a manner that provides dehydration tolerance to cells, consistent with studies that have delivered trehalose into the intracellular space by other methods. The ATP poration technique does not involve genetic manipulation of cells or the insertion of foreign materials into the cell membrane, and is therefore believed to have certain practical benefits over other loading techniques. Also, the sugar loaded via these non-selective pores is believed to be uniformly distributed throughout the cytoplasm (Coutinho-Silva, 1999), making it fully accessible to protect membranes, cellular proteins, and cell organelles.

In this work, it was found that stimulation of J774 cells with ATP could induce the formation of pores that were permeable to trehalose, presumably by activating P2X₇ receptors. Decreasing the incubation temperature during the exposure period to ATP made it possible to extend the exposure time to ATP prior to onset of cytolysis, with negligible impact on the loading efficiency of trehalose. Furthermore, it was shown that under optimized loading conditions, the levels of intracellular trehalose that were achieved were adequate to confer improved dehydration tolerance to J774 macrophages. The findings in this work demonstrate the overall utility of using ATP stimulation of native P2X₇ receptors to load trehalose and other protectants that otherwise cannot effectively permeate the cellular membrane. Further work is needed to look at long term survivability and function of cells that have been porated using ATP stimulation of P2X₇ receptors, as well as the long-term functionality of cells dried to various critical moisture levels.

CONCLUSIONS

The time course of trehalose uptake following ATP stimulation of J774.A1 mouse macrophages was explored in this study. The pattern of trehalose uptake revealed that although it was necessary to maintain samples at 37 °C for a short period of time (~5 min) during ATP exposure to achieve formation of a non-selective pore, the pore appeared to remain open when the sample was moved to ice. This was a critical finding in that the next day recovery of cells that were porated and exposed to trehalose at 37 °C for extended periods of time (>30-min) resulted in zero next-day survival of cells. By using a two-step protocol that involved a short time period at 37 °C to allow pore formation, and then continuing the incubation period on ice, trehalose loading could progress for up to 2 h before the next day survivability was significantly affected. This low temperature incubation allowed a 6-fold increase in the amount of trehalose that could be loaded before survival was adversely affected, compared to isothermal 37°C incubation. Cells that had been ATP-porated and loaded with trehalose were also tested for dehydration tolerance over a range of final moisture contents. Next day survival of cells was better for trehalose-loaded cells at all levels of dehydration.

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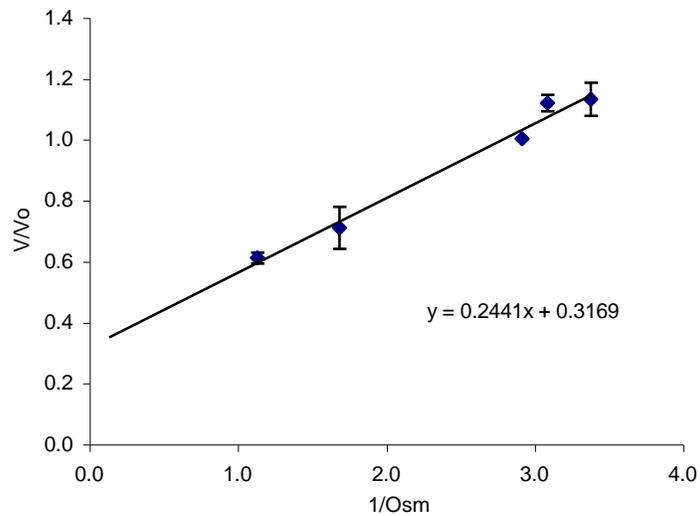


Figure 1. Normalized cell volume as a function of inverse extracellular osmolality. Cells were suspended in sodium chloride solutions of varying osmolality and allowed to equilibrate volumetrically for 15 min. The cell volume was normalized to the isotonic volume and plotted as a function of inverse osmolality. The intercept represents the osmotically inactive fraction of the cell volume (n=3).

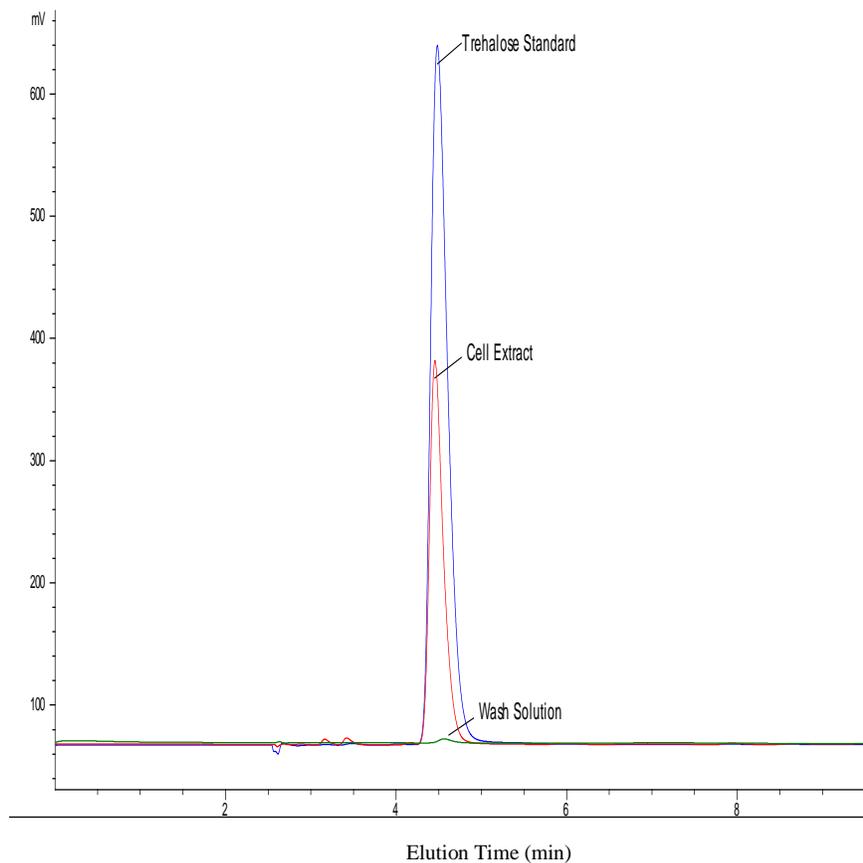


Figure 2. Characteristic HPLC chromatograms of sample, standard, and final PBS wash solutions. Based on signals from final wash solutions, residual extracellular trehalose was found to contribute insignificantly to the signals recorded for cell extracts. Signal-to-noise was >400 .

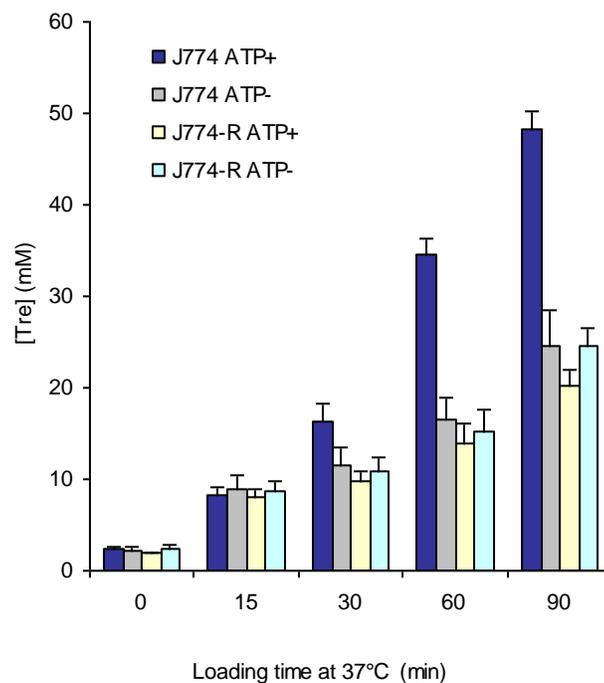


Figure 3. Time course of intracellular trehalose uptake in J774 and J774.R cells under isothermal conditions (37 °C). Cells were incubated in an intracellular-like trehalose buffer containing 225 mM trehalose. ATP+ cells were exposed to 5 mM ATP. ATP- samples were exposed to an equivalent volume of PBS. The J774.R cell line is a subclone of the J774 line that is resistant to ATP-poration and expresses greatly reduced levels of the functional P2X₇ receptor channel. ATP stimulated J774 cells showed a significantly higher amount of intracellular trehalose compared to non-treated controls (mean ± SEM; p<0.01). Stimulated and non-

stimulated J774.R cells had equivalent amounts of intracellular trehalose, consistent with the absence of pore formation.

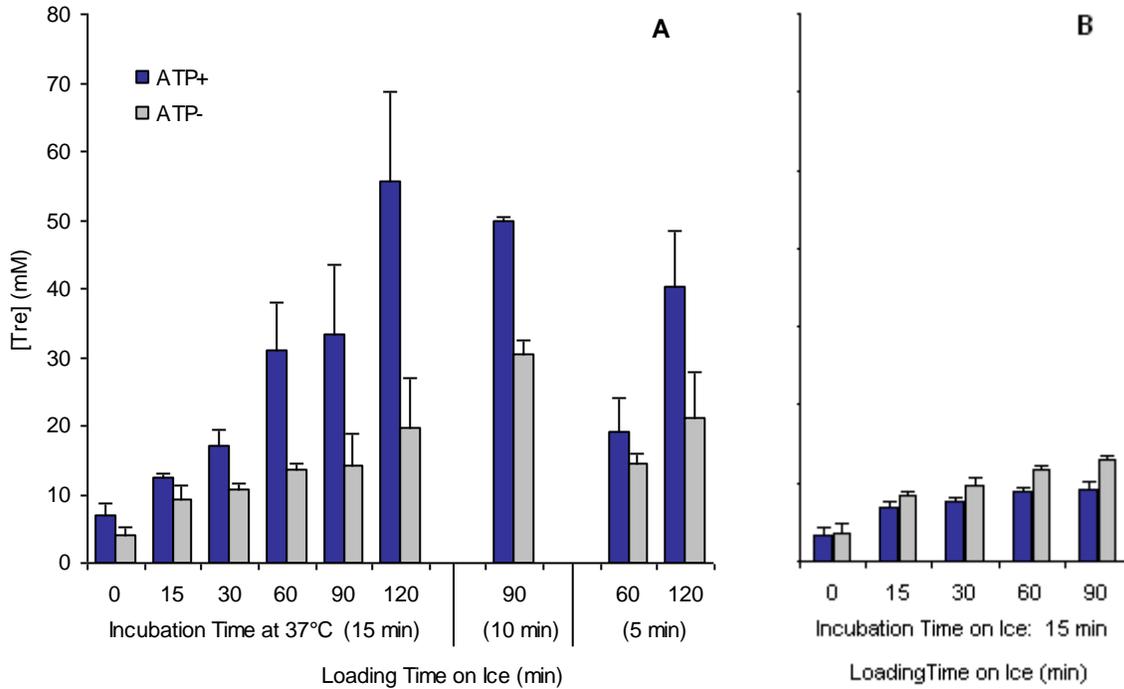


Figure 4. Time course of intracellular trehalose uptake in J774 cells using a 2-step loading procedure with 225 mM extracellular trehalose. Cells were incubated in an intracellular-like trehalose buffer containing 225 mM trehalose for various times (A) at 37°C to open the P2X₇ receptor channel, followed time periods on ice for additional loading, or (B) on ice alone. ATP+ cells were exposed to 5 mM ATP. ATP- samples were exposed to an equivalent volume of PBS. ATP-stimulated J774 cells showed a significantly higher amount of intracellular trehalose compared to non-treated cells for all incubation times at 37°C (mean + SD; p<0.01). Appreciable levels of trehalose were not observed for any sample incubated only on ice.

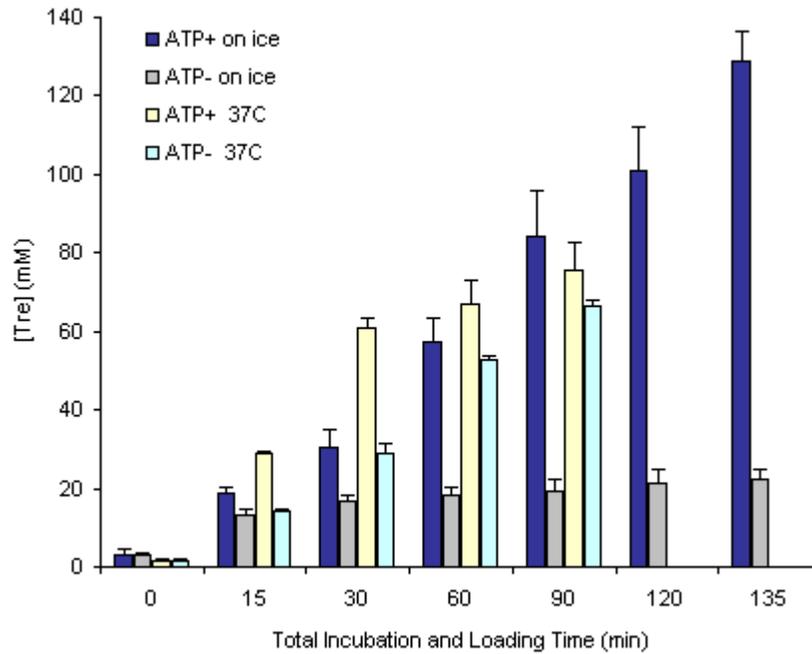


Figure 5. Time course of intracellular trehalose uptake in J774 cells using a 2-step loading procedure with 500 mM extracellular trehalose. Cells were incubated in an intracellular-like trehalose buffer containing 225 mM trehalose for 15 min at 37 °C. ATP+ cells were exposed to 5 mM ATP. ATP- samples were exposed to an equivalent volume of PBS. After this pre-incubation period the concentration of extracellular trehalose was increased to 500 mM and samples were either held at 37°C or transferred to an ice bath. ATP-stimulated cells showed a significantly higher amount of intracellular trehalose compared to non-treated cells for both loading temperatures (mean + SEM; $p < 0.01$). Both the isothermal and 2-step treatment resulted in equivalent amounts of intracellular trehalose at 60 and 90-min following ATP stimulation.

TABLE 1. Survival and growth of porated cells. For the Trypan Blue assay survival is expressed as the percentage of cells with an intact membrane at the indicated time period post-poration compared to the number of intact cells immediately following poration. For the Alamar Blue assay survival is expressed as the percent reduction of Alamar Blue compared to control at the indicated time following poration. n=3-5 replicates

	Survival and Growth (%)						
	Mean (SD)						
	Trypan Blue exclusion				Alamar Blue™		
	Immediate	24-h	48-h	72-h	22-h	46-h	70-h
Control	100	141 (47)	324 (75)	640 (87)	100	100	100
15'@37°C/0' ice		59 (4)	86 (44)				
30'@37°C/0' ice		11 (5)	9 (8)		25 (6)	15 (10)	15 (9)
5'@37°C/60' ice	89 (16)	54 (7)	135 (37)		54 (16)	49 (8)	49 (17)
5'@37°C/120' ice	96 (9)	45 (16)	125 (16)		56 (12)	46 (11)	51 (16)
10'@37°C/90' ice	90 (19)	49 (12)	132 (43)	233 (104)	56 (20)	45 (29)	48 (31)
15'@37°C/60' ice	89 (16)	24 (9)	85 (52)				
15'@37°C/120' ice	83 (9)	11 (5)	9 (8)				

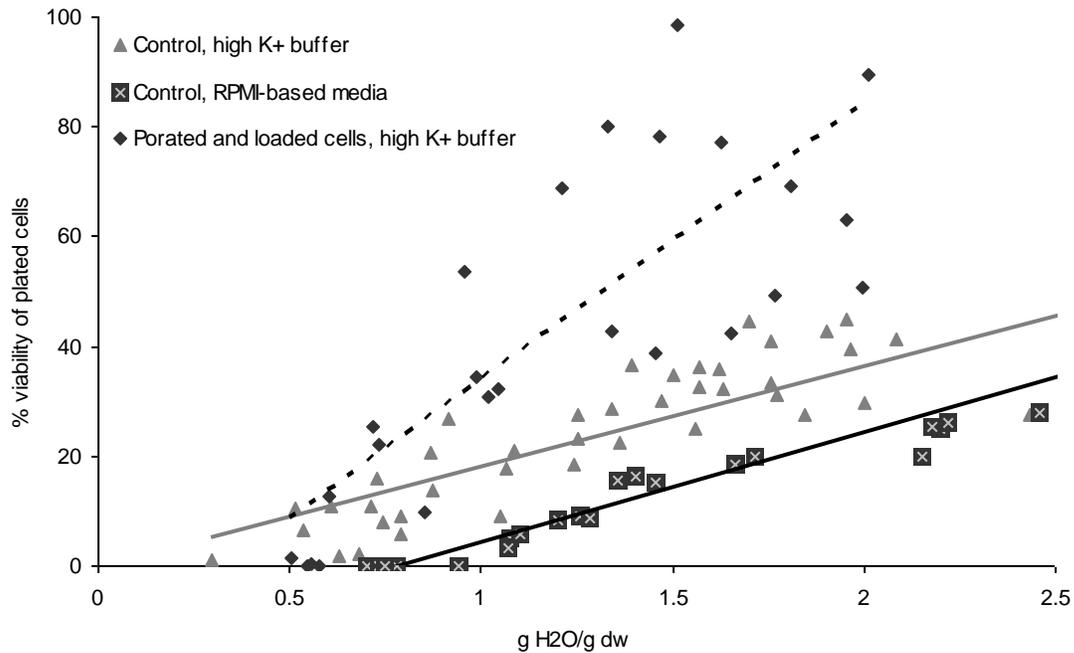


Figure 6. Recovery of J774 cells after drying to various moisture contents. Cells were dried in 15 μ l droplets in a desiccation cabinet to a range of final moisture contents. Cells were porated at 37C for 10 min followed by loading on ice for 90 min. Cells were returned to overnight culture for a recovery period prior to drying in high K⁺ buffer. Control cells received no treatment prior to drying and were dried either in RPMI-based buffer with 200 mM trehalose or in high K⁺ buffer with 250 mM trehalose. All slopes were statistically different from one another based on slope analysis ($p < 0.05$).