doi:10.4149/gpb_2012_054

Short Communication

NGF-induced neurite outgrowth in PC12 cells is independent of calcium entry through L-type calcium channels

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Abstract. Neuronal growth factor (NGF) induces neurodifferentiation of PC12 cells into cholinergic neurons-like cells. It was shown that intracellular Ca^{2+} ions participate in regulation of the differentiation of PC12 cells. We tested whether L-type calcium channels contribute to Ca^{2+} entry which supports neurite outgrowth accompanying NGF-activated differentiation process. Development of morphological changes did correlate with increase of functional expression of L-type calcium channels. However, inhibition of L-type calcium channels by 1 μM of isradipine did not affect significantly an NGF-activated neurite outgrowth.

Key words: PC12 cells — L-type calcium channels — Dihydropyridines — Neurite outgrowth

PC12 cell line was established almost 50 years ago by Greene and Tischler (Greene and Tischler 1976) from a rat adrenal pheochromocytoma. Nerve growth factor (NGF) initiates rapid phenotypic change of these cells manifested by the outgrowth of neurites, induction of a number of genes and development of electrical excitability (Greene and Tischler 1976; Pollock et al. 1990). PC12 cells are commonly used as a model system for study of signaling pathways controlling neurodifferentiation.

An initial step in signaling cascade which mediates NGF-dependent neurodifferentiation is binding of NGF to tyrosin kinase receptor type 1 or TrkA (Vaudry et al. 2002). This pathway encompasses number of protein kinases including protein kinase A (PKA), protein kinase C (PKC) and inositol (1,4,5) trisphosphate 3-kinase (IP3K) which eventually regulate activity of extracellular signal-regulated kinase (ERK) phosphorylation (Vaudry et al. 2002). Activation of both PKA and PKC supports NGF-driven neurite outgrowth (Kolkova et al. 2000; Hansen et al. 2003). In contrast, IP3K attenuates ERK phosphorylation and so opposes NGF driven neurite outgrowth (Eva et al. 2012).

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Signaling pathways which mediate NGF-driven neurodifferentiation are modulated by free intracellular Ca^{2+} ions. Increase in cytoplasmic calcium concentration *via* activation of intracellular sigma-1 and IP3 receptors potentiated NGF-activated neurodifferentiation of PC12 cells (Ishima and Hashimoto 2012). Calcium ions from extracellular environment may enter PC12 cells *via* TRP (transient receptor potential) channels (Puntambekar et al. 2005; Meng et al. 2008), voltage-dependent L- and N-type calcium channels (Usowicz et al. 1990; Cavalie et al. 1994; Liu et al. 1996; Colston et al. 1998) or Na^+-Ca^{2+}-exchanger (Meng et al. 2008; Oda et al. 2011; Sirabella et al. 2012). Influx of Ca^{2+} through the TRPC1 but not through the TRPC5 channels potentiated NGF-driven neurite outgrowth (Heo et al. 2012; Kumar et al. 2012). Activity of Na^+-Ca^{2+}-exchanger facilitated NGF-driven neurite outgrowth (Oda et al. 2011). Increased extracellular calcium concentration facilitated also staurosporine-induced neuronal differentiation of PC12 cells (Zhaleh et al. 2011), however, entry pathway for Ca^{2+} in these experiments was not identified by the authors. In current work we analyzed the role of calcium entry *via* L-type calcium channels (LTCC) in NGF-dependent neurite outgrowth in PC12 cells.

PC12 cells were 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. After plating out into 35 mm Petri dishes (day 0) cell differentiation was initiated by supplementing the culture medium with 50 ng/ml of NGF. Transcription and expression of LTCCs and neurite outgrowth was evaluated on the day 2, 5, 7 and 9 of the NGF treatment.

For evaluation of neurite outgrowth PC12 cells were seeded into 35 mm Petri dishes with scored coordinate system approximately 10 days after thawing and divided into three groups (50 ng/ml NGF; 50 ng/ml NGF + 20 nl/ml DMSO; 50 ng/ml NGF + 1 μM isradipine). On the day 2, 5, 7 and 9 culture

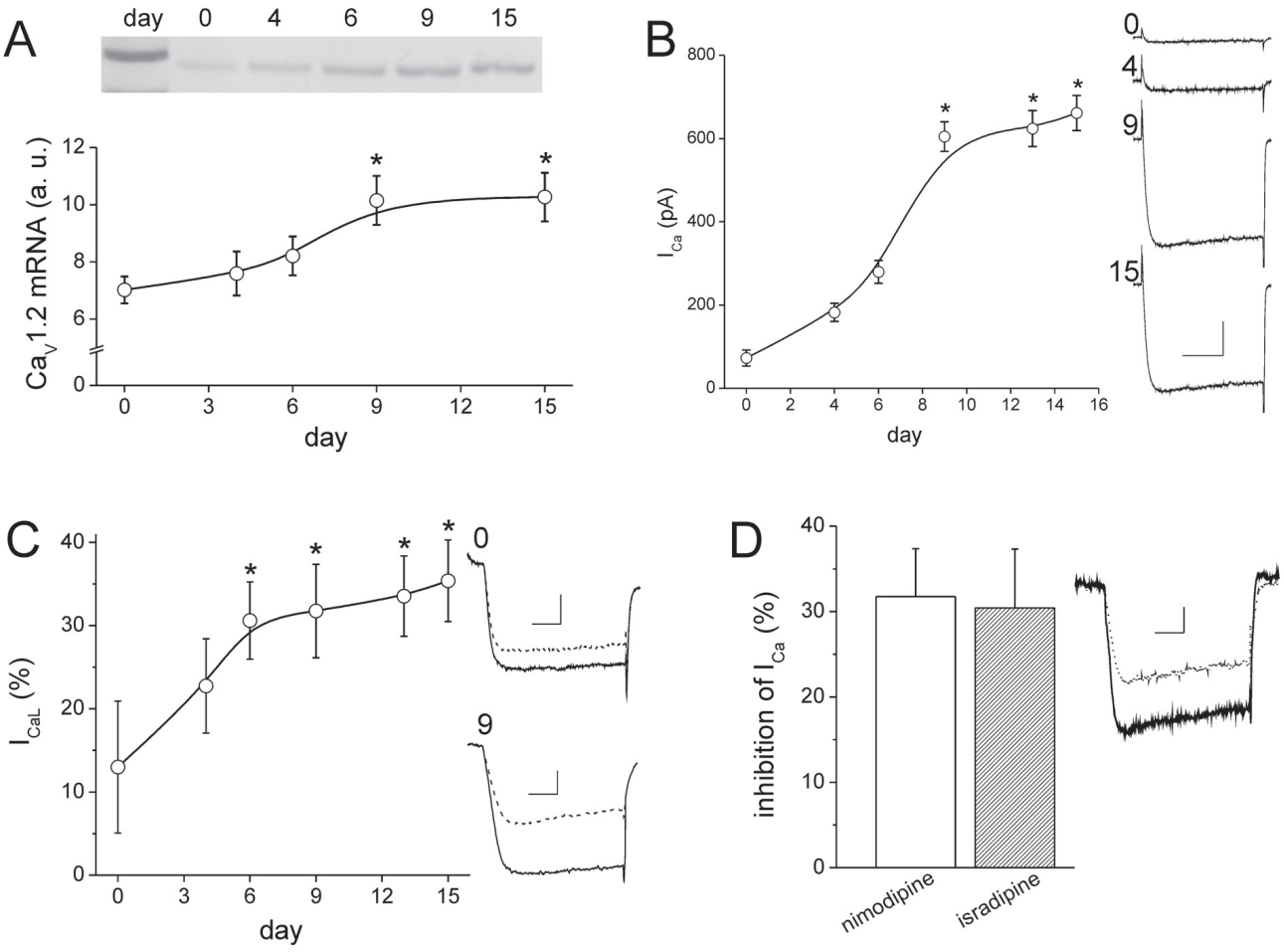

Figure 1. A. Concentration of mRNA for the *CACNA1C* gene encoding the α_1 subunit of the $Ca_V1.2$ calcium channel was evaluated relative to the concentration of mRNA for housekeeping *GAPDH* gene. Day 0 represents PC12 cells not treated with NGF. Axis x represents time of cell culture in the presence of 50 ng/ml NGF. Each point represents a mean ± SEM from 5 independent experiments. * significantly different from the day 0, $p < 0.05$. An example of a gel with bands corresponding to an amplified fragment of CACNA1C mRNA is shown on the top. **B.** Total calcium current amplitude measured from cells not treated with NGF (day 0; $n = 23$) and cells cultured for 4 ($n = 4$), 6 ($n = 9$), 9 ($n = 11$), 13 ($n = 11$) and 15 ($n = 9$) days in presence of 50 ng/ml NGF. Each point represents a mean ± SEM. * significantly different from the day 0, $p < 0.05$. Examples of current traces recorded at experimental days as marked are shown on the right. Common scale bars represent 50 ms and 500 pA. **C.** Percentage of L-type calcium current in the total calcium current was evaluated as a percentage of total calcium current blocked by 10 μM nimodipine. Each point represents a mean ± SEM. Number of individual experiments at each day was the same as in the panel B. * significantly different from the day 0, $p < 0.05$. Examples of current traces recorded at experimental days as marked are shown on the right. Solid black lines correspond to the current trace recorded under the control conditions. Dashed lines show the current trace amplitude measured in equilibrium with 10 μM nimodipine. Scale bars represent 10 ms and 100 pA. **D.** Comparison of the inhibition of I_{Ca} by 10 μM nimodipine and 1 μM isradipine. PC12 cells were conditioned for 9 days with NGF. Columns represent mean ± SEM inhibition of current amplitude by each respective drug. 11 and 7 cells were tested with nimodipine and isradipine, respectively. Data for nimodipine are the same as in the panel C day 9. Example of current traces measured under the control conditions (solid line) and in equilibrium with 1 μM isradipine (dashed line) is given on the right. Scale bars represent 10 ms and 100 pA.

dishes were photographed on Olympus X71 microscope for an evaluation of neurite outgrowth. After photographing 50% of culture medium with additives as listed above was exchanged. Cells exposed to isradipine were preserved from light. Whole experiment was repeated three times. Each experiment consisted of 12 replicates (3 Petri dishes/4 fields each). Figure 2 demonstrates a representative field cultured at condition as marked photographed at days 2, 5, 7, and 12.

Expression of the *CACNA1C* gene encoding the α_1 subunit of the $Ca_V1.2$ channel was evaluated by RT-PCR analysis from total cell lysates of PC12 cells harvested on indicated days. Total RNA was isolated by TRI Reagent (Sigma) and homogenized for 5 minutes at a room temperature. Total RNA was extracted by chloroform/isopropanol precipitation (SERVA). After centrifugation RNA pellet was washed with 70% ethanol and precipitated in 96% ethanol (SERVA) at –20°C overnight. Samples were stored for longer time at –70°C. The purity and integrity of isolated RNAs was checked spectrophotometricaly on nanophotometer (IMPLEN). Reverse transcription was performed using 1.5 μg of total RNAs and Ready-To-Go You-Prime First-Strand Beads (GE Healthcare) with Random Hexamer Primer (Fermentas). For subsequent PCR amplification following primers were used: CACNA1C forward primer 5′-GAC CGG GGA GGA CTG GAA TT-3′ and reverse primer 5′-GGC CAG CTT CTT CCT CTG CTT-3′. GAPDH primers: forward 5′-AGA TCC ACA ACG GAT ACA TT-3′ and reverse: 5′-TCC CTC AAG ATT GTC AGC AA-3′ were used to amplify a housekeeper gene for semi-quantitative evaluation of PCR products. Each PCR program started with initial denaturation at 94°C for 5 min, followed by 33 (for $Ca_V1.2$) or 20 (for GAPDH) cycles of denaturation at 94°C for 1 min, annealing at 61°C for 1 min (60°C for GAPDH) and polymerization at 72°C for 1 min. PCRs were terminated by a final polymerization at 72°C for 5 min. All PCR products were analyzed on 2% agarose gels (Jurkovicova et al. 2007).

Expression of functional calcium channel proteins was assessed by measuring of inward calcium currents. Whole-cell configuration of the patch-clamp technique with the HEKA-10 patch-clamp amplifier (HEKA Electronic, Lambrecht, Germany) was used. 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). The osmolarity of the internal solution was approximately 300 mOsm; the osmolarity of the external solution was adjusted by adding glucose to a final value 2–3 mOsm lower than the osmolarity of the internal solution. Extracellular solutions were exchanged by a gravity-driven flow system with manually controlled valves. Patch pipettes were manu-

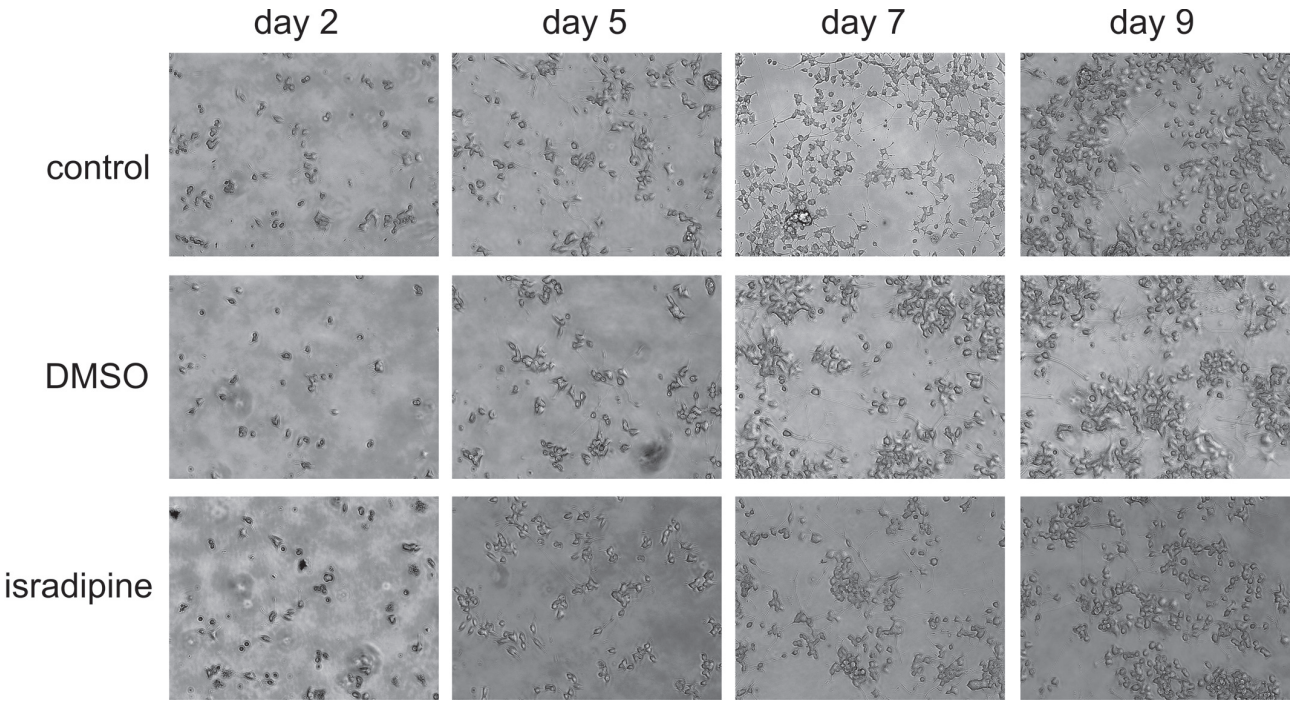

Figure 2. Example of photographs taken from cell culture dishes maintained for 2, 5, 7 and 9 days as marked in the presence of 50 ng/ml NGF (upper row), NGF and 20 nl/ml DMSO (middle row) and NGF and 1 μM isradipine (lower row). All photograps in each row were taken from the same cell culture dish. Photographs are representatives for 3 independent experiments. Actual size of photographed field was 920 μm × 680 μm.

factured from borosilicate glass (Sutter Instrument, Novato, CA) with input resistance ranging from 1.6 to 2.0 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 mean ± SEM. Values measured at individual days were compared by one-way ANOVA with Tukey post-test and $p < 0.05$ was considered significant.

Concentration of the mRNA for CACNA1C gene evaluated by the RT-PCR analysis followed sigmoidal time course reaching a plateau after the day 8 (Figure 1A). Same time course followed the expression of functional calcium channel proteins as is documented by sigmoidal increase of a total calcium current amplitude (Figure 1B).

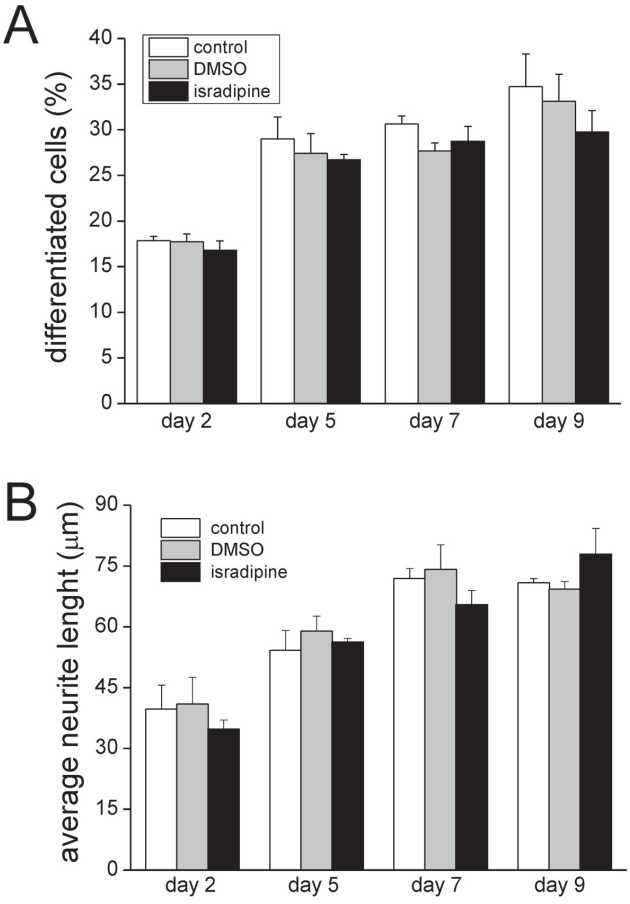

Figure 3. A. Percentage of differentiated cells after 2, 5, 7 and 9 days (as marked at the axis x) of conditioning with 50 ng/ml NGF. Differentiated cell was defined as a cell with neurite length greater that the diameter of a cell body. Values are mean ± SEM from three independent experiments each consisting of 12 replicates (3 dishes/4 fields). **B.** Average neurite length measured from differentiated cells after 2, 5, 7 and 9 days (as marked at the axis x) of conditioning with 50 ng/ml NGF. Values are mean ± SEM from three independent experiments each consisting of 12 replicates (3 dishes/4 fields). Control, cells cultured with NGF only; grey, cells cultured with NGF and DMSO; black, cells cultured with NGF and 1 μM isradipine.

It is known that differentiated PC12 cells express predominantly L-type and N-type calcium channels (Usowicz et al. 1990; Cavalie et al. 1994; Liu et al. 1996; Colston et al. 1998). To evaluate the portion of the total calcium current carried through LTCCs we blocked these channels by 10 μM nimodipine (Figure 1C). In these experiments current was activated by 50 ms long depolarizing pulses from a holding potential of –80 mV to a membrane potential corresponding to the peak of a current-voltage relationship of each investigated cell (+10 or +20 mV). Holding potential (V_H) of –80 mV was chosen to minimize current run-down. Inhibition of L-type calcium current by dihydropyridine channel blockers is known to be voltage-dependent and is by approximately one decimal orders less effective at a V_H –80 mV compare to a V_H –40 mV (Lacinova 2005). Nevertheless, 10 μM nimodipine is sufficiently high concentration to achieve a complete block of L-type calcium current even at a V_H –80 mV (Lacinova et al. 2000). Therefore a portion of the total calcium current blocked by nimodipine represents the L-type calcium current. Amplitude of the nimodipine-blocked current, i.e., an L-type calcium current expressed in % of total calcium current amplitude is shown in the Figure 1C. The synthesis and membrane targeting of functional L-type calcium channel proteins increased sigmoidaly with a similar time course as the concentration of the CACNA1C mRNA and the total calcium current amplitude (compare Figure 1A, B and C).

LTCCs can be an entry pathway for sustained calcium influx at resting membrane potentials between –45 mV and –25 mV ($Ca_V1.2b$; Fleischmann et al. 1994) or between –40 mV and 0 mV ($Ca_V1.2a$; Hirano et al. 1992). To test whether such windows current may be active in PC12 cells during our cell culture conditions we measured resting membrane potential of these cells using bath solution mimicking ionic composition of culture media. In these experiments pipette solution contained (in mM): MgATP 3, HEPES 10, EGTA 10, KCl 130, NaGTP 0.4, pH 7.4 (CsOH). Bath solution 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). NGF-activated neurodifferentiation was accompanied by statistically significant hyperpolarization of resting membrane potential (day 2: –24.2 ± 2.1 mV; $n = 9$; day 5: –28.1 ± 2.0 mV; $n = 8$; day 7: –33.4 ± 3.4 mV; $n = 10$; day 9: –35.3 ± 4.7 mV; $n = 10$; all values are means ± SEM). These membrane potentials may allow window calcium current through LTCCs.

To test if the potential spontaneous activity of LTCCs does support NGF-activated differentiation we cultured the PC12 cells in a presence of dihydropyridine channel blocker isradipine in a concentration of 1 μM. To see if such concentration

is sufficient for complete block of L-type calcium current in PC12 cells we compared the inhibition of total calcium current after 9 days of NGF treatment by 10 μM nimodipine and by 1 μM isradipine (Figure 1D). Both dihydropyridines were equally effective. Stock solution of isradipine was prepared in DMSO in 50 mM concentration and was dissolved to final concentration in a culture medium prior to each medium exchange. To exclude possible artifact caused by DMSO we cultured in parallel PC12 cells in presence of corresponding concentration of the DMSO only.

Neurite outgrowth was evaluated using Photoshop CS2 9.0. Figure 3 shows results of this analysis. Cell with at least one neurite longer than the cell body diameter was considered to be differentiated. To correct for variation of an initial number of cells in each field the number of differentiated cells was expressed in percentage of all cells in the investigated field. Neither DMSO nor isradipine affected proportion of differentiated cells (Figure 3A). Average neurite length was evaluated by measuring total length of all neurites in a field and dividing this number by a number of differentiated cells in that field. Average neurite length was not altered by isradipine or by vehicle alone (Figure 3B) as well.

Our finding extends current knowledge about the role and source of intracellular calcium ions in neurodifferentiation of PC12 cells. Lu and coauthors (Lu et al. 2009) reported that an activity of voltage-gated calcium channels is necessary for Numb-mediated neurite outgrowth in PC12 cells. Zhaleh and coauthors (Zhaleh et al. 2011) showed that calcium entry from the extracellular space through an unidentified pathway supports neurodifferentiation of PC12 cells activated by a protein kinase C inhibitor staurosporine. From our results we can conclude that while NGF-driven differentiation is accompanied by enhanced expression of functional L-type calcium channels, activity of these channels is not required for neurite outgrowth. In our model NGF activated neurite outgrowth by a pathway which does not include Numb protein. We cannot exclude that L-type calcium channels may contribute to staurosporine-induced neurite outgrowth, because we did not control the activity of protein kinases in our model.

Acknowledgements. This work was supported by the Slovak Research and Development Agency under the contract No. APVV-0212-10 and VVCE-0064-07. Authors wish to thank Dana Jurkovicova for a helpful discussion and Emilia Kocurova for an excellent technical assistance. Authors declare no conflict of interest and no financial interest in the publication of this manuscript.

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Received: October 10, 2012
Final version accepted: November 8, 2012