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Comparative Evaluation of the Susceptibility of Neuronal (N1E-115) and Non-neuronal (HeLa) Cells to Acetylsalicylic Acid (ASA) Cytotoxicity by Confocal Microscopy

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Abstract—A voltage-sensitive probe, tetramethylrhodamine methyl ester (TMRM) and digital imaging (confocal microscopy) were used to quantify differential membrane potential alteration in neuronal (murine neuroblastoma, N1E-115) and non-neuronal (human epithelial-like, HeLa) cells, after 1 and 24 hr of exposure to a known *in vivo* neurotoxic agent, acetylsalicylic acid (ASA), as a first step in substantiating the relevance of alteration in resting membrane potential (V_m) as a neurotoxic endpoint. After 1 hr of exposure, ASA (5.0 mM) hyperpolarized both HeLa and N1E-115 cells. V_m decreased from -57.6 mV $\pm$ 2.8 (n = 20 cells) to -74.7 mV $\pm$ 1.9 (n = 20 cells) and from -64.0 mV $\pm$ 2.1 (n = 20 cells) to -82.5 mV $\pm$ 3.4 (n = 20 cells) for HeLa and N1E-115 cells, respectively. The extent of hyperpolarization was found to be ASA concentration dependent. There was no significant difference ($P < 0.05$) between the cell lines with respect to ASA sensitivity, suggesting that under these experimental conditions, ASA exhibited no selective cytotoxic activity for the neuronal cells. In comparison with control cultures, 24-hr ASA (5.0 mM) exposure did not affect the surviving cell V_m. The results of the present study were inconclusive with respect to the suitability of V_m alteration as an indicator of neurotoxic potential.

INTRODUCTION

The complexity and diversity of the nervous system has precluded the rapid development of *in vitro* alternatives for neurotoxicity testing (Williams *et al.*, 1994). However, recent commercial pressures and regulatory requirements have stimulated interest in this area. For example, the US EPA has stated that neurotoxicology should have high priority and that the development of methods for the identification of neurotoxic risk must be intensified in order to improve human risk assessment (EPA, 1991). To date, guidelines for neurotoxicity testing involve animal-based models only and therefore are expensive and time consuming. Besides, some chemicals (e.g. antibodies and peptides) may be human specific (not detectable by animal models). Consequently the EPA, as well as other regulatory agencies, are examining ways to integrate *in vitro* testing approaches in the risk assessment process (Veronesi, 1992). These efforts are based on the assumption that neurotoxic effects in nervous system cellular models are important for toxicological prescreening, identification of potential neurotoxic compounds and for elucidation of the mode of action.

Because of the low regenerative capacity of the nervous system, Walum *et al.* (1993) have suggested that cellular tests included in multiple system primary screens should be based on determinations of cellular dynamic physiological parameters, such as alterations in membrane function, rather than measurement of single biochemical reactions. Endpoints consistent with this approach have included alteration in action potential (Quandt *et al.*, 1982) and inward currents (Oortgiesen *et al.*, 1993). Despite the suitability of alteration in excitable cell membrane properties as a neurotoxic endpoint, few studies have used electrophysiological techniques to assess neurotoxicology. This has been attributed to the laboriousness and time-consuming nature of traditional electrophysiological techniques (Xie and Harvey, 1993). However, recent developments in chemical probes (Tsien and Waggoner, 1995) and computer-controlled instrumentation should pave the way for use of alteration in excitable membrane properties such as resting membrane potential as neurotoxic endpoints. In the present study, we used a voltage-sensitive probe, tetramethylrhodamine

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Abbreviations: AOI = area of interest; ASA = acetylsalicylic acid; DMEM = Dulbecco's modified Eagle's medium; EBSS = Earle's balanced salt solution; FBS = foetal bovine serum; LDH = lactate dehydrogenase; TMRM = tetramethylrhodamine methyl ester.

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methyl ester (TMRM) and digital imaging (confocal microscopy) to quantify differential membrane potential alteration in the commonly available neuronal (N1E-115 neuroblastoma) and non-neuronal (HeLa) cells, after exposure to a known *in vivo* neurotoxic agent, acetylsalicylic acid (ASA), as a first step in substantiating the relevance of alteration in resting membrane potential (V_m) as a neurotoxic endpoint.

MATERIALS AND METHODS

Chemicals

TMRM was chosen in the present study for several reasons: (1) TMRM exhibits low non-specific binding and therefore readily display reversible potential dependent uptake; (2) TMRM is not quenched when taken up by mitochondria and therefore cytoplasmic and mitochondrial fluorescence are readily distinguishable; and (3) TMRM is non-toxic to most cells (Ehrenberg *et al.*, 1988; Emaus *et al.*, 1986). The choice of ASA was also based on several reasons: (1) ASA is one of the first 10 standard chemicals in the Multicenter Evaluation of *In Vitro* Cytotoxicity Tests (MEIC) program (Bernson *et al.*, 1987); (2) ingestion of ASA in humans and animals has been reported to lead to malformations of the nervous system and other congenital abnormalities (Joschko *et al.*, 1993); and (3) Xie and Harvey (1993) recently assessed the acute toxicity of ASA in a neuroblastoma × glioma hybrid (NG108-15) and reported an ASA concentration-dependent depolarization over 1- and 24-hr exposure times. TMRM and ASA were obtained from Molecular Probes (Eugene, OR, USA) and Sigma Chemical Co. (St Louis, MO, USA), respectively.

Cell culture

HeLa cells of passage 105 were obtained from the American Type Culture Collection (Rockville, MD, USA). N1E-115 cells of passage 12 were obtained from Dr M. Nirenberg, Laboratory for Biochemical Genetics, Bethesda, MD, USA. Both cell lines were grown to confluence in 75 cm^2 culture flasks in Dulbecco's modified Eagle's minimal essential medium (DMEM) containing 0.12% $NaHCO_3$, supplemented with 10% (HeLa) and 13% (N1E-115) foetal bovine serum (FBS), 2% L-glutamine, 120 U/ml penicillin and 12 U/ml streptomycin. The cultures were maintained at 37 °C in a humidified atmosphere containing 10% CO_2.

Lactate dehydrogenase (LDH) assay

The purpose of LDH assay was to identify an ASA concentration that caused minimal cytostatic effect or cytotoxicity in terms of loss of cell viability in both N1E-115 and HeLa cells, for use in neurotoxicity evaluation. A commercial kit (Sigma) for LDH activity determination was used. The kit comprised of LDH reagents A (NADH and phosphate buffer, pH 7.5) and B (pyruvate) was prepared according to the manufacturer's instructions (Sigma Procedure no. DG-1340-UV). "Sample Start" reagent was prepared by adding 8 ml LDH reagent B to 20 ml LDH reagent A. N1E-115 cells were mechanically removed from the culture flasks and replated in six-well clusters (Costar) at 120×10^3 cells/well. HeLa cells were dislodged with a mild trypsin (0.25%) treatment and similarly replated at 80×10^3 cells/well. N1E-115 cells were grown in normal growth medium (13% FBS) for 24 hr, after which the growth medium was replaced by low-serum medium (2.5% FBS). 2 days following replanting, N1E-115 cells were incubated with fresh low-serum medium containing different concentrations of ASA (in triplicate): 0.0 (control), 2.5, 5.0, 10.0, 20.0, 30.0 and 40.0 mM. After 1 and 24 hr of exposure, the test medium was removed and the cells were washed with 0.15 M Tris buffer. Total intracellular LDH activity for the washed cells was determined by lysing the cells with 0.05% Triton X-100 in 5 ml isotonic saline at 37 °C for 4 hr. To a cuvette labelled test, 2.5 ml Sample Start reagent were added. 100 µl of the lysate were added and mixed immediately by inversion for 30 sec. Absorbance at 340 nm *v.* water as reference was recorded at 1, 2 and 3 min following initial absorbance reading. The mean absorbance change per minute (ΔA/min) was determined and LDH activity in U/litre was calculated by multiplying ΔA/min by a temperature-dependent constant of 4180, recommended by the kit manufacturer. All measurements were performed at 25 °C with a Beckman DU650 spectrophotometer. Total intracellular LDH activity for HeLa cells was determined in a similar manner, except that cells were maintained in normal growth medium (10% FBS) for the 2 days before exposure to ASA. The data from LDH activity experiments was expressed as a percentage of the control. Rationale for 1- and 24-hr exposure times was based primarily on the pharmacokinetics of aspirin in humans (Thiessen, 1982).

Confocal microscopy

The confocal microscopy procedure has been described in detail elsewhere (M. Hernandez and W. S. Kisaalita, unpublished data, 1996). Briefly, a confocal imaging system (MRC-600, Bio-Rad Laboratories, Cambridge, MA, USA), equipped with fast photon counting electronics, was used. A schematic diagram of the experimental set-up is shown in Fig. 1. The flow chamber used is similar to that previously described by Berg and Block (1984). A distribution system was used in conjunction with a peristaltic pump to allow rapid flow of medium through the longitudinal chamber channel along its entire width. Temperature control of the samples during microscopic observation was achieved with an air-stream stage incubator (ASI-400, Nicholson Precision Instruments, Gaithersburg, MD, USA). Unless stated otherwise, all experiments were carried out at 18 °C.

The imaging system was linked to an upright Nikon Optiphot microscope, equipped with a X60 Apochromat, oil immersion, high numerical aperture (1.40) objective lens. The imaging system's krypton/argon mixed-gas laser was used to excite the fluorescent dye at 568 nm, and emission scans were recorded as relative fluorescence intensities at 610 nm.

3 days prior to ASA exposure, N1E-115 cells were mechanically removed from the culture flasks, counted and replated in six-well clusters, each holding one 12-mm round glass coverslip, at a density of 120×10^3 cells/well. Cultures were incubated overnight in growth medium and the next day the growth medium was exchanged with low-serum medium (2.5% FBS), to minimize cell growth without initiating electrophysiological differentiation. After 48 hr under low-serum conditions the medium was refreshed with the same medium containing 5.0 mM ASA. ASA exposure lasted for 1 and 24 hr.

2 days prior to ASA exposure, HeLa cells were removed from culture flasks by a brief treatment with 0.25% trypsin and were also replated in six-well clusters each holding one 12-mm round glass coverslip at a density of 80×10^3 cells/well. Cultures were maintained in growth medium (10% FBS) for 48 hr followed by exposure to 5.0 mM ASA for 1 and 24 hr.

For both cell lines, ASA exposure was terminated by removal of the medium, followed by washing of the cells on the coverslips with Earle's balanced salt solution (EBSS). After wiping their edges and reverse sides dry, the coverslips were placed with the cells facing the interior of the flow chamber filled with EBSS and 0.5 μM TMRM for V_m measurement. Coverslips were held in position by means of a thin film of high-vacuum silicon grease applied to the top seat of the flow chamber. Complete equilibration was taken as the time at which fluorescence measured from a cell had attained a steady intensity. This was achieved in approximately 2 and 5 min for HeLa and N1E-115 cells, respectively. On complete dye equilibration, the flow chamber was placed under the Nikon Optiphot upright microscope stage and the MRC-600 system was set up for fast photon counting. The krypton/argon mixed-gas laser was switched on and an appropriate focal plane (3–9 μm from the coverslip–cell interface) for a single cell loaded with fluorescent probe was focused upon. The fluorescence sample was recorded and the image was digitized with 512×512 pixel spatial and eight-bit intensity resolution by a frame grabber and stored directly to a Panasonic optical disk (940 MB). Approximately 20 cells were measured from each coverslip. Data for a given set of conditions were obtained from at least two coverslips.

Data analysis was performed with MRC-600 system software package. A rectangular area of interest (AOI) was defined by the image processor's cursor controls. This AOI was placed inside the cell in a region devoid of mitochondria and the fluorescence intensity histograms were generated. The same size AOI was then placed outside the cell, in a region short of processes or any other glowing debris and the fluorescence intensity histograms were generated in a similar manner. The ratio of the mean values of the fluorescent intensities measured from inside and outside the cell (Fl_i/Fl_0) was related to V_m by the Nernst equation:

$$V_m = -2.3(RT/ZF)\log_{10}(Fl_i/Fl_0)$$

where V_m is the resting membrane potential in mV, Z is the charge on the TMRM ion, F is Faraday's constant, R is the ideal gas constant, T is the absolute temperature. Considering that the stage temperature was maintained at 18°C and assuming a Z of 1 for TMRM ion, the constant (-2.3 RT/ZF) was found to be equal to -58.0 mV. All individual cell V_m data were presented as means ± 5 SEM. Unless specified otherwise, the significance of the differences between treatments were analysed using Student's *t*-test (mean

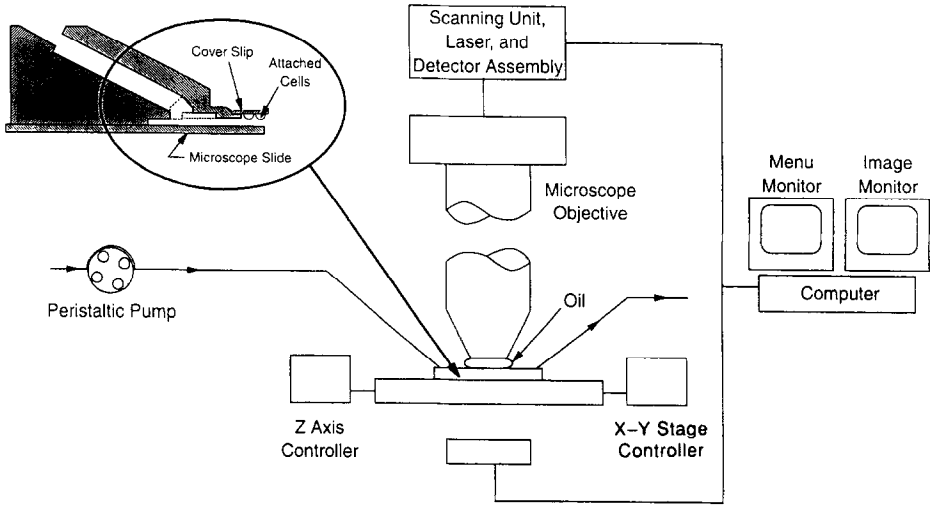

Fig. 1. Schematic diagram of the experimental set-up for confocal microscopy.

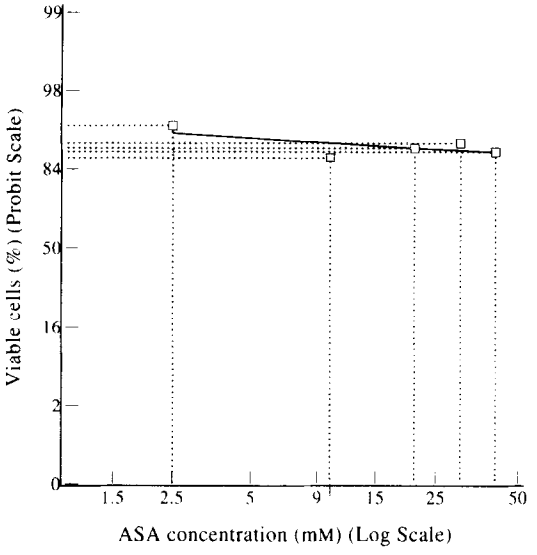

Fig. 2. Effect of ASA concentration on N1E-115 cell viability (in terms of intracellular LDH) after 1 hr of exposure. Viability is expressed as percentage of control.

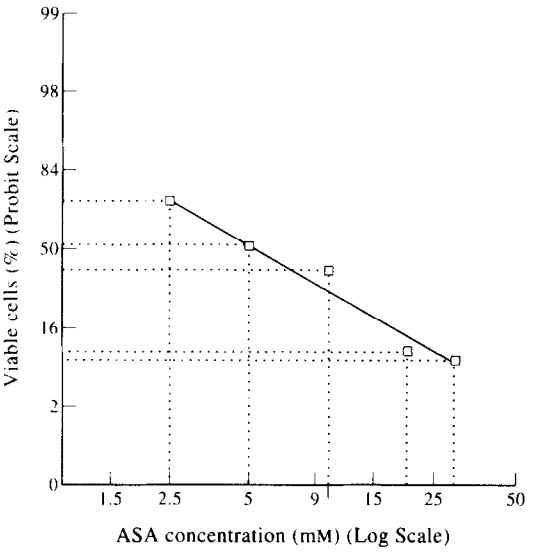

Fig. 3. Effect of ASA concentration on N1E-115 cell viability (in terms of intracellular LDH) after 24 hr of exposure. Viability is expressed as percentage of control.

of three identical control groups was processed to provide a baseline or reference point for all other groups). P values less than 0.05 were accepted as being significant.

RESULTS AND DISCUSSION

LDH assay

Normally, LDH leakage rather than intracellular LDH is the activity most frequently measured in cytotoxicity studies. However, this has a serious drawback in that not all enzymes are stable in culture medium conditions, and the measured activity of leaked LDH may decrease during long-term incubations depending on the cell type (Ponsoda *et al.*, 1991). The approach adopted in the present study was to measure intracellular LDH after ASA exposure. Figures 2–5 show the ASA cytotoxicity results over a concentration range of 2.5–40.0 mM for 1- and 24-hr exposure times. Concentration–response data are represented on probability (response) and logarithmic (concentration) value scales. The effects of ASA on N1E-115 and HeLa cells during 1 hr of exposure are shown in Figs 2 and 4. Student's t-test analysis showed that there was no significant difference between control (no ASA exposure) and all experiments for N1E-115 ($P < 0.005$) and HeLa ($P < 0.05$) cells. This was consistent with the 'flatness' of the concentration–response curve fit, which implied that a large change in ASA concentration was required before a significant loss of viability could be observed.

A significant concentration-dependent decrease in intracellular LDH ($P < 0.05$) for both cell lines was observed after 24 hr of exposure. The toxic response in N1E-115 was evident even at the lowest concentration used (2.5 mM), while in HeLa, 5.0 mM was needed to exert a significant toxic reaction ($P < 0.05$). As shown in Figs 3 and 5, the slope of the concentration–response curve is steeper when compared with 1 hr of exposure, suggesting that a relatively small change in concentration caused a significant change in viability. The ASA concentrations required to cause 50% reduction in intracellular LDH (EC_{50}) after 24 hr of exposure were 5.74 mM for N1E-115 and 6.88 mM for HeLa cells. Taken together, these results suggested that N1E-115 cells (neuronal) were more susceptible to ASA insult in comparison with HeLa cells (non-neuronal). A dramatic reduction in intracellular LDH was

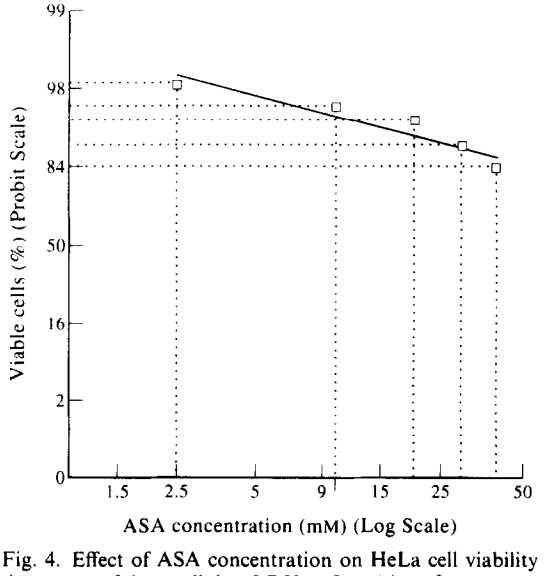

Fig. 4. Effect of ASA concentration on HeLa cell viability (in terms of intracellular LDH) after 1 hr of exposure. Viability is expressed as percentage of control.

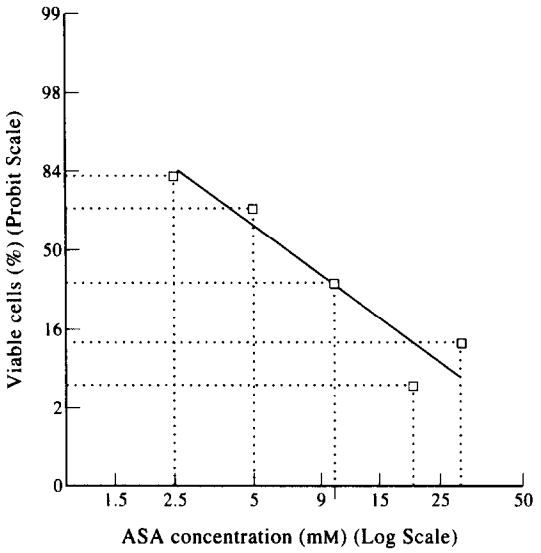

Fig. 5. Effect of ASA concentration on HeLa cell viability (in terms of intracellular LDH) after 24 hr of exposure. Viability is expressed as percentage of control.

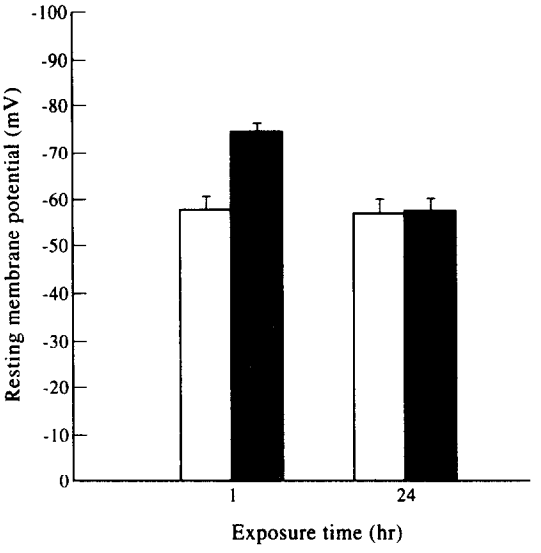

Fig. 7. Effect of ASA (5.0 mM) on HeLa resting membrane potential after 1 and 24 hr of exposure. Open bars represent control (not exposed). Values are means (n = 20) ± standard error of the mean (SEM).

observed at the highest ASA concentration after 24 hr of exposure, indicating severe cytotoxicity. Minimal reduction in intracellular LDH was observed at the lowest ASA concentration for both 1 or 24 hr of exposure, suggesting mild or negligible cytotoxicity.

Because of our focus on neurotoxicity as opposed to general cytotoxicity, it was necessary to select an ASA concentration with minimal cytotoxic effects (in terms of cell death) for the main objective of this study (i.e. comparative evaluation of the susceptibility of neuronal and non-neuronal cells to ASA with alteration in V_m as the endpoint). On the basis of the 1-hr concentration–response profiles, an ASA concentration of 5.0 mM was selected for both cell lines.

Effect of ASA exposure on V_m

As shown in Figs 6 and 7, 1 hr of exposure to 5.0 mM ASA hyperpolarized both HeLa and N1E-115 cells. V_m decreased from -57.6 mV $\pm$ 2.8 (n = 20 cells) to -74.7 mV $\pm$ 1.9 (n = 20 cells) and from -64.0 mV $\pm$ 2.1 (n = 20 cells) to -82.5 mV $\pm$ 3.4 (n = 20 cells) for HeLa and N1E-115 cells, respectively. This effect was found to be concentration dependent (Fig. 8). There was no significant difference ($P < 0.05$) between the cell lines with

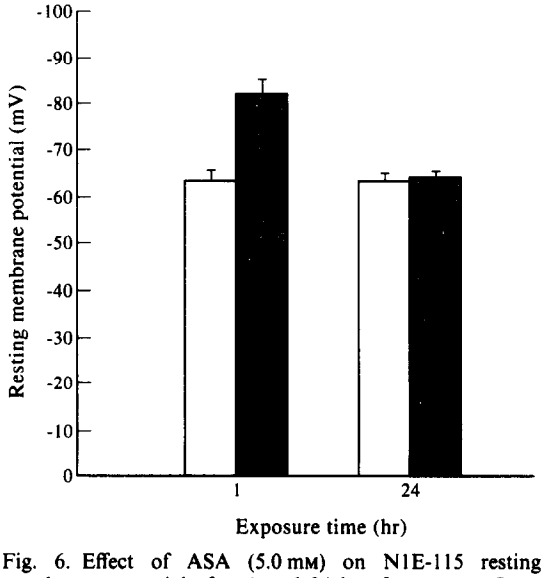

Fig. 6. Effect of ASA (5.0 mM) on N1E-115 resting membrane potential after 1 and 24 hr of exposure. Open bars represent control (not exposed). Values are means (n = 20) ± standard error of the mean (SEM).

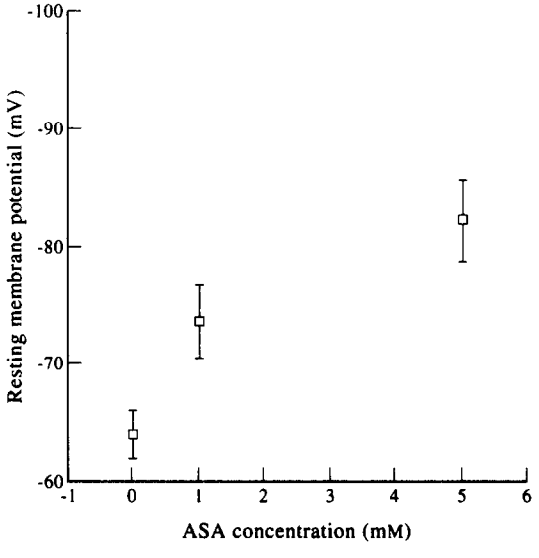

Fig. 8. Concentration–response curve for N1E-115 cells exposed to 5.0 mM ASA for 1 hr. Values are means (n = 20) ± standard deviation (SD).

respect to ASA sensitivity, suggesting that under the experimental conditions in this study, ASA exhibited no selective cytotoxic activity for the neuronal cells. In comparison with control cultures, 24-hr ASA (5.0 mM) exposure did not result in any significant hyperpolarization. It is possible that the hyperpolarized cells (observed after 1 hr of exposure) were all dead within the 24-hr period, leaving behind a segment of the cell population that was immune to ASA effects. This is consistent with the observed reduction in cell viability shown in Figs 3 and 5. It is also possible that all cells were hyperpolarized, but a compensatory or protective mechanisms in the surviving cells may have been activated, leading to re-establishment of normal V_m.

Fast hyperpolarization of molluscan neurons (in seconds) by salicylate has been previously observed by Barker and Levitan (1971), who speculated that the changes in V_m in presence of salicylate were due to increase and decrease in K^+ and Cl^- permeability, respectively. The well documented salicylate interference with oxidative phosphorylation (Roos and Boron, 1981; Spenney and Bhown, 1977) was considered unlikely since this effect requires at least 30 min. In other studies, Wieth (1970) observed increase and decrease in cation and anion permeability, respectively, in erythrocyte membranes exposed to salicylate. Also, Ashley *et al.* (1989) reported ASA-mediated hyperpolarization of epithelial cells of *Necturus antrum*. In the N1E-115 and HeLa cases, further work is needed to determine the predominant hyperpolarization mechanism.

Xie and Harvey (1993) reported a concentration-dependent depolarization of NG108-15 (rat glioma × neuroblastoma hybrid cell line) by ASA. The discrepancy between the findings of Xie and Harvey (1993) and our results is probably due to differences in cell lines. These opposite effects of ASA in two different cell lines provide a unique opportunity to further elucidate the hyperpolarization/depolarization mechanism of ASA and related drugs.

Conclusions

In the present study, the neurotoxicity of ASA was evaluated in a comparative analysis of sensitivities between a murine neuroblastoma (N1E-115) cell line and a human epithelial-like (HeLa) cell line, by monitoring alteration in intracellular LDH and V_m over 1- and 24-hr exposure periods. This work was initiated to substantiate the relevance of alteration in V_m as a neurotoxic endpoint. The results support the following conclusions: (1) neuronal N1E-115 and non-neuronal HeLa cells are equally sensitive to the toxic effects of ASA as discerned by their inability to maintain a normal V_m during 1 hr of exposure; and (2) a short period (1 hr) exposure of neuronal N1E-115 and non-neuronal HeLa cells to ASA causes a concentration-dependent hyperpolarization; however, a long period (24 hr) of exposure does not affect the surviving cells. Although the results of the present study indicated that V_m alteration may not be suited to the detection of neurotoxic potential, further studies with ASA and other related drugs (non-steroidal anti-inflammatory drugs) at lower exposure times (seconds to minutes), to further investigate the validity of V_m alteration as a neurotoxic endpoint, is a main objective of ongoing research in our laboratory.

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