Calcium-Dependent Proliferation of NG108-15 Neuroblastoma Cells

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Abstract. While there is increasing evidence that Ca^{2+} plays an important role in regulating cell proliferation, the precise mechanisms have not been clearly elucidated so far. In order to gain insight into how Ca^{2+} controls cell division, the rate of proliferation, cell volume, viability and attachment to the culture support were measured in NG108-15 neuroblastoma cells in the presence of various extracellular Ca^{2+} concentrations ($[Ca^{2+}]_o$). Culture medium $[Ca^{2+}]_o$ was decreased from 1.8 mmol/l to various values down to 1 μmol/l with EGTA. The rate of cell proliferation was almost independent of $[Ca^{2+}]_o$ between 1.8 mmol/l and 45 μmol/l. It was decreased by about 50 % at 12 μmol/l Ca^{2+} and was almost zero in the presence of 1 μmol/l Ca^{2+}. As we hawe shown previously (Rouzaire-Dubois and Dubois 1998) long term hypertonicity increased the cell volume and decreased the rate of proliferation. The effects of hypertonicity and decrease in $[Ca^{2+}]_o$ on cell proliferation were synergistic and can be described by cell size-dependent and independent mechanisms, respectively. Relative to control conditions (1.8 mmol/l Ca^{2+}), decreases in $[Ca^{2+}]_o$ to 12 and 1 μmol/l decreased the cell viability to 76 and 52 % and the cell adhesion to dishes to 16 and 3 %, respectively. Altogether, these results indicate that the effects of alteration in $[Ca^{2+}]_o$ and cell size on neuroblastoma cell proliferation are independent and act on different signalling pathways controlling cell division.

Key words: Cell volume — Neuroblastoma cells — Proliferation — Adhesion

Introduction

It has been known for more than three decades that a physiological extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) and Ca^{2+} influx are indispensable for normal mammalian cell growth and proliferation (Santella 1998; Munaron 2002). By contrast, the proliferation of neoplastic cells and especially the anchorage-independent proliferation of cancer cells shows a markedly reduced dependence on $[Ca^{2+}]_o$ (Balk

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1971; Durham and Walton 1982; Takuwa et al. 1993). It has been suggested that the role(s) of Ca^{2+} in DNA synthesis and cell cycle progression is mediated by the activity of several calcium-dependent proteins, including calmodulin and integrins (Takuwa et al. 1995; Santella 1998; Mizejewski 1999). In addition to Ca^{2+} influx from the external medium and Ca^{2+} release from intracellular stores, cell proliferation is dependent on plasma membrane transport of monovalent ions. In particular, it has been shown in a large number of normal and tumoural cell types that the activity and expression of K^+ and Cl^- channels control the cell cycle progression (reviewed by Dubois and Rouzaire-Dubois 1993; Nilius and Droogmans 1994; Wonderlin and Strobl 1996; Nilius and Droogmans 2001; Fraser et al. 2002). Two different but not mutually exclusive hypotheses have been developed to take into account the role of these channels in cell proliferation, namely: regulation of Ca^{2+} influx *via* the control of membrane potential and regulation of cell volume (Nilius and Droogmans 1994; Rouzaire-Dubois and Dubois 1998). Recently, we showed that both cell size-dependent and independent mechanisms control the rate of proliferation (P) of C6 glioma cells, which can be described by the equation (Rouzaire-Dubois et al. 2004):

$$P = P_{\max} A\ I \tag{1}$$

In this equation, $P_{\max}$ is the maximal rate of proliferation, independent of cell volume and A (activation) and I (inhibition) are dependent of cell volume, so that P is optimal within a cell volume window.

Given that the regulation of cell volume is controlled by intra- and extracellular Ca^{2+} (Balk 1971; Olson et al. 1990; Bostel et al. 2002), it was of interest to know whether the two above mechanisms proposed to explain the role of K^+ and Cl^- channels in cell proliferation (control of cell Ca^{2+} or volume) are connected or independent. In order to answer this question, we measured cell number and volume of neuroblastoma x glioma hybrid NG108-15 cells cultured in the presence of various $[Ca^{2+}]_o$. Furthermore, the proportions of viable and attached cells to dishes were determined under the same conditions.

Materials and Methods

Cell culture and materials

The experiments were performed on undifferentiated hybrid neuroblastoma × glioma NG108-15 cells cultured in 9.2 cm diameter Petri dishes containing 10 ml Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 100 μmol/l hypoxanthine, 0.4 μmol/l aminopterin, 16 μmol/l thymidine, 2 mmol/l glutamine, 3 μmol/l glycine, 100 i.u. ml^{-1} penicillin and 100 $\mu g \cdot ml^{-1}$ streptomycin. The cultures were maintained at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. The $[Ca^{2+}]_o$ in culture medium was decreased with EGTA (Sigma) and was measured with a Ca^{2+} selective electrode connected to a

713 pH Meter (Metrohm, Herisau, Switzerland). After calibration with solutions containing Ca^{2+} at known concentrations, this electrode allows to measure $[Ca^{2+}]_o \geq 1$ μmol/l. EGTA was added to the culture medium at concentrations of 1.73, 1.80, 1.84 and 1.95 mmol/l. Under these conditions, $[Ca^{2+}]_o$ was decreased from an initial value of 1.81 ± 0.09 mmol/l to 98 ± 5 μmol/l, 45 ± 5 μmol/l, 12 ± 1 μmol/l and ≤ 1 μmol/l (mean ± SEM of 12 to 29 determinations in each condition). In all cases, EGTA was added to the culture medium one day after the cells were seeded at a density of 300,000/dish.

Cell proliferation and volume determinations

Cell number and mean cell volume were electronically determined with a Coulter channelizer (model Z2, Beckman-Coulter, Inc.). Prior to cell number and volume measurement, the cells were mechanically detached from Petri dishes, centrifuged at $100 \times g$ for 5 min and resuspended in Isoton (Coulter). The rate of cell proliferation P in the different experimental conditions was calculated from the cell number N at day 1 (D1) and D3 and expressed as: $P = \log N_{D3} - \log N_{D1}$. For each individual experiment, cell volume and number were determined in triplicate.

Cell viability and attachment to dishes

Cell viability was determined by counting the proportion of Trypan blue excluding cells with a Mallasez cell. In order to count all the cells, these counts were performed without centrifugation. To determine the percentage of attached cells to dishes, the culture medium was gently removed from dishes and cells in this medium were counted. Then, the cells remaining in dishes were mechanically detached with a cell free medium and counted.

Results

Effects of $[Ca^{2+}]_o$ on cell number and proliferation

Fig. 1 shows the evolution with time of cell number and rate of proliferation in the presence of various $[Ca^{2+}]_o$. The rate of proliferation was almost independent of $[Ca^{2+}]_o$ between 1.8 mmo/l and 45 μmol/l. It was decreased by about 50 % in the presence of 12 μmol/l Ca^{2+} and was almost zero when $[Ca^{2+}]_o$ was ≤ 1 μmol/l. In the presence of 1 μmol/l Ca^{2+}, it should be noted that the cell number slightly increased between D1 and D2 and then decreased between D2 and D3 (Fig. 1A). This decrease reflects a delayed cell death (see below).

Effects of $[Ca^{2+}]_o$ on cell volume

Fig. 2 shows the evolution with time of cell volume as a function of $[Ca^{2+}]_o$. The cell volume increased between D0 and D1 of culture and then decreased (Fig. 2A). This decreasing was similar to that observed on glioma cells (Rouzaire-Dubois et al. 2004) and attributed to the production by the cells of inhibitory factors. However, in contrast to that observed on glioma cells, the volume of neuroblastoma cells

slightly but not significantly ($p > 0.1$) reincreased between D2 and D3. By contrast to observations on other cell types or under different experimental conditions (Balk 1971; Olson et al. 1990; Bostel et al. 2002), the cell volume was almost independent of $[Ca^{2+}]_o$ measured at D2 and D3 (Fig. 2B).

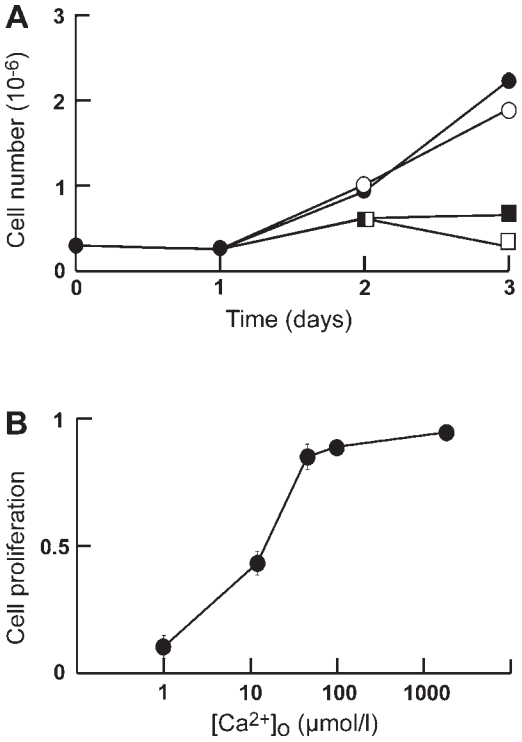

Figure 1. Cell number and rate of cell proliferation as a function of $[Ca^{2+}]_o$. **A.** EGTA was added to the culture medium at day 1 (D1) and the cell number was determined in control conditions (1.8 mmol/l Ca^{2+}) (filled circles) and in the presence of 98 μmol/l (open circles), 12 μmol/l (filled squares) and $\leq$1 μmol/l (open squares) Ca^{2+}. **B.** The rate of cell proliferation was calculated from cell number (see Materials and Methods) at D1 and D3. Each point is the mean $\pm$ SEM of 4 to 12 experiments.

Differential effects of $[Ca^{2+}]_o$ and hypertonicity on cell proliferation

The preceding results suggest that the effects on cell proliferation of low $[Ca^{2+}]_o$ are not mediated by cell size alterations. In order to confirm this conclusion, the effects on proliferation of hypertonicity and decrease in $[Ca^{2+}]_o$ were studied in parallel cell cultures. Hypertonicity was performed by adding 100 mmol/l sucrose to the culture medium. As has previously been shown on neuroblastoma and glioma cells, hypertonicity induces a paradoxical delayed increase in cell size associated with a decrease in cell proliferation (Rouzaire-Dubois and Dubois 1998; Rouzaire-Dubois et al. 2000, 2004). This is confirmed when cell volumes were measured after 48 h in isotonic and hypertonic media containing 1.8 mmol/l Ca^{2+} (Fig. 3, filled symbols). Similar experiments made in the presence of 12 μmol/l Ca^{2+} (Fig. 3, open symbols) revealed that the effects of a decrease in $[Ca^{2+}]_o$ and hypertonicity are synergistic.

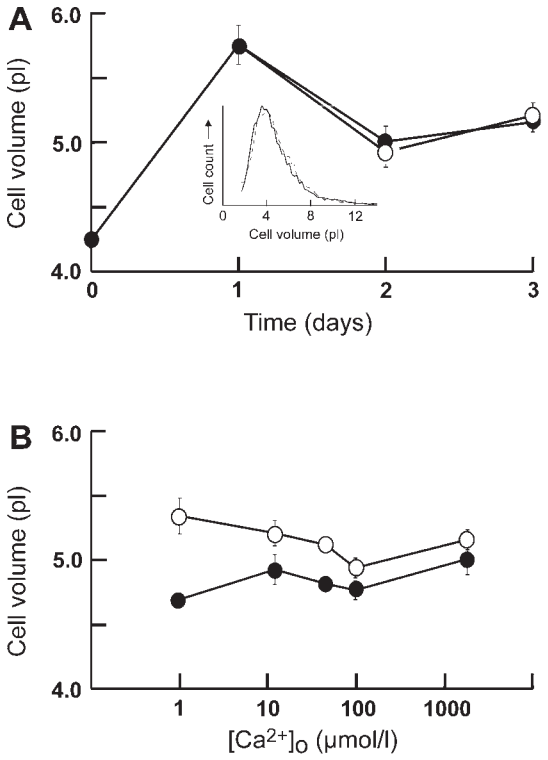

Figure 2. Cell volume as a function of $[Ca^{2+}]_o$. Same experiments than in Fig. 1. In **A.** the mean cell volume was determined in the presence of 1.8 mmol/l (filled circles) and 12 μmol/l (open circles) Ca^{2+}. The insert shows the cell volume distribution at D2 of one representative experiment in 1.8 mmol/l (continuous curve) and 9 μmol/l (interrupted curve) Ca^{2+}. In **B.** the mean cell volume was determined at D2 (filled circles) and D3 (open circles). Mean ± SEM of 4 to 12 experiments in each condition.

Table 1. Effects of low $[Ca^{2+}]_o$ on cell viability and attachment to dishes

$[Ca^{2+}]_o$	Viable cells (%)	Attached cells (%)
1.8 mmol/l	97.4 ± 0.4	98.4 ± 0.0
12 μmol/l	74.3 ± 3.4	15.6 ± 3.1
$\leq$1 μmol/l	50.6 ± 3.0	2.9 ± 0.3

The percentages of viable and attached cells were determined at D3 of culture in control conditions (1.8 mmol/l Ca^{2+}) and 48 h after addition of EGTA to the culture medium. Mean ± SEM of 3 experiments in each condition.

While in these experiments, the low $[Ca^{2+}]_o$ slightly decreased the cell volume (see also Fig. 2B), its effect on proliferation was independent of that of hypertonicity.

Effects of $[Ca^{2+}]_o$ on cell adhesion and death

Since the proliferation of NG108-15 cells is anchorage-dependent and we observed that low $[Ca^{2+}]_o$ induced cell death (Fig. 1A), the proportions of viable and dish-attached cells were determined in the presence of 1.8 mmol/l, 12 μmol/l and

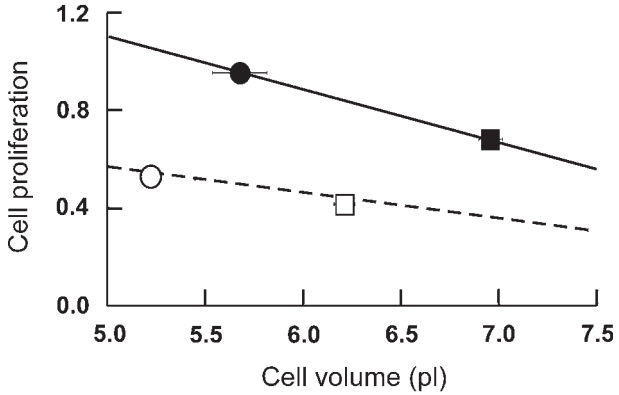

Figure 3. Differential effects of hypertonicity and low $[Ca^{2+}]_o$ on cell proliferation. The rate of cell proliferation, calculated from cell number at D1 and D3 and the cell volume at D2 were determined in the presence of 1.8 mmol/l (filled symbols) and 12 μmol/l (open symbols) Ca^{2+}. In parallel experiments, the same parameters were determined in isotonic (circles) and hypertonic (squares) media. Mean ± SEM of 3 experiments in each condition. The continuous straight line represents the linear regression through the filled symbols. The interrupted straight line was drawn with the same equation as that of the continuous line but with parameter values (ordinate at zero volume and slope) multiplied by 0.51.

1 μmol/l Ca^{2+} (Table 1). Relative to control conditions (1.8 μmol/l Ca^{2+}), decrease in $[Ca^{2+}]_o$ to 12 and 1 μmol/l decreased cell viability by 24 and 48 % and cell attachment to dishes by 84 and 97 %, respectively.

Discussion

In a large number of cell types, it has been shown that cell proliferation is controlled by the activity of Ca^{2+}, K^+ and Cl^- channel. Several authors have suggested that the common effect of K^+ and Ca^{2+} channel blockade is a decrease in Ca^{2+} influx, which is indispensable for cell cycle progression. Concerning K^+ channels, their blockade induces, in most cell types, a membrane depolarization that has been shown to reduce Ca^{2+} influx through voltage-independent Ca^{2+} channels (Nilius and Droogmans 1994; Kamouchi et al. 1999). In contrast, it has been shown in hepatocytes that inhibition of proliferation induced by the blockade of K_{ATP} channels altered neither the basal intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) nor the ATP-induced increase in $[Ca^{2+}]_i$ (Malhi et al. 2000). Another explanation for the role of K^+ (and Cl^-) channels in cell proliferation is the control of cell volume (Rouzaire-Dubois and Dubois 1998; Rouzaire-Dubois et al. 2000). This hypothesis is supported by the observations that cell volume increase induced by K^+ and Cl^- channel blockade or down regulation of K^+ channels and hypertonicity decreases the rate of proliferation of several cell types (Xu et al. 1996; Rouzaire-Dubois and

Dubois 1998; Wondergem et al. 2001; Koegel et al. 2003). The fundamental problem investigated in the present work was as follows: were the effects on cell proliferation of a decrease in $[Ca^{2+}]_o$ mediated by or independent of an alteration in cell volume? Recently, we have furnished experimental arguments supporting the new concept that cell proliferation is dependent on three parameters (Eq. (1)), namely: a maximal rate of proliferation (P_{max}) – independent on cell size, an activator (A) and an inhibitor (I) parameter dependent on cell size (Dubois and Rouzaire-Dubois 2004; Rouzaire-Dubois et al. 2004). The present results indicate that, at least in neuroblastoma cells, the effects of Ca^{2+} and cell volume on proliferation are independent and synergistic. According to Eq. (1), $[Ca^{2+}]_o$ influences P_{max} while increases in cell volume, induced here by hypertonicity decreases the volume-dependent parameter I. The decrease in P_{max} induced by low $[Ca^{2+}]_o$ seems to be essentially due to cell detachment from the culture support and cell death (Table 1). As compared with cell number determinations made for proliferation assays after cell centrifugation (Fig. 1), the effects observed without cell centrifugation could be overestimated by the presence of cell debris and dead cells. In spite of this restriction, they strongly suggest that low $[Ca^{2+}]_o$ and/or decrease in Ca^{2+} influx reduces cell proliferation *via* a decrease in the activity of Ca^{2+}-dependent proteins involved in cell anchorage rather than in cell volume regulation.

While the above conclusion appears clear and simple, the reality may be more complex because of the interconnection between ion channel activity, membrane potential, $[Ca^{2+}]_i$ and cell volume (Rouzaire-Dubois et al. 2002; Dubois and Rouzaire-Dubois 2004). Furthermore, because of the interaction between extracellular Ca^{2+} and phospholipids and proteins located at the external face of the membrane, the effects of a decrease in $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ may not be equivalent. While it is very likely that a decrease in $[Ca^{2+}]_o$ should reduce $[Ca^{2+}]_i$, it also increases the surface potential, and consequently modifies the activity of voltage-dependent channels (Hille 1992) and it may alter the activity of proteins involved in cell anchorage (Mizejewski 1999). Consequently and depending on cell types and experiment conditions, $[Ca^{2+}]_o$ and the activity of Ca^{2+}, K^+ and Cl^- channels may control cell proliferation *via* several mechanisms, including regulation of $[Ca^{2+}]_i$ or cell volume and cell adhesion.

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