Background Osmolyte Current Involved in Cell Volume Regulation of Neuroblastoma × Glioma Hybrid NG108-15 Cells

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Abstract. Recently, we showed that at constant extracellular osmolarity, the volume of NG108-15 cells was dependent on the external NaCl concentration and we assumed that the responsible mechanism was mediated by background channels (Rouzaire-Dubois et al 1999) In order to confirm this view, the mean cell volume and the background current of NG108-15 cells were measured under different experimental conditions, after blockade of specific volume regulating mechanisms and ion channels When the external NaCl concentration was decreased, the reversal potential of the background current was shifted toward negative values and the membrane conductance decreased Opposite effects were observed when the NaCl concentration was increased Substitution of external Na^+ with various monovalent cations altered the mean cell volume by Rb^+, +17%, Cs^+, +15%, K^+, +10%, Li^+, −6%, choline, −9%, N-methylglucamine, −25% The reversal potential of the background current and the membrane conductance were altered by these Na^+ substitutes in such a way that the cell volume increased linearly with the background current at −60 mV Substitution of external Cl^- with various monovalent anions altered the mean cell volume by I^-, +4%, Br^-, 0%, NO_3^-, −3%, F^-, −5%, isethionate, −30%, gluconate, −50% Cl^- substitutes did not significantly alter the background current at −60 mV, except F^- which increased it by 39% These results suggest that 1 the cell volume is dependent on ion fluxes through background channels, 2 electrogenic cation fluxes are larger than anionic ones and the background current is proportional to the difference between these fluxes, 3 whereas external cations do not interfere with anion fluxes, external anions alter cation fluxes

Key words: Cell volume — Background channels — Neuroblastoma cells

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Introduction

It is now widely recognized that alterations of cell volume and volume regulating mechanisms participate in a variety of cellular functions (see review by Lang et al. 1998), including cell proliferation and apoptosis (Rouzaire-Dubois and Dubois 1998; Maeno et al. 2000). Following cell volume changes due to alterations of the extracellular osmolarity, rapid (within minutes) cell volume regulations are due to passive movements of solutes and osmotically obligate water across the plasma membrane through selective or non-selective channels (see reviews by Hoffmann and Simonsen 1989; Kirk and Strange 1998). In such a case, the sequence of events are: flux of water – cell volume alteration – activation of ion channels – flux of solutes and osmotically obligate water – cell volume regulation. In contrast, at constant extracellular osmolarity, rapid cell volume changes are the direct consequence of alteration of ion channel activities with the following sequence of events: change of ion channel activities – change in ion and osmotically obligate water fluxes – cell volume alteration. In both of the above cases, the sequences of events are the corollary of two fundamental principles: electroneutrality of the bulk intracellular solution and equality between intra and extracellular osmolarities.

Different types of ion channels involved in cell volume regulating mechanisms have been described both under anisotonic conditions and at constant extracellular osmolarity. Of these channel types, it is generally assumed that the most important and ubiquitous ones are cation and anion channels sensitive to intracellular Ca^{2+} or cell volume (see Lang et al. 1998). Recently, we showed on neuroblastoma × glioma hybrid NG108-15 cells that, when the activity of these specific channels was inhibited, there remained a residual volume regulating mechanism sensitive to the extracellular NaCl or Cl^- concentration (Rouzaire-Dubois et al. 1999). This mechanism being insensitive to classical channel blockers, we assumed that it was mediated by leakage background channels similar to "osmolyte" or non-specific channels described in other cell types (Franciolini and Nonner 1987; Vaca and Kunze 1992; Hall et al. 1996). Since the properties of background channels are almost unknown, the aim of this paper was to describe their ionic selectivity and sensitivity. For this purpose we measured, under different experimental conditions and after blockade of specific regulating volume mechanisms and ion channels, the mean cell volume and membrane leakage current of NG108-15 cells.

Materials and Methods

Cell culture and Materials

The experiments were performed at room temperature on undifferentiated hybrid neuroblastoma × glioma NG108-15 cells. Cell culture was as previously described (Rouzaire-Dubois et al. 1999). Chemical agents used were from Sigma (Saint Quentin Fallavier, France) except 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) which was purchased from Tocris (Bristol, UK).

Cell volume determinations

Cells were sized electronically using a Coulter counter (model ZM) coupled to a Coulter channelizer (model 256) (Coultronics, France) The mean cell volumes were calculated as previously described (Rouzaire-Dubois et al 1999) Prior to cell volume measurement, the cells were mechanically detached from Petri dishes and centrifuged at 100 × *g* for 5 min They were then resuspended in control or test solutions and mechanically stirred Control cell volumes were measured in a standard external solution containing (in mmol/l) NaCl , 140, KCl, 5, $CaCl_2$, 1, $MgCl_2$, 2, caffeine, 10 (to reduce cell shrinkage induced by cell stirring, see Rouzaire Dubois et al 1999), NPPB, 0 1, HEPES, 10, pH adjusted to 7 3 with NaOH Test media were derived from this control standard solution as described in the text

Electrophysiological recordings

Membrane currents were recorded with the standard whole-cell patch-clamp method The bath solution was the standard external solution In order to block voltage dependent K^+, Na^+ and Ca^{2+} currents, this solution was supplemented with 10 mmol/l tetraethylammonium, 1 μmol/l tetrodotoxin and 100 μmol/l $CdCl_2$ The pipette solution contained (in mmol/l) $CsNO_3$, 140, $MgCl_2$, 2, HEPES, 10, pH adjusted to 7 3 with NaOH Cs^+ and NO_3^- were used to block respectively residual voltage dependent K^+ currents and an outward current attributed to the activity of a vacuolar type H^+ pump (see Gerard et al 1994, Rouzaire-Dubois and Dubois 1997) The pipette resistances were 3–5 MΩ when filled with the standard solution and the seal resistances were at least 10 GΩ The zero current was obtained with the electrode in the bath prior to formation of the cell-attached patch configuration Changes in the liquid junction potential at the reference electrode that occurred when the bath solution was changed were minimized by the use of a 140 mmol/l NaCl agar bridge as the reference electrode Currents were recorded during ramp potentials (100 mV/s) applied from −60 mV to +60 mV Under these conditions, the capacity current (C dV/dt) was constant and appeared at the beginning of the ramp potential as an almost instantaneous positive current of 5–10 pA It was subtracted from the total current

Results

Background current

Under control conditions, the leakage or background current was almost linear with voltage (Fig 1) and reversed at +1 ± 1 mV membrane potential (mean ± S E M of 55 cells) At −60 mV, its amplitude was −27 ± 1 pA and the conductance was 0 44 ± 0 02 nS The membrane conductance and the reversal potential or zero current potential were dependent on the external NaCl concentration When it was decreased to 70 mmol/l, the current changed within 1 to 5 s After this period of time, the zero current potential was shifted towards negative values by 5 ± 2 mV and the conductance was decreased by 10 ± 2% (n = 4) (Fig 1A) When the

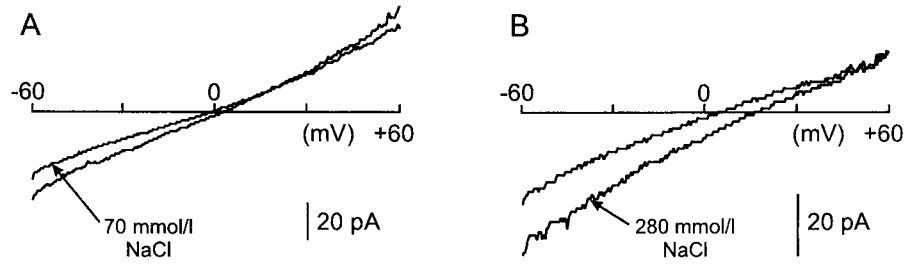

Figure 1. Effects of external NaCl concentration on the background current Currents were recorded during ramp potentials (100 mV/s), applied from −60 mV to +60 mV, in external solutions containing 140 mmol/l NaCl and 70 mmol/l NaCl (**A**) or 280 mmol/l NaCl (**B**) Specific currents were blocked and capacity current was subtracted as described in Methods

external NaCl concentration was increased to 280 mmol/l, the zero current potential was shifted towards positive values by 10 ± 1 mV and the conductance was increased by 47 ± 12% (n = 4) (Fig 1B) These effects indicate that the background current is both cationic and anionic and the permeability to Na^+ is higher than that for Cl^- However, it should be noted that in the above experiments, changes of the NaCl concentration were associated with changes of the external osmolarity Consequently, the effects on the current could be due to cell volume changes and/or alterations of intracellular osmolyte concentrations rather than to changes in extracellular NaCl concentration To test this possibility, the osmolarity of the standard external solution was increased twofold with 280 mmol/l sucrose Under this condition, the current was modified as it was when the osmolarity was increased with NaCl, i e the zero current potential shifted towards positive values and the conductance increased However, in contrast with the effects of NaCl concentration changes, which developed within few seconds, the effects of sucrose addition developed within 30–40 s This suggests an indirect effect of sucrose mediated by a water efflux and a subsequent increase of anion efflux This conclusion was confirmed by the lack of effect of the addition of 280 mmol/l sucrose to the external solution when 110 mmol/l NO_3^- of the pipette solution was equimolarly substituted with aspartate

Effects of cation substitution on cell volume and background current

In a previous work (Rouzaire-Dubois et al 1999), we showed that at constant external osmolarity, the cell volume was dependent on the external NaCl concentration and we suggested that this effect was mediated by the background current In order to confirm this conclusion, we studied here the effects on cell volume and background current of Na^+ substitution with Li^+, K^+, Rb^+, Cs^+, N-methylglucamine (NMG), and choline In NaCl solution, the mean cell volume was 3 69 ± 0 03 pl (mean ± S E M of 17 experiments) The effects of Na^+ substitution on the cell volume were very fast This is illustrated in Fig 2A where RbCl replaced 80% of

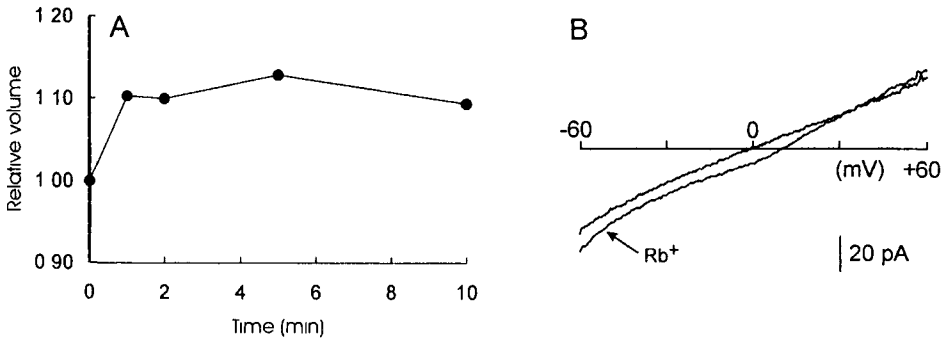

Figure 2. Substitution of external Na^+ with Rb^+ increased the cell volume and the background current **A.** At time zero, the mean cell volume was determined in an external solution containing 140 mmol/l NaCl Then the external solution was diluted with a solution containing 140 mmol/l RbCl in such a way that 80% of Na^+ was substituted with Rb^+ **B.** Current-voltage curves obtained in external solutions containing 140 mmol/l NaCl or 140 mmol/l RbCl

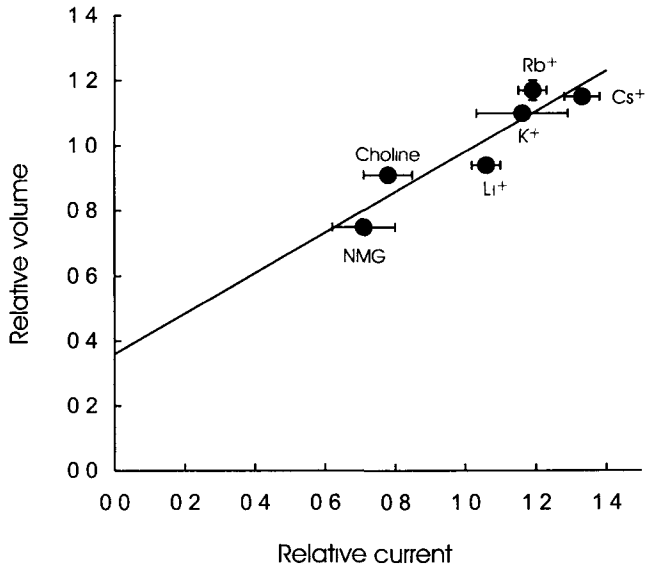

Figure 3. Mean cell volume as a function of background current in the presence of various external Na^+ substitutes Mean cell volume and background current at −60 mV membrane potential were determined in external solutions containing 140 mmol/l of different Cl^- salts and were expressed relative to their respective value determined in a solution containing 140 mmol/l NaCl Means ± S E M of 3 to 7 experiments in each condition The straight line is a linear regression through the points It crosses the vertical axis at 0 36 and has a slope of 0 62

NaCl After this substitution, the mean cell volume increased within 1 min and then remained stable for at least 10 min In the following experiments, cell volumes were measured in parallel cell cultures incubated for 10 min in solutions containing 140 mmol/l Na^+ or 140 mmol/l of its substitutes When expressed relative to its value in Na^+ solution, the mean cell volume was Rb^+, 1 17 ± 0 03, Cs^+, 1 15 ± 0 01, K^+, 1 10 ± 0 00, Li^+, 0 94 ± 0 01, choline, 0 91 ± 0 00, NMG, 0 75 ± 0 01 (means ± S E M of 3 experiments in each condition) Na^+ substitution altered the background current Depending on the substitute, its reversal potential shifted in positive or negative direction and the conductance increased or decreased This is illustrated in Fig 2B which shows the effects of Na^+ substitution with Rb^+ From the zero current potential, the permeability sequence was $P_{Rb} = P_K > P_{Li} = P_{Na} = P_{Cs} > P_{choline} > P_{NMG}$ (Table 1) Since the cell volume was likely dependent on the current in the negative voltage range, we measured the current at −60 mV in the presence of Na^+ and then of one of its substitutes Expressed relative to its value in Na^+ solution, the background current was Cs^+, 1 33 ± 0 05, Rb^+, 1 19 ± 0 04, K^+, 1 16 ± 0 13, Li^+, 1 06 ± 0 04, choline, 0 78 ± 0 07, NMG, 0 71 ± 0 09 (means ± S E M of 3 to 7 experiments in each condition) These results indicate that cell volume changes are associated with changes in the amplitude of the background current This is clear in Fig 3 where the relative mean cell volume was plotted against the relative current From this graph, it can be seen that the cell volume increases linearly with the current, which suggests that the influx of cation through background channels is a key determinant in the control of cell volume

Effects of anion substitution on cell volume and background current

Since, to maintain electroneutrality, influx of cation is necessarily accompanied by anion influx, we studied the effects on cell volume and background current of Cl^-

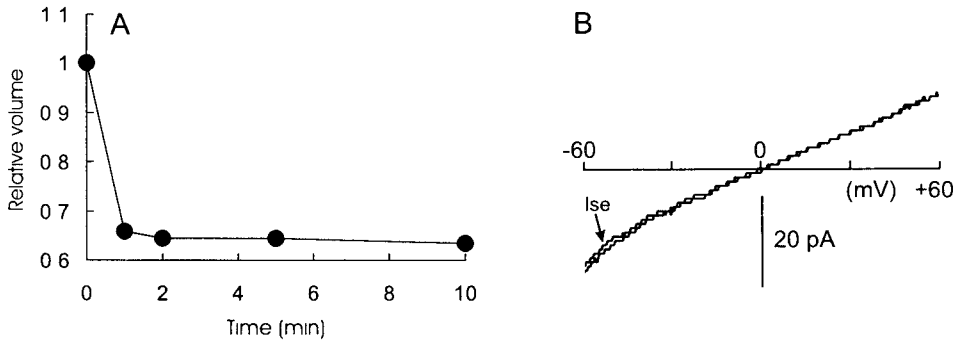

Figure 4. Substitution of external Cl^- with isethionate (Ise) decreased the cell volume but did not alter the background current **A.** The mean cell volume was determined in external NaCl solution (time zero) and then in a solution containing 28 mmol/l NaCl and 112 mmol/l Na^+-isethionate (see experimental procedure in the legend of Fig 2) **B.** Current-voltage curves obtained in external solutions containing 140 mmol/l NaCl or 140 mmol/l Na^+-isethionate

Table 1 Reversal potential of the background current in the presence of various Cl^- or Na^+ salts

Cation	ΔV_{rev} (mV)	Anion	ΔV_{rev} (mV)
Rb^+	+17 ± 2	I^-	−15 ± 2
K^+	+17 ± 1	NO_3^-	−11 ± 1
Li^+	+2 ± 1	Gluconate	−4 ± 1
Cs^+	+1 ± 1	Isethionate	+2 ± 1
Choline	−9 ± 2	Br^-	+3 ± 2
NMG^+	−12 ± 3	F^-	+15 ± 3

Shifts of the reversal potential (ΔV_{rev}) of the background current when 140 mmol/l of external Na^+ or Cl^- were substituted with the indicated ions Mean ± S E M of 3 to 10 experiments in each condition

substitution with F^-, Br^-, I^-, NO_3^-, isethionate (ise) and gluconate (gluc) The effects of Cl^- substitution on cell volume were as fast as that of Na^+ substitution (see Fig 4A) With an experimental protocol similar to that we had used for Na^+ substitution, the mean cell volume relative to its value in Cl^- solution was I^-, 1 04 ± 0 02, Br^-, 1 00 ± 0 00, NO_3^-, 0 97 ± 0 01, F^-, 0 95 ± 0 01, ise, 0 70 ± 0 01, gluc, 0 50 ± 0 01 (means ± S E M of 3 experiments in each condition) Most of the Cl^- substitutes altered the current reversal potential and the conductance From the zero current potential, the permeability sequence was $P_I > P_{NO3} > P_{gluc} > P_{ise} = P_{Cl} = P_{Br} > P_F$ (Table 1) However, with the exception of F^-, the current at −60 mV was poorly dependent on the anion species (Figs 4B and 5) Expressed relative to its value in Cl^- solution, the background current was F^-, 1 39 ± 0 05, Br^-, 1 05 ±0 03, NO_3^-, 0 98 ± 0 06, I^-, 0 97 ± 0 04, ise, 0 90 ± 0 04, gluc, 0 89 ± 0 06 (mean ± S E M of 3 to 10 experiments in each condition)

Discussion

The present results show that an increase of the external NaCl concentration shifts the reversal potential of the background current towards positive values and increases the conductance and that opposite effects are observed when the NaCl concentration is decreased This indicates that background channels are more permeable to Na^+ than to Cl^- In order to study the ionic selectivity and sensitivity of these channels, we have measured the mean cell volume and the background current in the presence of various Cl^- or Na^+ salts

Cation substitution reveals a linear relationship between the cell volume and the current at −60 mV (Fig 3), a membrane potential closed to the resting potential under physiological conditions (Rouzaire-Dubois and Dubois 1997) This observation raises several questions First, one can ask whether the background current at −60 mV is an indication of ion fluxes at rest The measured current is the sum of the membrane current and the shunt current flowing through the seal

resistance (Dubois 2000) Fischmeister et al (1986) and Sachs and Qin (1993) have shown that the seal between a patch pipette and a cell or an insulating Sylgard surface had an ionic selectivity and its resistance was voltage dependent According to Sachs and Qin, the seal exhibits the following permeability ratios P_{Cs}/P_{Cl} = 51 1 and P_{Cs}/P_{Na} = 4 64 Given that in most of our experiments, pipette and external media were different, one can ask whether the ionic selectivity of the seal cannot take account of changes in background current observed during ion substitutions This seems unlikely for the following 1 under control conditions (external Na^+ and intrapipette Cs^+), the reversal potential of the background current was +1 mV while according to Sachs and Qin it should be −39 mV, 2 the cationic selectivity of the background current (see results) was different than that obtained by Sachs and Qin ($P_{Na} > P_K > P_{Cs} > P_{Rb} > P_{Li}$) and 3 if one assumes that the seal resistance (> 10 GΩ) did not decrease when establishing the whole-cell configuration, the shunt current at −60 mV was < 6 pA and the membrane current represented at least 78% of the measured current (27 pA) Moreover, while the background conductance in NaCl solution (0 44 nS) should be the major conductance at rest (0 29–0 71 nS) (Rouzaire-Dubois and Dubois 1997), it is no longer so in KCl solution and also presumably in RbCl solution, where voltage-dependent K^+ channels are activated However, it should be noted that while voltage dependent K^+ channels contribute to the genesis of membrane potential and volume regulation of NG 108-15 cells, their blockade has only small effects on membrane potential and induces cell swelling which develops within several hours (Rouzaire-Dubois and Dubois 1997, 1998), i e , much slower than cell volume changes reported here All these considerations are in favour of the idea that the background current at −60 mV can be considered as an indication of ion fluxes at the resting potential Assuming a proportional relationship between the cell volume and the background current recorded in the presence of various cation species, a point that deserves attention is what is the proportionality factor? Taking into account that cation fluxes are accompanied by anion fluxes, the background current should be proportional to the difference between these fluxes In external NaCl solution containing NPPB, the mean cell volume was 3 69 pl (see Results) With an intracellular cation concentration of 140 mmol/l, the quantity of cations within a cell was $3\ 69 \times 10^{-12} \times 0\ 14 \times 6\ 02 \times 10^{23} = 3\ 11 \times 10^{11}$ In the presence of Rb^+, the mean cell volume increased by 17% , which corresponds to a net influx of $3\ 11 \times 10^{11} \times 0\ 17 = 0\ 53 \times 10^{11}$ cations or $0\ 53 \times 10^{11} \times 1\ 6 \times 10^{-19} = 0\ 85 \times 10^{-8}$ coulomb Within 1 min, this gave an additional inward cation current of 141 pA whereas the additional background current was only $27 \times 0\ 19 = 5\ 13$ pA, i e 27 fold smaller If one applies the same reasoning with NMG, we find that cell shrinkage corresponded to an additional outward cation current of 207 pA and an additional outward background current of 7 83 pA, i e 26 fold smaller The most likely explanation for these discrepancies is that the electrogenic cation flux is larger than the anion one and the background current is proportional to the difference between these fluxes

In contrast with cation substitution, anion substitution does not reveal any relationship between the cell volume and the current (Fig 5) or the permeability

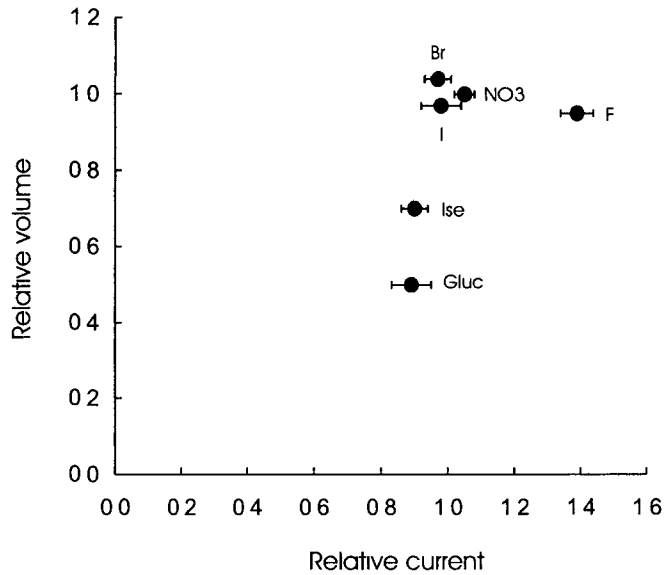

Figure 5. Mean cell volume as a function of background current in the presence of various external Cl^- substitutes Mean cell volumes and background current at −60 mV membrane potential were determined and expressed as in Fig 3 in the presence of different Na^+ salts Means ± S E M of 3 to 10 experiments in each condition

coefficient For instance, with F^-, the cell volume was decreased by 5% while the current was increased by 39% and with gluconate, the volume was decreased by 50% while the current was decreased by 11% This can be explained because either anions are transported by electroneutral systems or anions interact with cation fluxes The fact that the effects of Cl^- substitution on cell volume are very fast (see Fig 4A) favours the second hypothesis, supported by previous observations On one hand, the activities of selective or non-selective cation channels of various cell types have been shown to be dependent on the Cl^- concentration and blocked by organic anions (Franciolini and Nonner 1987, Laurienti and Blankenship 1996, Wang et al 1999, Kilb and Luhmann 2000, Yuan et al 2000) On the other hand, fluoride has been shown to increase the open probability of selective or non-selective cation channels in red blood cells (Varecka et al 1994), platelets (Stamouli et al 1993) and osteoblastic cells (Gofa and Davidson 1996) If these conclusions apply to NG108-15 cells, they may explain that organic anions reduce the cell volume while having very small effects on the background current Indeed, if the poorly permeant organic anions reduce the cation influx, they should induce cell shrinkage but have a small effect on the background current which is proportional to the difference between cation and anion fluxes (see above) With the same reasoning, if F^- is less permeant than Cl^- but increases the cation current, the cell volume

should be hardly altered while the background current at negative voltages should be increased. In such a case, it should be noted that the positive shift of the background current reversal potential reflects both the decrease in anion influx and the increase in cation influx.

The present results do not allow to conclude whether the background current is carried by either a single type of channels permeable to both cations and anions or by parallel cation and anion channels. However, they show that the cell volume is very sensitive to the extracellular ionic composition and they suggest that non-selective channels mediate this property. These channels not only control the cell volume but also probably the membrane potential and the free intracellular Ca^{2+} concentration (Kamouchi et al. 1999; Dubois 2000). Since these parameters are of importance in a variety of cellular functions, including cell proliferation and apoptosis (Maeno et al. 2000; Rouzaire-Dubois et al. 2000), it would be interesting to find therapeutic agents which act specifically on background channels

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