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ELECTROPHYSIOLOGY AND PHARMACOLOGY STUDY OF A HUMAN NEUROBLASTOMA CELL LINE*

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Abstract. *Via* patch-clamp experiments on undifferentiated human neuroblastoma cells we identified tetrodotoxin-sensitive Na^+ channels, delayed rectifier K^+ channels, while Ca^{2+} channels, transient outward K^+ channels and hyperpolarization-activated channels were absent.

Key words: SH-SY5Y neuroblastoma cell line, whole-cell patch-clamp, delayed rectifier K^+ current, voltage-dependent Na^+ current, steady-state inactivation, recovery from inactivation.

1. INTRODUCTION

Neuroblastoma is a tumour formed by neuronal precursors of the sympathetic nervous system (SNS), derived from the neural crests, present at birth or detected during the first years of childhood, usually before the age of 10. The primary tumour can be located in any SNS structure, most frequently in one of the adrenal glands, but also in the sympathetic nerve tissue of the neck, chest, abdomen or pelvis. During embryonic development, a common sympathoadrenal precursor differentiates into three distinct cell lineages: chromaffin cells, that in turn generate the adrenal gland and paraganglia, and may degenerate into pheochromocytoma and sympathetic paraganglioma, respectively, small intensely fluorescent or SIF cells, and sympathetic neuroblasts. The SIF cells further condense into sympathetic ganglia, while sympathetic neuroblasts generate sympathetic neurons with bodies within sympathetic ganglia and the developing adrenal gland [1]. The latter two lineages can both generate neuroblastomas, lobular with neuroendocrine differentiation capacity and adrenal/extraadrenal, respectively. Neuroblastomas

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present different degrees of differentiation, being classified into 5 stages according to the degree of undifferentiation and invasiveness. Stage 1 and 2 tumors are usually cured by surgery alone, while more advanced stages require combined methods including radio- and chemotherapy, bone marrow/hematopoietic stem cell transplantation, differentiation agents, antibodies and cytokines GM-CSF and IL-2. From a historical perspective, the first description of such a tumour with abdominal location in a child belongs to the famous founder of cell pathology, Rudolf Virchow, in 1864, when the tumour was classified as "glioma". In 1891, Felix Marchand established the origin from the SNS and adrenal medulla. In 1901, William Pepper described the distinctive stage 4S in infants, with dissemination limited to liver, skin or bone marrow, and in 1910, James Homer Wright demonstrated the origin of the tumour in the neural crests and named it neuroblastoma, also describing "Homer-Wright pseudorosettes" in bone marrow samples. The etiology of neuroblastoma is far from being understood. Although most cases do not show familial inheritance, in the 1–2 % familial cases several rare germline mutations have been described in the genes ALK (anaplastic lymphoma kinase) [2], PHOX2A and KIF1B. Familial neuroblastomas are also associated with neurofibromatosis type 1 and the Beckwith-Wiedemann syndrome. Other genetic abnormalities include amplification of the N-myc oncogene in advanced stages of disease [3], duplicated segments of the LMO1 gene [4], copy-number variation in the NBPF10 gene resulting in the 1q21.1 deletion syndrome or 1q21.1 duplication syndrome [5].

Neuroblastoma cells show variable degrees of differentiation, ranging from undifferentiated cells, which are round and small cells with scant cytoplasm, to differentiated cells with larger nuclei and cytoplasm. Certain tumours, classified as ganglioneuroblastoma, contain a mixture of these two phenotypes, while other benign tumours contain exclusively ganglion-like cells and are known as ganglioneuromas. In the 1920s, Cushing and Wohlbach found a case with benign ganglioneuroma cells in a lymph node, assuming they were derived from a neuroblastoma metastasis *via* spontaneous differentiation. In the 1970s, several stabilized *in vitro* neuroblastoma cell lines were established. Thus, in 1970, June L. Biedler at the Memorial Sloan-Kettering Cancer Center in New York developed a cell line from a bone marrow aspiration sample containing neuroblastoma metastases removed from a 4-year old female patient (L.S.), named SK-N-SH, because it contained cells with 3 different phenotypes: sympathoadrenal neuroblasts (N), substrate-adherent non-neuronal (S), and intermediate (I). The S population includes large epithelioid cells (SK), and small "spiny" cells with scant cytoplasm forming focal aggregates, predominant in aged cultures (SH) [6]. This cell line was cloned thrice (SK-N-SH → SH-SY → SH-SY5 → SH-SY5Y), resulting in the SH-SY5Y cell line, characterized by clusters of neuroblastic cells with multiple short fine neurites, that aggregate, form clumps and float (Fig. 1).

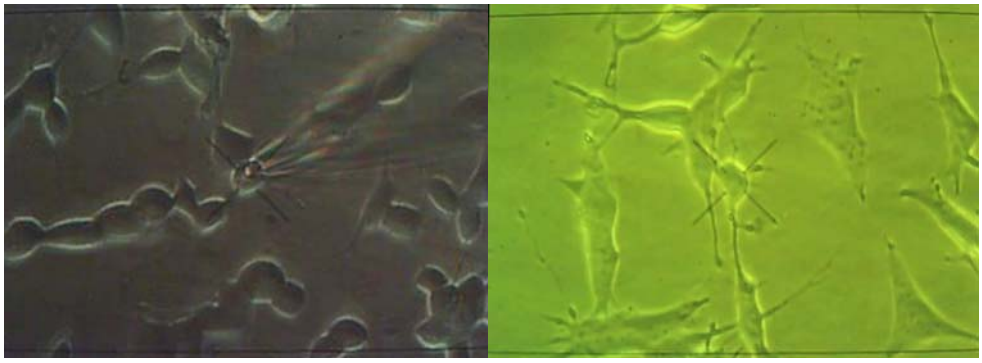

Fig. 1 – Undifferentiated SH-SY5Y cells in culture (left) and neuron-like morphology after treatment with all-trans retinoic acid (right); in the left field, the cell in the middle is approached with a patch pipette.

The SH-SY5Y cell line was studied extensively as a model for neuronal differentiation. The first attempt to differentiate *in vitro* these cells was performed in the early 1980s by the group of Sven Påhlman, using a phorbol ester derivative, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) [7]. The induction of neurites was paralleled by accumulation of norepinephrine and neuron-specific enolase (NSE), markers of differentiation used in diagnosis of neuroblastoma. Since then, many other methods have been used to differentiate this neuroblastoma cell line, including biologically active phorbol esters [8], *all-trans* retinoic acid (ATRA) [9], growth factors such as NGF and BDNF [10], dibutyryl cyclic AMP (dBcAMP) [11], purines [12], staurosporine [13], and even serum-free culture media [14, 15].

Differentiation induced in *in vitro* experiments fueled hope to use this method as a clinical treatment for neuroblastoma and other tumours. So far, natural and synthetic retinoids have shown the highest clinical effectiveness in curing neuroblastoma patients with residual disease, as well as other hematological malignancies, most notably acute promyelocytic leukemia [16]. On the other hand, hypoxia has been shown to induce neuroblastoma dedifferentiation and reduction in expression of neuronal and neuroendocrine markers such as neurofilaments, neuropeptide Y (NPY), growth-associated protein (GAP43), and chromogranins A and B [1].

Retinoids and other differentiation agents induce morphology changes and expression of several markers, such as the microtubule-associated proteins doublecortin (DCX) and lissencephaly-1 (LIS1), semaphorin 3B and SPARC (osteonectin) (reduction in expression), cytoskeletal proteins vimentin (VIM), neurofilament-68 (NF-68), and tau, GAP-43, NeuN (neuronal nuclear antigen), receptors for neurotrophic factors, neuropeptides, neurosecretory granulae and associated proteins such as NSE and neurophysin [17].

Beyond changes in various phenotype markers, differentiation induces a mature adrenergic neuron-like ion channel expression pattern, including higher densities and modified molecular identities of voltage-dependent Na^+ and K^+

channels [18, 19] leading to increased frequencies of spontaneous firing of action potentials and, most remarkably, appearance of voltage-gated Ca^{2+} channels, located almost exclusively in growth cones and varicosities [13]. These channels are predominantly of N and L-type [20, 21], confirming earlier findings of increased radioactive $^{45}Ca^{2+}$ uptake in neuroblastoma cells upon depolarization and application of dihydropyridine agonist BAY K8644 [22]. The ω-conotoxin-sensitive N-type current, inhibited by NPY [23] or muscarine [24], has been since extensively used in pharmacology experiments to titrate effectiveness of novel computer-aided designed drug blockers [25]. In addition to such a purpose, the present study aims to refine experimental methods, consisting in combinations of adequate voltage protocols, solutions and pharmacological compounds, in order to apply them for electrophysiology characterization of bone marrow-derived mesenchimal stem cells cultured in undifferentiating conditions.

2. MATERIALS AND METHODS

2.1. SH-SY5Y CELLS

The cell line used in experiments, kindly provided by Dr. Bogdan Popescu and Teddy Regalia, was cultured in standard conditions (Dulbecco's Modified Eagle's Medium supplemented with 10 % fetal bovine serum and 1 % penicillin-streptomycin, weekly passage *via* detachment with trypsin-EDTA, 5-min centrifugation at 1000 rcf, resuspension of the pellet resulted from a 25 cm^2 flask in 2 mL of medium, and seeding of a new flask with 100-200 μL of suspension). The cells were plated on Petri dishes with an outer diameter of 35 mm (Nunc Intermed, Roskilde, DK).

2.2. ELECTROPHYSIOLOGY EXPERIMENTS – PATCH-CLAMP

Patch pipettes were manufactured from borosilicate glass capillaries with internal filament (GC150F-10, Harvard Apparatus Ltd., Edenbridge, UK), having an outer diameter of 1.50 mm and an inner diameter of 0.86 mm, *via* pulling in 4 steps with a computerized vertical puller (PUL-100, WPI, Sarasosta, FL), and thermal polishing of the tip up to a resistance of 1-3 MΩ when immersed in the working solution. Experiments were performed at room temperature, on the platform of an Olympus IMT-2 inverted microscope, equipped with a Peltier-driven temperature controller (model TC202A, Harvard Apparatus). Currents or potentials were processed with a WPC-100 amplifier (ESF electronic, Göttingen, DE), low-pass filtered at 3 kHz with the built-in 4-pole Bessel filter, numerically converted with a Digidata 1322A interface controlled by the Clampex8.2 module of the pClamp software, and stored on the recording computer hard disk for further analysis with the Clampfit module.

2.3. SOLUTIONS

The external solution was K^+ Tyrode, with the following composition (in mM): NaCl 135, KCl 5.4, NaH_2PO_4 0.33, HEPES 10, and pH 7.40 at 25 °C titrated with NaOH 1 M. $CaCl_2$ 1.8 mM, $MgCl_2$ 0.9 mM and D-glucose 10 mM were freshly added from 1 M stock solutions during the day of experiment. We used several pipette solutions. We started with a complex solution: K aspartate 130, KCl 25, EGTA 1, MgATP 5.5, LiGTP 0.1, HEPES 5, pH 7.21 at 25 °C with KOH 1M, a solution that features physiological concentrations of intracellular chloride. Subsequently we switched to simplified solutions: CsCl 140, EGTA 5, HEPES 10, pH 7.21 at 25 °C with CsOH, to study voltage-dependent Na^+ currents, or the equivalent solution with K^+ instead of Cs^+ for pharmacology assays on K^+ currents, noting an easier formation of seals compared to the initial solution. For certain experiments we used Na^+-free external solution, with Na^+ replaced by N-methyl-D-glucamine ($NMDG^+$) at the same concentration. The applied pharmacological compounds were 4-aminopyridine (4-AP) 3 mM, tetraethylammonium (TEA) 5 mM, and nifedipine 0.1 or 100 μM, freshly prepared from stock solutions, either aqueous (4-AP and TEA) or in pure ethanol (nifedipine), directly in the syringes of the application system. The application system was home-made, composed of a 8-way manifold built from G18 syringe needles ended in a low-dead-volume mixing chamber adapted from an Eppendorf gel-loader. The 8 ways were connected to 20-mL syringes *via* plastic catheters, and perfusion was gravity-driven, at a flow rate of ~ 1 mL/min.

2.4. DATA PROCESSING

All data are reported as mean ± SEM or mean ± SD, as appropriate, *n* indicating in each case the number of experiments (*i.e.* the number of approached cells). Data series were non-linearly fitted with exponential or Boltzmann charge-voltage functions using a Levenberg-Marquardt algorithm.

3. RESULTS

3.1. GENERAL ELECTRICAL PROPERTIES OF THE CELLS

The electrical access in the whole-cell configuration was generally very good; access resistances were often below 10 MΩ due to the use of large tip pipettes, with thick rims, manufactured from hard borosilicate glass with special adhesion properties to the cell membrane. The holding currents were often below –100 pA. For this reason we did not apply series resistance compensation, performing just capacitive transient cancellation by setting the G_{series}, C_{slow}, C_{fast} and

τ C_{fast} dials on the front panel of the amplifier. Seal stability was satisfactory, recording conditions were kept steady until the end of experimental protocols in 44 of 60 cells (73.33 %). The approached cells were small in size, with a membrane capacity C_m = 15.2 ± 8.8 pF (mean ± SD, n = 60), and a membrane resistance R_m = 637.9 ± 614.4 MΩ (n = 53). The pipette resistance was 2.6 ± 0.8 MΩ (n = 53), the access resistance R_a = 12.5 ± ±7.9 MΩ (n = 60), the time constant of the equivalent electrical circuit 205 ± 228 μs (n = 60), and the holding current at -80 mV -147 ± 310 pA (n = 60).

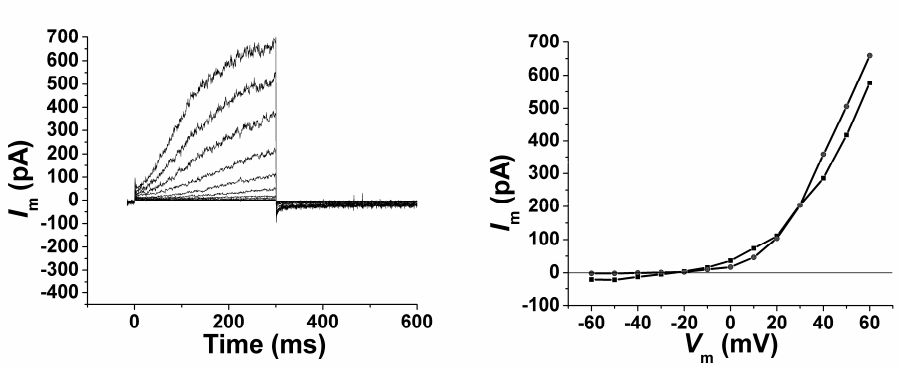

Fig. 2 – Delayed rectifier K^+ currents recorded with a complex pipette solution (left) and the *I-V* plot of this current component in two typical experiments (right).

Part of the SH-SY5Y cell culture dishes were pretreated with *all-trans* retinoic acid (ATRA – Sigma R2625 50 mg) at concentrations of 10 μM, 1 μM, and 0.1 μM, added to the culture medium from a stock solution in DMSO and kept for 24 hours, but these dishes were not currently used for electrophysiology experiments. Examination of ATRA-treated cell cultures with phase contrast microscopy revealed a relative abundance of neuronal cell phenotypes, with round soma and multiple dendrite-like neurites, while in untreated cultures the spindle-like phenotype with reduced prolongations, both numerically and in length, was predominant (Fig. 1). The cells treated with high ATRA concentrations featured increased mechanical resistance at the approach with a patch pipette; pipette attachment and patch rupture were difficult, probably due to overexpression of membrane-associated cytoskeletal components leading to increased membrane rigidity.

3.2. DELAYED RECTIFIER K^+ CURRENTS

Using the complex pipette solution containing K^+ aspartate, ATP, GTP, and Mg^{2+}, we could record delayed-rectifier K^+ currents that were insensitive to 4-AP 3 mM and nifedipine 100 μM (Fig. 2). The I–V curve of these currents shows a pronounced outward rectification and a reversal potential more positive than the K^+

equilibrium potential for the combination of external and internal solutions, possibly due to contamination with non-selective cation current components. We did not record transient outward (I_{to}) or hyperpolarization-activated (funny – I_f) currents. In further experiments we used the simple K^+-based pipette solution. By applying 5 mM TEA in the external solution we noticed a slightly voltage-dependent block (slight increase at depolarized potentials), amounting to 51 ± 8 % of the initial plateau current level at +60 mV (mean ± SEM, n = 5), reversible upon washout of the blocker. Varying the external Mg^{2+} concentration in $NMDG^+$ Na^+-free solutions we obtained a slowdown in activation kinetics as $[Mg^{2+}]_o$ was increased from 0 to 1.8 and further to 5 mM (Fig. 3).

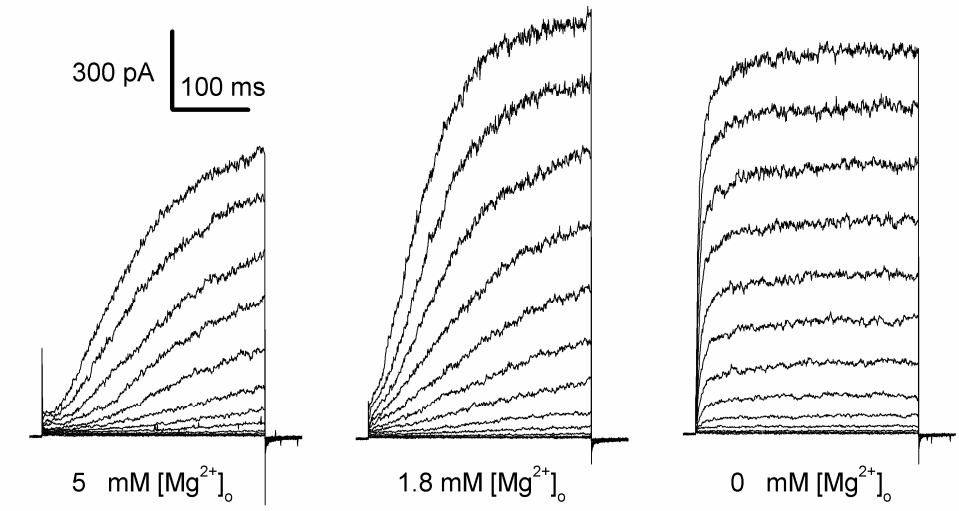

Fig. 3 – Dependence of the activation of delayed rectifier K^+ current on extracellular Mg^{2+} concentration; simple K^+-based pipette solution.

3.3. VOLTAGE-DEPENDENT Na^+ CURRENTS

In a large number of SH-SY5Y cells approached in the whole-cell patch-clamp configuration we retrieved voltage-dependent Na^+ currents, using a Cs^+-based pipette solution to block the majority of outward K^+ current components. The proof of a genuine Na^+ current was provided by its reversible disappearance when using an extracellular solution where Na^+ was replaced by an impermeant organic cation, $NMDG^+$ (Fig. 4), and by its insensitivity to nifedipine 0.1 μM. In Fig. 5 (left) we show a typical recording using a double pulse protocol with a long (1000 ms) holding at different potentials followed by a second activating pulse to assess steady-state inactivation and its voltage dependence. In the right panel of the same figure, we show average data of n = 7 experiments representing the steady-state inactivation variable (h_∞), computed by normalization of peak current elicited by

the final depolarizing pulse relative to its value when the potential between the two depolarizing pulses was –120 mV. Data were fitted with a Boltzmann charge-voltage function that yielded a half-inactivating potential $V_{1/2}$ = –84.8 mV and a slope factor of 5.6 mV/*e*-fold change.

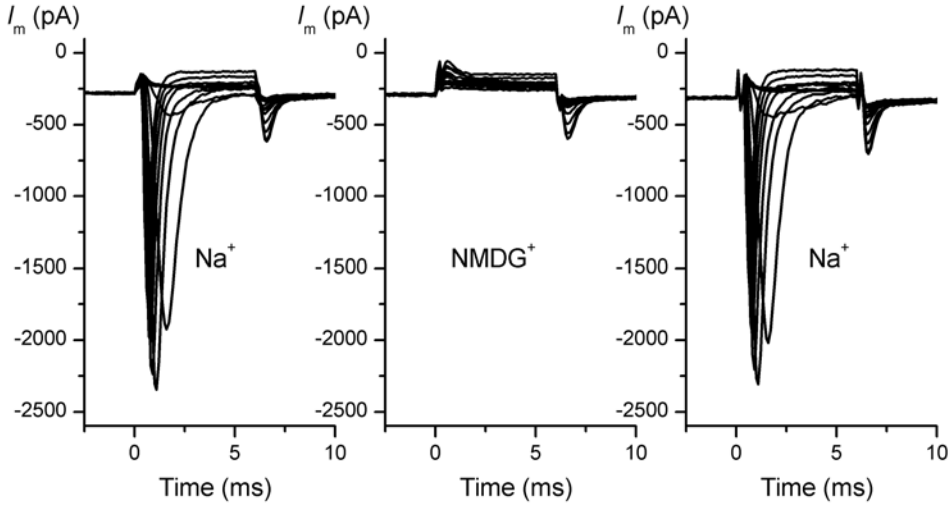

Fig. 4 – Reversible removal of inward currents recorded in a SH-SY5Y cell upon external Na^+ replacement with $NMDG^+$. This experiment, along with insensitivity to nifedipine 0.1 μM, is a proof of a genuine voltage-dependent Na^+ current.

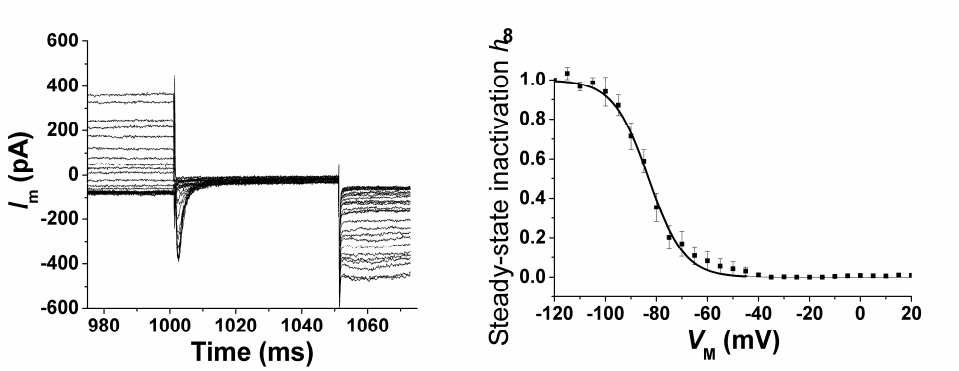

Fig. 5 – Na^+ currents recorded after a long pre-pulse at different potentials to assess steady-state inactivation (left) and the values of steady-state inactivation variable h_∞ (averages ± SEM of n = 7 experiments) fitted with a Boltzmann charge-voltage equation (right).

In Fig. 6 we show another complex voltage protocol used to assess recovery from inactivation at –120 mV. Starting from a holding potential of –120 mV, a first depolarizing pulse to –30 mV lasting 1000 ms elicited a maximal Na^+ current

activation, because all channels were removed from inactivation. This long depolarization at –30 mV completely inactivated all channels, and was followed by a second briefer depolarization at the same potential (–30 mV), after a variable period spent at –120 mV, whereby a variable proportion of Na^+ channels recovered from inactivation, yielding a second Na^+ current with variable peak amplitude, proportional to the percentage of recovered channels. The series of peak current amplitudes was fitted with an exponential function, and thus we obtained a time constant τ of recovery from inactivation in the range of 12.4–17.5 ms.

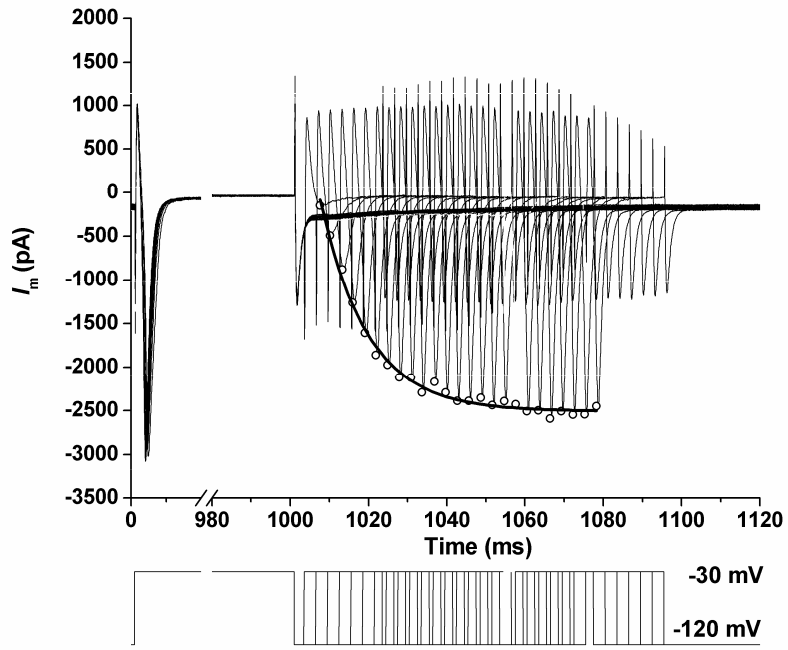

Fig. 6 – Repeated double-pulse voltage-clamp protocol used to study recovery from inactivation of Na^+ channels, and an exponential fit of peak Na^+ currents elicited by the second pulse used to assess the time constant of recovery from inactivation. Upper traces: whole-cell currents; lower traces: command voltage. During the 1-s holding at –30 mV Na^+ channels inactivate completely, and recover progressively during the variable period at –120 mV before the second depolarizing pulse; thus, the peak inward current amplitude at the second pulse reflects the degree of recovery form inactivation.

We also studied electrical excitability of SH-SY5Y cells *via* current-clamp recordings, using pulses of injected current with duration of 100 ms and amplitudes between 100 and 500 pA in 100 pA increments. In most cells we found a relative deficit of repolarizing outward currents (K^+ and anion currents), and thus depolarization spikes elicited by injected current stimuli were not followed by a fast repolarization, like in a normal neuronal action potential, but, after a brief overshoot, a plateau at positive potentials persisted over the entire duration of the current stimulus.

4. DISCUSSION

Using appropriate experimental protocols in undifferentiated SH-SY5Y neuroblastoma cells approached in the ruptured whole-cell patch-clamp configuration we identified and characterized several voltage-dependent ion current components: a delayed rectifier K^+ current (I_K), a TTX-sensitive-like Na^+ current (I_{Na}), and, within a unique experiment, a maxi-K-like single-channel current.

The properties of the delayed rectifier current are similar to a certain extent to those described in SH-SY5Y and other neuroblastoma cell lines in previous studies. Thus, in 1995, Lesser and Lo described a delayed rectifier K^+ current (I_K) in SK-N-SH cells, the precursors of SH-SY5Y [26]. These currents were up-regulated upon cell differentiation with NGF (nerve growth factor) or CTNF (ciliary neurotrophic factor). Their I-V curves are very similar to those retrieved in our study, while their macroscopic activation time constants (2 ms at +50 mV and 11 ms at +10 mV), are somewhat similar to the fast time constants in our experiments in 0 external Mg^{2+}, using double exponential fits (8.7 ms at +50 mV and 11.0 ms at +10 mV). However, in their study the delayed rectifier current was completely blocked by external 1 mM 4-AP and 5 mM TEA in undifferentiated and CTNF-treated cells, while in NGF-treated cells a small TEA-resistant component appeared. In 2003, Friederich *et al*. identified the main delayed rectifier I_K component in SH-SY5Y cells as Kv3.1, using electrophysiology and RT-PCR methods [27]. Actually, there should be a variable mixture of main and auxiliary subunits largely depending on cell phenotype and culture conditions. An interesting finding was the variation in I_K current activation kinetics with external Mg^{2+} concentration. This effect may be accounted for by changes in the transbilayer electrical field intensity due to surface charge effects [28]. Such effects may occur due to higher membrane surface densities of polysialated neural cell adhesion molecules (PSA-NCAM) in these cells [29], but most likely result from direct interactions of Mg^{2+} with charged regions of the voltage-sensing domain. Increase of external Mg^{2+} concentration reveals sigmoidicity in the current activation curves (Fig. 3), indicating that a more complex multistep activation model should be used to fit the data instead of simple exponential functions.

Voltage-dependent Na^+ currents have been also extensively studied in various neuroblastoma cell lines [18]. In fact, these cell lines represent a traditional and convenient preparation employed for characterizing the intricate details of Na^+ channel gating. Thus, in a classical study, Aldrich and Stevens used deconvolution methods to explore latency to first closure distribution and the reopening phenomenon in single-channel recordings on N1E115 cultured neuroblastoma cells [30]. Later, Chinn and Narahashi explored the effects of the pyrethroid insecticide deltamethrin on Na^+ channels from mouse neuroblastoma cells, identifying temperature-dependent subconductance states and changes in kinetics [31]. It is usually assumed that differentiation of neuroblastoma cells increases the density and changes the phenotype of voltage-dependent Na^+ currents, leading to an

improved excitability [19, 26]. We found very encouraging the fact that the parameters of steady-state inactivation and recovery from inactivation of I_{Na} are in very good agreement with previous measurements performed in 1994 by Brown, Kemp and Seabrook on the same cell line [32]. Thus, the $V_{1/2}$ for inactivation in their study was –86 ± 3 mV (n = 11), compared to –84.8 mV (n = 7) in our study; the time constant of recovery from inactivation was 15.9 ± 2.6 ms (n = 4) at -100 mV, while in our study it was in the range 12.4–17.5 ms (n = 3) at –120 mV. These authors found that most of the voltage-dependent Na^+ current was blocked by TTX in the low nanomolar range (K_D of 4 nM and complete block at 40 nM), and explained the peculiar voltage dependency of inactivation by absence of auxiliary regulatory subunits. In fact, in our undifferentiated SH-SY5Y cell line a small proportion of TTX-resistant Na^+ channels may persist [19, 33], explaining the slight deviations from a sigmoid in the lower inflexion of the h_∞ *vs*. voltage graph shown in Fig. 6 (right). Our steady-state inactivation parameters are also in very good agreement with those reported in the 1997 study of Gu, Waxman *et al*. [33], who retrieved a $V_{1/2}$ for h_∞ of –81.8 mV for normalized peak Na^+ currents in B105 cells, and a slope factor of 5.1 mV/*e*-fold change compared to 5.6 mV/*e*-fold change in our study.

Another surprising finding of our study was the complete lack of voltage-dependent Ca^{2+} currents, either of N type or L type. These channels may be only expressed upon differentiation of the cell line [21], although certain studies reported the same N and L-type current components in undifferentiated SH-SY5Y cells [34]. There may be also other inhibitory effects on Ca^{2+} currents in this cell line, like those of NPY [23] or muscarine [24]. A recent extensive study using molecular biology and electrophysiology methods screened in depth the expression and pharmacology of different endogenous voltage-dependent Ca^{2+} currents in the SH-SY5Y cell line [35].

In conclusion, using complex electrophysiology protocols we have provided a preliminary exploration of membrane ion currents in undifferentiated SH-SY5Y human neuroblastoma cells, identifying and characterizing delayed rectifier K^+ currents, the major component of which is presumably Kv3.1, as well as TTX-sensitive-like Na^+ currents, and possibly maxi-K Ca-dependent swelling-activated K^+ currents, and failed to identify transient outward currents (I_{to}) or hyperpolarization-activated funny currents (I_f). The study can be further extended by exploration of still other current components such as non-selective cation currents or chloride currents, by exploring changes induced by differentiation of these neuroblastoma cells with various agents. The protocols refined herein can be easily applied to characterize other cell lines of interest, such as bone-marrow derived mesenchimal stromal cells exposed to different environmental conditions.

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