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$Ca_V1.2$ and $Ca_V1.3$ L-type calcium channels regulate the resting membrane potential but not the expression of calcium transporters in differentiated PC12 cells

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Abstract. PC12 cells differentiated under the influence of the neuronal growth factor (NGF) serve as a model of both sympathetic neurons and chromaffin cells. NGF-induced differentiation critically depends on elevated intracellular calcium concentration. Main pathway for Ca^{2+} entry in excitable cells is represented by voltage-dependent calcium channels including L-type calcium channels (LTCC). We investigated role of $Ca_V1.2$ and $Ca_V1.3$ LTCC subtypes in NGF-differentiated PC12 cells. The expression of LTCC subtypes was downregulated by transfection of NGF-differentiated PC12 cells with siRNA for either *CACNA1C* or *CACNA1D* gene. Efficiency of gene silencing was verified by RT-PCR and by functional essay. The dominant LTCC subtype in PC12 cells was $Ca_V1.2$. Downregulation of either LTCC significantly hyperpolarized the resting membrane potential. Expression of mRNA for intracellular calcium transporters inositol trisphosphate receptor type 1, 2 and 3, ryanodine receptor type 1 and 2 and sarco/endoplasmic reticulum Ca^{2+} ATPase type 2 as well as plasma membrane transporters Na^+-Ca^{2+} exchanger type 1 and 2 was not altered in the absence of either LTCC subtype. In conclusion, Ca^{2+} influx through $Ca_V1.2$ or to $Ca_V1.3$ channel subtypes contributes to maintenance of the resting membrane potentials of NGF-differentiated PC12 cells but is not required for regulation of expression of genes for calcium-transporting proteins.

Key words: PC12 cells — $Ca_V1.2$ — $Ca_V1.3$ — IP_3 receptor — Ryanodine receptor — Na^+-Ca^{2+}-exchanger — Resting membrane potential

Introduction

PC12 cell line was established by Greene and Tischler (1976) from a rat adrenal pheochromocytoma. These cells are commonly used as a model of neurodifferentiation and are considered as a sympathetic neuron-like and/or chromaffin cell-like. Differentiation of PC12 cells can be activated by multiple external factors including neuronal growth factor (NGF). Ca^{2+} entry from extracellular space was required for differentiation and significant upregulation of L-type calcium current accompanied it (Lievano et al. 1994; Lichvarova et al. 2012).

Regulation of calcium homeostasis in NGF-differentiated PC12 cells is more close to that of sympathetic neurons than that of chromaffin cells (Duman et al. 2008). Similar to chromaffin cells (Morgado-Valle et al. 1998), NGF upregulated the expression of L-type calcium channels (LTCC) in sympathetic neurons, as well (Lei et al. 1997; Ford et al. 2008). Calcium influx through LTCC significantly contributes to activity-dependent gene transcription in both sympathetic and CNS neurons in spite that these channels contribute only a lesser part of total calcium current (Morgan and Curran 1986; Bito et al. 1996; Zhao et al. 2007). It is tempting to expect that calcium influx through LTCC plays a role in neurodifferentiation of PC12 cells. Indeed, an L-type but not N-type calcium current was necessary for Numb-mediated neurite outgrowth in PC12 cells (Lu et al. 2009). On the other hand, mid-term (Lu et al. 2009) or long-term (Lichvarova et al. 2012) inhibition of LTCC by dihydropyridines did not affect NGF-induced neurite outgrowth in PC12 cells, which is one of hallmarks of their neurodifferentiation.

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Calcium homeostasis in PC12 cells is regulated by a crosstalk of multiple calcium channels and calcium transporters in both plasma membrane and sarco/endoplasmic reticulum membrane (Duman et al. 2008). LTCC directly control gene transcription in excitable cells (Dolmetsch et al. 2001; Gomez-Ospina et al. 2006) and are functionally and transcriptionally coupled to ryanodine receptor (RyR) type 2 in rat hippocampus (Kim et al. 2007) and to inositol-1,4,5-trisphosphate receptors (IP_3R) in mouse hippocampal neurons (Kato et al. 2012) and in vascular smooth muscle (Abou-Saleh et al. 2013). It is possible that altered expression level of other calcium transporting proteins in differentiated PC12 cells may compensate lack of calcium influx *via* LTCC.

The aim of our work was to analyze contribution of $Ca_V1.2$ and $Ca_V1.3$ subtypes to total LTCC in NGF-differentiated PC12 cells and to elucidate whether activity of these channels is required for expression of calcium transporters in both plasma membrane (Na^+-Ca^{2+} exchanger, NCX1 and NCX2) and sarco/endoplasmic reticulum membrane (RyR1 and RyR2, IP_3R1-3, and sarco/endoplasmic reticulum calcium transport ATPase – SERCA2). Expression of both LTCC subtypes was suppressed by siRNA and absence of functional channel protein was confirmed by measuring corresponding calcium current by means of patch clamp.

Material and Methods

Cell maintenance and transfection

PC12 cell line was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and grown in DMEM with L-glutamin, 20% fetal bovine serum and 100 U/ml Penicillin-streptomycin in an atmosphere of 5% CO_2 and 95% air at 37°C. Cell differentiation was initiated at Day 0 by supplementing the culture medium with 50 ng/ml of NGF. At the Day 9 of the NGF treatment cells were assigned into four groups. First group was cultured with NGF only for further 48 hours (control). For cell transfection nanoparticle siRNA Transfection System N-TER™ (Sigma-Aldrich Slovakia) was used. Second control group was treated by transfection reagent N-TER only to exclude possible effect of this reagent itself (N-TER). Third control group was transfected with a commercially available MISSION® siRNA Universal Negative Control (siRNA – UNC; Sigma-Aldrich Slovakia) (NC). Fourth group was transfected with a mixture of three commercially available MISSION® siRNAs (Sigma-Aldrich Slovakia) specific either for the *CACNA1C* gene encoding for the $Ca_V1.2$ channel (CACNA1CsiRNA) or for the *CACNA1D* gene encoding for the $Ca_V1.3$ channel (CACNA1DsiRNA). For electrophysiological experiments siRNAs were fluorescently labeled with 6-Carboxyfluorescein (6-FAM) to allow visual identification of transfected cells. Total concentration of siRNA was 80 nM in all cases.

Molecular analysis

Expression of genes encoding the α_1 subunits of the $Ca_V1.2$ channel (*CACNA1C* gene), $Ca_V1.3$ channel (*CACNA1D* gene) and calcium transporters NCX1, NCX2, IP_3R1, IP_3R2, IP_3R3, RyR1, RyR2 and SERCA2 was evaluated by RT-PCR analysis from total cell lysates of PC12 cells harvested 48 hours after transfection as described previously (Lichvarova et al. 2012). Briefly, total RNA was isolated by TRI Reagent (Sigma-Aldrich Slovakia), homogenized for 5 minutes at a room temperature and extracted by chloroform/isopropanol precipitation (SERVA GmbH Germany). After centrifugation RNA pellet was washed with 70% ethanol and precipitated in 96% ethanol (SERVA GmbH Germany) at –20°C overnight. Samples were stored for longer time at –70°C. Reverse transcription was performed using 1.5 μg of total RNAs and Ready-To-Go You-Prime First-Strand Beads (GE Healthcare UK) with Random Hexamer Primer (Fermentas Germany). GAPDH was used as a housekeeper gene for semi-quantitative evaluation of PCR products. All PCR products were analyzed on 2% agarose gels.

Electrophysiological experiments

HEKA-10 patch clamp amplifier (HEKA Electronic, Lambrecht, Germany) was used for whole-cell patch clamp experiments. The extracellular solution contained (in mM): NaCl 130, KCl 4, $BaCl_2$ 20, $MgCl_2$ 1, HEPES 10, glucose 10, pH 7.4 (with NaOH). The intracellular solution contained (in mM): CsCl 100, EGTA 14, NaCl 10, TEA-Cl 20, Mg-ATP 5, Na-GTP 0.3, HEPES 20, pH 7.4 (with CsOH). For measurement of a resting membrane potential (RMP) bath solution mimicked the ionic composition of culture media and contained (in mM): NaCl 109.51, KCl 5.36, HEPES 10, $CaCl_2$ 1.36, $MgSO_4$ 0.81, $NaHCO_3$ 44.04, NaH_2PO_4 0.91, D-glucose 24.98, pH 7.4 (NaOH). Pipette solution contained (in mM): MgATP 3, HEPES 10, EGTA 10, K-gluconate 130, KCl 20, NaGTP 0.4, pH 7.4 (KOH). RMP was measured using perforated patch clamp with 50 μg/ml amphotericin B.

Osmolarity of experimental solutions was measured with Osmomat 030 (Gonotec GmbH, Germany). The osmolarity of pipette solutions was approximately 300 mOsm. The osmolarity of bath solution was adjusted by varying glucose concentration to a value by 3–4 mOsm lower, then the osmolarity of pipette solution.

Expression of L-type calcium channel proteins was assessed by measuring of inward calcium currents and inhibiting part

carried by LTCC by 1 μM of isradipine (Lichvarova et al. 2012). Stock solution (10 mM) of isradipine (Sigma-Aldrich Slovakia) was prepared in ethanol, stored at –20°C and dissolved to a final concentration of 1 μM in a bath solution prior to the experiment. Extracellular solutions were exchanged by a gravity-driven flow system with manually controlled valves. Patch pipettes were manufactured from borosilicate glass (Sutter Instrument, Novato, CA) with input resistance ranging from 2.0 to 2.5 MΩ. The cell capacitance ranged between 20 and 80 pF. Capacity transient and series resistance were compensated up to 70%. Residual linear leak current was subtracted by built-in procedure of the EPC 10 amplifier.

Data are presented as a mean ± S.E.M. Values measured at individual days were compared by one-way ANOVA with Tukey post-test and $p < 0.05$ was considered significant.

Results

Previously, we have shown that L-type calcium current is upregulated in PC12 cells during differentiation activated by NGF and that this process reaches plateau after 8 days of NGF treatment (Lichvarova et al. 2012). Therefore the transfection was scheduled at the Day 9. Effects of transfection were evaluated after 48 hours. As a first, we evaluated efficiency of siRNA-downregulation of the expression of $Ca_V1.2$ (*CACNA1C* gene) and $Ca_V1.3$ (*CACNA1D* gene) channels. In PC12 cell transfected with CACNA1C-specific siRNA relative mRNA level for the *CACNA1C* gene decreased significantly while in cells transfected with CACNA1D-specific siRNA the *CACNA1C* gene was not affected (Fig. 1A). Transfection reagent itself (N-TER) or transfection

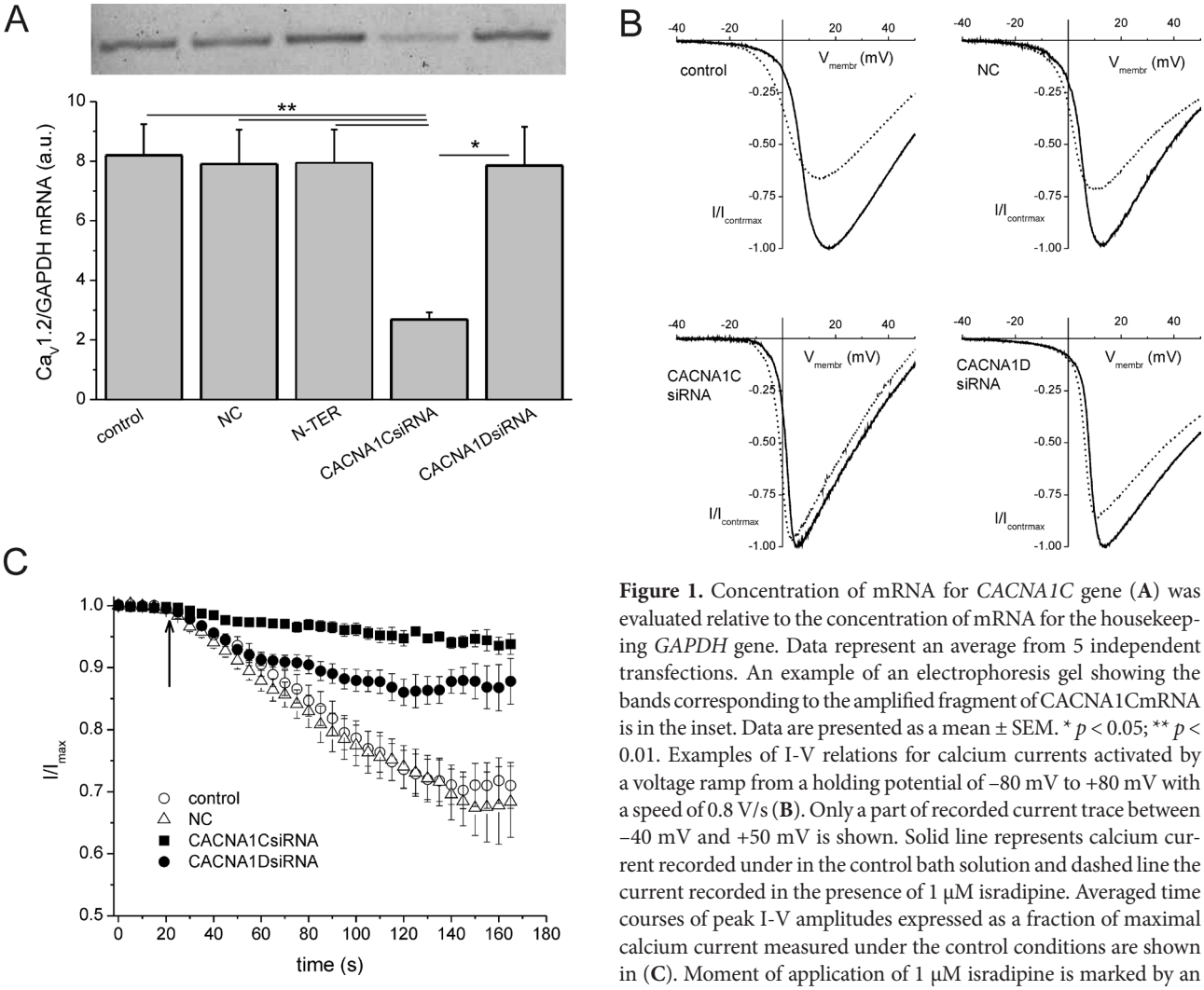

Figure 1. Concentration of mRNA for *CACNA1C* gene (**A**) was evaluated relative to the concentration of mRNA for the housekeeping *GAPDH* gene. Data represent an average from 5 independent transfections. An example of an electrophoresis gel showing the bands corresponding to the amplified fragment of CACNA1CmRNA is in the inset. Data are presented as a mean ± SEM. * $p < 0.05$; ** $p < 0.01$. Examples of I-V relations for calcium currents activated by a voltage ramp from a holding potential of –80 mV to +80 mV with a speed of 0.8 V/s (**B**). Only a part of recorded current trace between –40 mV and +50 mV is shown. Solid line represents calcium current recorded under in the control bath solution and dashed line the current recorded in the presence of 1 μM isradipine. Averaged time courses of peak I-V amplitudes expressed as a fraction of maximal calcium current measured under the control conditions are shown in (**C**). Moment of application of 1 μM isradipine is marked by an arrow. Cells originated from 5 independent transfections. Data are presented as a mean ± SEM. In all panels control denotes group of cells cultured in the presence of NGF without any other treatment. Other groups were transfected with transfection reagent only (N-TER), with scrambled siRNAs (NC), with a mixture of three siRNAs specific for *CACNA1C* gene (CACNA1CsiRNA) and with a mixture of three siRNAs specific for *CACNA1D* gene (CACNA1DsiRNA).

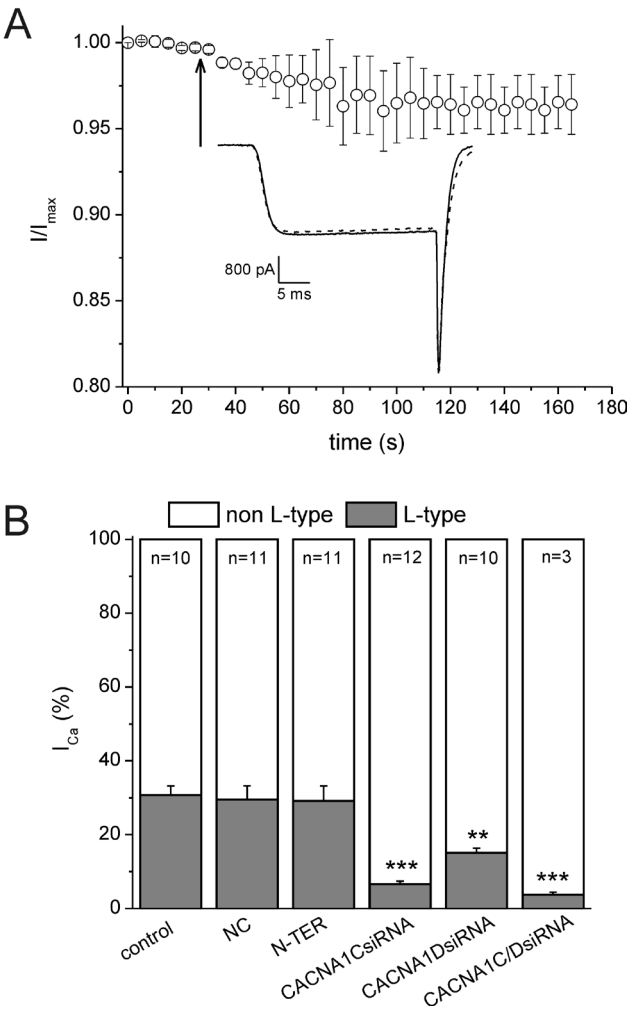

Figure 2. A. Averaged time courses of current amplitudes expressed as a fraction of maximal calcium current measured under the control conditions. Moment of application of 1 μM isradipine is marked by an arrow. Current was activated by a series of depolarizing pulses from –80 mV to +20 mV. Cells were transfected with siRNAs for both *CACNA1C* and *CACNA1D* genes. Example of original current recordings measured under the control conditions (solid line) and in the presence of isradipine (dashed line) is shown in the inset. **B.** Average amplitude of L-type calcium current expressed in percentage of total calcium current was calculated from a current blocked by isradipine in experiments documented in the Figure 1C and 1A. Number of cells measured in each group is marked in the graph. Except for the last column cells originated from 5 independent transfections. Data are presented as a mean ± SEM. ** $p < 0.01$; *** $p < 0.001$ *versus* all control groups. Control denotes group of cells cultured in the presence of NGF without any other treatment. Other groups were transfected with transfection reagent only (N-TER), with scrambled siRNAs (NC), with a mixture of three siRNAs specific for *CACNA1C* gene (CACNA1CsiRNA), with a mixture of three siRNAs specific for *CACNA1D* gene (CACNA1DsiRNA) and with a mixture of six siRNAs specific for either *CACNA1C* (three) or for *CACNA1D* (three) genes (CACNA1C/DsiRNA).

with scrambled siRNAs (NC) did not alter relative mRNA level for the *CACNA1C* gene (Fig. 1A). To detect mRNA level for the *CACNA1D* gene use of nested RT-PCR was necessary (Michna et al. 2003), therefore we could not evaluate relative mRNA concentration for this gene and we relied solely on the functional test for protein expression.

As the amplitude of L-type calcium current is directly proportional to the number of functional channels expressed in the cell membrane, it was used as a measure for quantification of expression of functional channel proteins (Hille 2001). To minimize rundown, calcium current was activated by voltage ramps. 1 μM isradipine is capable completely inhibit L-type calcium current in PC12 cells (Lichvarova et al. 2012). Relative contribution of blocked, i.e., an L-type, current to the total current was calculated from amplitudes of ramp currents recorded in the control solution and in equilibrium with 1 μM isradipine (Fig. 1B, C). Currents recorded in control, N-TER and NC groups did not differ. Further, we tested whether transfection with siRNAs for both *CACNA1C* and *CACNA1D* genes will fully suppress LTCC. For this experiment we have decreased concentration of siRNA for individual genes to 40 nM so that total concentration of all siRNAs was again 80 nM. Higher concentration may have cytotoxic effect. In these cells (Fig. 2A) LTCC represented only 3.7 ± 0.6% of total calcium current, i.e., efficiency of gene silencing was 88%. As in this experiment concentration of siRNAs for individual *CACNA1C* and *CACNA1D* genes was lowered, actual efficiency of individual gene silencing may be even higher.

Transfection with siRNA specific for either $Ca_V1.2$ or $Ca_V1.3$ channel significantly suppressed amplitude of L-type calcium current (Fig. 2B). It was apparent that a dominant isoform of L-type calcium channel in PC12 cells is $Ca_V1.2$ channel. Density of total calcium current was not affected by transfection with either siRNAs (Fig. 2B and Table 1). Silencing of the gene for the $Ca_V1.2$ channel caused a mild decrease of average total current amplitude, nevertheless, this decrease was not significant (Table 1). Cell capacity, which is a measure of a cell size, was not altered by a transfection with CACNA1CsiRNA or CACNA1DsiRNA (Figure 3A). RMP was significantly hyperpolarized when the expression either of both channels was downregulated (Figure 3B). Hyperpolarization was slightly more prominent in CACNA1CsiRNA group. To test whether block of LTCC by 1 μM isradipine can mimick this effect we continuously monitored RMP and applied the drug to a PC12 cells from all three control groups. Example of such experiment is in the Figure 3C (a cell from a control group). However, we only observed minor depolarization of RMP by about 1 mV. This effect was not statistically significant.

PC12 cells exposed for 11 days to NGF express mRNA for surface membrane calcium transporters NCX1 and NCX2 as well as for intracellular ion channels and transporters IP_3R1, IP_3R2, IP_3R3, RyR1, RyR2 and SERCA2 (Fig. 4). Relative

Table 1. Densities of total, L-type, and non-L-type calcium current

	NC	CACNA1CsiRNA	CACNA1DsiRNA
$I_{Ca\text{-}L}$ (pA/pF)	−10.6 ± 3.2 (11)	−1.8 ± 0.5 (12)*	−6.6 ± 1.8 (10)
$I_{Ca\text{-}non\text{-}L}$ (pA/pF)	−26.9 ± 4.6 (11)	−31.0 ± 5.7 (12)	−32.1 ± 4.2 (10)
I_{Ca} (pA/pF)	−36.7 ± 7.0 (11)	−33.4 ± 6.0 (12)	−38.7 ± 5.6 (10)

Average current density was measured in each of three control groups and in cells transfected with siRNAs for either $Ca_V1.2$ or $Ca_V1.3$ channel. L-type calcium current density was identified as a density of current blocked by 1 μM isradipine and non-L-type calcium current density was established as a density of current remaining in the presence of 1 μM isradipine. Values obtained from control groups (control, N-TER, NC) were not significantly different. For a sake of clarity only NC group is listed in the table. I_{Ca}, $I_{Ca\text{-}L}$ and $I_{Ca\text{-}non\text{-}L}$ represent total calcium current, L-type calcium current, and non-L-type calcium current density, respectively. Number of cells tested is indicated in brackets. * $p < 0.05$ *vs.* NC.

mRNA concentration for none of those was altered when cells were treated for 48 hours with transfection reagent N-TER or transfected with scrambled siRNA (NC). For a sake of clarity, only NC group was included into graphs of Fig. 4. Downregulation of the expression of $Ca_V1.2$ channel did not alter expression level of none of these channels and transporters (Fig. 4). Most prominent effects of CACNA1D downregulation were enhancement of mRNA level for IP_3R2 and suppression of mRNA level for NCX1, nevertheless, neither of them was significant (Fig. 4).

Discussion

PC12 cells are commonly used as a model of sympathetic neurons and/or chromaffin cells. Duman and collabora-

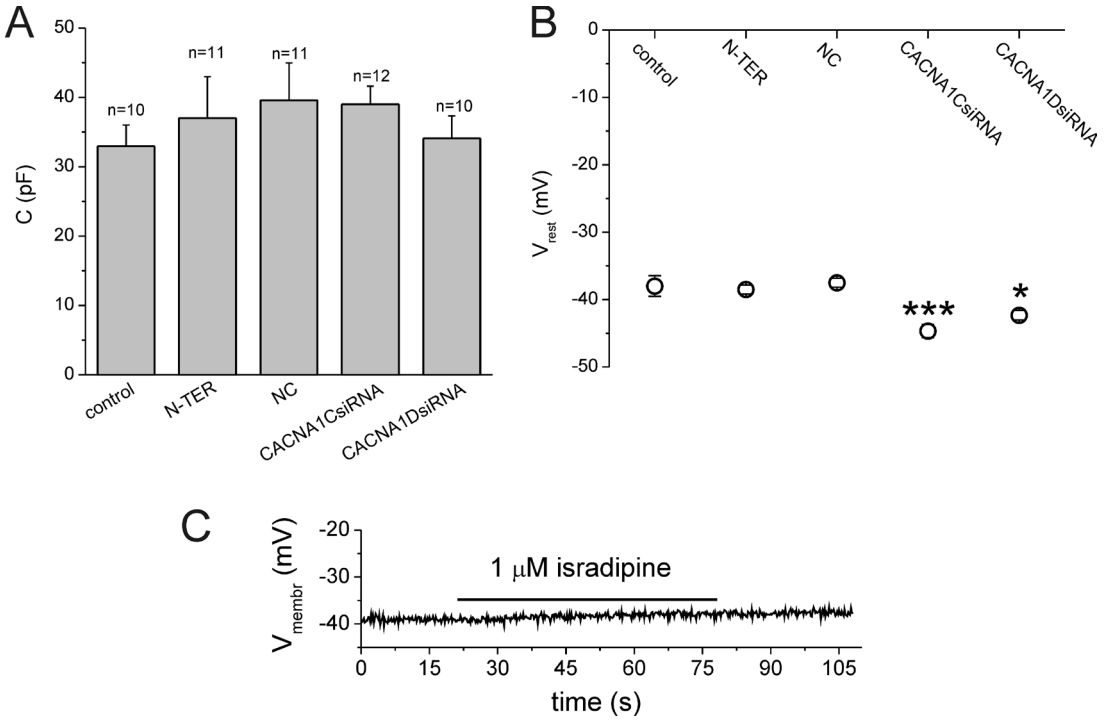

Figure 3. Average cell capacity (**A**) and resting membrane potential (**B**) measured from cells cultured in the presence of NGF without any other treatment (control), cells transfected with transfection reagent only (N-TER), with scrambled siRNAs (NC), with a mixture of three siRNAs specific for *CACNA1C* gene (CACNA1CsiRNA) and with a mixture of three siRNAs specific for *CACNA1D* gene (CACNA1DsiRNA). Number of cells tested in each group is marked in the panel A. In the panel B number of cell is each group was 6. Data are presented as a mean ± SEM. * $p < 0.05$; ** $p < 0.01$ *versus* all control groups. Representative example of continuous resting membrane potential (V_{membr}) recording from a control cell is shown in the panel **C**. Duration of application of 1 μM isradipine is marked. C, average cell capacity; V_{rest}, resting membrane potential.

tors (Duman et al. 2008) analyzed calcium transport in PC12 cells mediated by transporters in plasma membrane (NCX) and in sarco/endoplasmic reticulum (SERCA). They concluded that mode of action of calcium transporters in NGF-differentiated PC12 cells resembles more closely that of sympathetic neurons (Duman et al. 2008). Our work was concentrated on the role of L-type calcium channels in the same cell model.

Voltage dependent calcium channels play important role in physiology of both chromaffin cells and sympathetic neurons. PC12 cells cultured in presence of NGF exhibit significant LTCC activity (Liu et al. 1996; Duman et al. 2008; Lichvarova et al. 2012) supported by both $Ca_V1.2$ and $Ca_V1.3$ channel subtypes (Colston et al. 1998). Contribution of these channel subtypes to total LTCC cannot be evaluated by pharmacological means as no selective channel blocker is available. Therefore, we have used siRNA technique to down-regulate separately expression of these proteins. Previously, we have shown that after 8 days of NGF exposure differentiation of PC12 cells manifested by neurite outgrowth, increased expression of LTCC, and percentage of differentiated cells reaches its plateau (Lichvarova et al. 2012). Therefore Day 9 was chosen for transfection of cells with a mixture of three specific siRNAs either for the *CACNA1C* gene or for the *CACNA1D* gene. Suppression of the expression of *CACNA1C* and/or *CACNA1D* genes did lead to suppression of the expression of functional channel proteins. Consistent with previous works on NGF-differentiated PC12 cells (McCullough et al. 1998; Lichvarova et al. 2012), $Ca_V1.2$ and $Ca_V1.3$ channels were together responsible for approximately 30% of total voltage-dependent calcium current. This is somehow less than in rat or mouse chromaffin cells in which LTCC carry about 40–50% of total calcium current (Marcantoni et al. 2008, 2010; Mahapatra et al. 2012; de Pascual et al. 2013). On the other hand, it is much more than in sympathetic neurons, where LTCC contribute only a minor part (8–16%) of total calcium current (Zhao et al. 2007; Hernandez-Ochoa et al. 2009; Vivas et al. 2014). Furthermore, in adult rat sympathetic neurons exposure to NGF reduced LTCC from 16 to 6% of the total calcium current (Vivas et al. 2014) while in chromaffin cells NGF-treatment increased calcium current amplitude (Morgado-Valle et al. 1998).

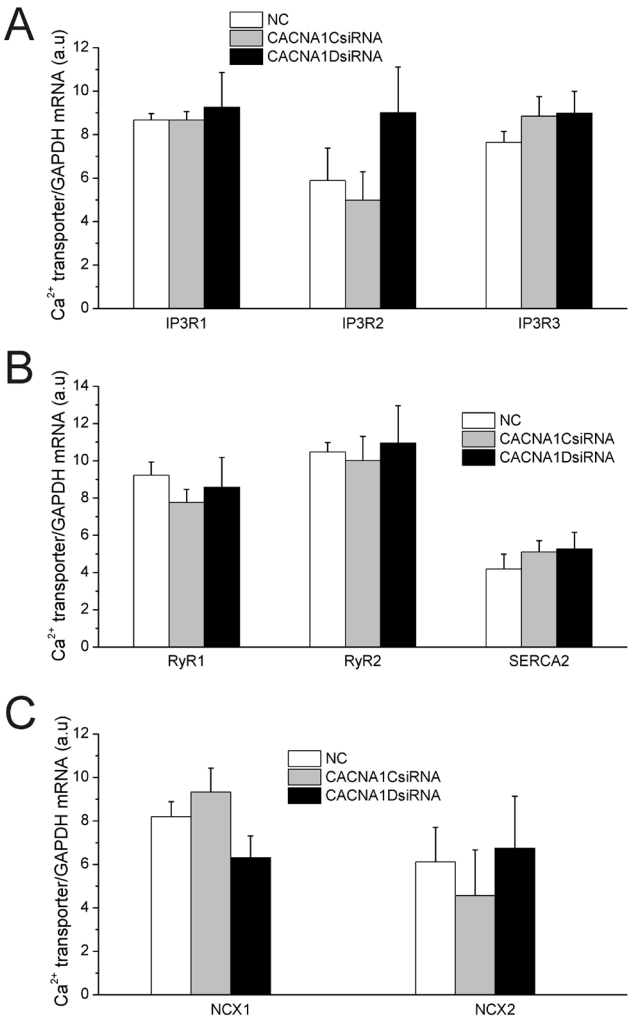

Figure 4. Concentration of mRNA for genes encoding IP_3R type 1, 2 and 3 (**A**); RyR type 1 and 2, SERCA type 2 (**B**) and NCX type 1 and 2 (**C**) was evaluated relative to the concentration of mRNA for the housekeeping *GAPDH* gene. Cells were transfected with scrambled siRNAs (NC), with a mixture of three siRNAs specific for *CACNA1C* gene (CACNA1CsiRNA) and with a mixture of three siRNAs specific for *CACNA1D* gene (CACNA1DsiRNA). Data are presented as a mean ± SEM and represent an average from 5 independent transfections.

At least 75% of L-type calcium current in PC12 cells was carried by the $Ca_V1.2$ channel. Contribution of channel subtypes to total LTCC in chromaffin cells was analyzed in $Ca_V1.3$-knockout mouse (Marcantoni et al. 2010; Mahapatra et al. 2012). In this model both channel subtypes contributed equally to the total LTCC (Marcantoni et al. 2010; Mahapatra et al. 2011, 2012). While both channel subtypes are equally important for LTCC regulation by protein kineses C and G (Mahapatra et al. 2012), $Ca_V1.3$ channel was more important with regard to pacemaking in mouse chromaffin cells than the $Ca_V1.2$ channel (Marcantoni et al. 2010; Mahapatra et al. 2011).

Absence of either $Ca_V1.2$ or $Ca_V1.3$ channel did not result in decrease in cell capacity suggesting that no recess of neuronal processes took place and therefore no calcium influx through LTCC was necessary for neurite maintenance. This observation is in line with previous finding that chronic inhibition of LTCC by dihydropyridines lasting 4 days (Lu et al. 2009) or 9 days (Lichvarova et al. 2012) did not prevent NGF-dependent neurite outgrowth (Lu et al. 2009; Lichvarova et al. 2012).

In nondifferentiated PC12 cells RMP measured by a conventional whole cell patch clamp was found to be –27.5 mV (Zhang et al. 2012), and –37 ± 1 mV (Zhu et al. 1996). Taylor and collaborators (Taylor et al. 2000) measured RMP in nondifferentiated PC12 cells using perforated patch clamp and reported a mean value of –35 mV. In PC12 cells differentiated by 100 ng/ml of NGF RMP measured by conventional whole-cell patch clamp at the day 3 and at the day 12 was –46 ± 1.7 mV and –50 ± 6.2 mV, respectively (Jeub et al. 2006). Rat chromaffin cells were reported to have RMP -60 mV (Akaike 1992) and –62 ± 3 mV (Hollins and Ikeda 1996). RMP of rat sympathetic neurons was –51.9 ± 2.3 mV (Doczi et al. 2008) and –55.6 ± 1.3 mV (Lamas et al. 2009). RMP in control PC12 cells found in our experiments was close to –40 mV. More depolarized value compared to that of Jeub (Jeub et al. 2006) may be caused by lower concentration of NGF which we used and by difference in composition of extracellular solution, which mimicked composition of culture medium in our experiments. Generally, RMP of PC12 cells is more close to that of sympathetic neurons.

Resting membrane potential was significantly hyperpolarized in cells with downregulated *CACNA1C* or *CACNA1D* genes, suggesting that LTCC do participate in its maintenance. The observation that when LTCC are expressed RMP is slightly depolarized supports our previous proposal that in PC12 cell cultured under standard conditions opening probability of these channels may be greater than zero and they may provide basal calcium influx (Lichvarova et al. 2012). Similarly, when cultured chromaffin cells, which have more negative RMP, were chronically depolarized by KCl, reported were attributed to activity of LTCC (Benavides et al. 2007). Effects of LTCC on RMP are chronic, as the acute inhibition of LTCC by isradipine did not have immediate effect on RMP.

Various potassium channels contribute to RMP regulation in excitable cells. NGF-differentiated PC12 cell express predominantly delayed-rectifier type of potassium current (Rhie et al. 1999; Castillo et al. 2001; Kim et al. 2004) which may be carried through the $K_V2.1$ channel (Sharma et al. 1993). Some authors reported also minor (Castillo et al. 2001) or moderate contribution of an A-type potassium current carried through the $K_V3.4$ channel (Pannaccione et al. 2007). Additionally, presence of calcium-dependent BK channels (Shimazu et al. 2005) and SK channels (Janbein et al. 2014) in PC12 cells was demonstrated. These channels may mediate effect of LTCC on RMP.

In our experiments inward calcium current was detectable in control cells at voltages positive to –30 mV. However, the actual concentration of Ca^{2+} in a culture medium is 1.36 mM while our experiments were done using 20 mM Ba^{2+} as a charge carrier. Such difference in concentration of divalent cations shifts the I-V relationship by approximately 20 mV towards more depolarized potentials.

Total calcium current amplitude was not significantly changed when expression of $Ca_V1.2$ or $Ca_V1.3$ channels was downregulated, suggesting that non-L-type calcium channels, which in PC12 cells are represented mostly by $Ca_V2.1$ and $Ca_V2.2$ channels (Colston et al. 1998), partly compensated for decreased calcium influx through LTCC. This suggestion is supported also by observed moderate, but insignificant increase in non-L-type current density in both CACNA1CsiRNA and CACNA1DsiRNA cell groups.

It is well known that calcium influx regulates gene transcription in excitable cells. LTCC are necessary for gene transcription in neuronal cells (Lipscombe et al. 2004) including sympathetic neurons (Zhao et al. 2007). This was not the case in NGF-differentiated PC12 cells. No significant changes in expression of mRNAs for various calcium transporters were observed in the absence of either $Ca_V1.2$ or $Ca_V1.3$ channels. Apparently, calcium influx through these channels is not necessary for regulation of gene expression of analyzed transporters.

In conclusion, we have shown that LTCC carry about 30% of all inward calcium current in NGF-differentiated PC12 cells and that most of this current is carried through the $Ca_V1.2$ channel. LTCC contribute to regulation of resting membrane potential but not to regulation of transcription of genes encoding plasma and intracellular membrane calcium transporters. Relatively high proportion of LTCC in total calcium current and an enhancement of LTCC due to NGF treatment (Lichvarova et al. 2012) resemble properties of chromaffin cells. Relative proportion of $Ca_V1.3$ channel in total LTCC in PC12 cells is smaller in PC12 cells than in chromaffin cells. Lack of contribution to the control of gene transcription contrasts the findings on sympathetic neurons. In regard to the role of LTCC in physiological regulations NGF-differentiated PC12 cells share some, but not all, features with chromaffin cells.

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