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Neuronal differentiation by analogs of staurosporine☆,☆☆

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ABSTRACT

RGC-5 cells are transformed cells that express several surface markers characteristic of neuronal precursor cells, but resemble glial cells morphologically and divide in culture. When treated with the apoptosis-inducing agent staurosporine, RGC-5 cells assume a neuronal morphology, extend neurites, stop dividing, and express ion channels without acute signs of apoptosis. This differentiation with staurosporine is similar to what has been described for certain other neuronal cell lines, and occurs by a mechanism not yet understood. Inhibition of several kinases known to be inhibited by staurosporine fails to differentiate RGC-5 cells, and examination of the kinome associated with staurosporine-dependent differentiation has been unhelpful so far. To better understand the mechanism of staurosporine-mediated differentiation of neuronal precursor cells, we studied the effects of the following structurally similar molecules on differentiation of neuronal and non-neuronal cell lines, comparing them to staurosporine: 9,12-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, 2,3,9,10,11,12-hexahydro-10-hydroxy-9-methyl-1-oxo-, methyl ester, (9S,10R,12R)-(K252a), (5R,6S,8S)-6-hydroxy-5-methyl-13-oxo-6,7,8,13,14,15-hexahydro-5H-16-oxa-4b,8a,14-triaza-5,8-methanodibenzo[b,h]cycloocta[jkl]cyclopenta[e]-as-indacene-6-carboxylic acid (K252b), staurosporine aglycone (K252c), 7-hydroxystaurosporine (UCN-01), and 4′-N-benzoylstaurosporine (PKC-412). Morphological differentiation, indicated by neurite extension and somal rounding, was quantitatively assessed with NeuronJ. We found that the critical structural component for differentiation in RGC-5 cells is a basic amine adjacent to an accessible methoxy group at the 3′ carbon. Given that UCN-01 and similar compounds are potent anti-cancer drugs, examination of molecules that share similar structural features may yield insights into the design of other drugs for differentiation.

The RGC-5 cell line expresses some neuronal markers characteristic of retinal ganglion cells (RGCs), but morphologically resembles glial cells and divides in culture (Krishnamoorthy et al., 2001). Unexpectedly, the phenotypic similarity between RGC-5 cells and primary RGCs can be increased by treating cells with the broad-spectrum kinase inhibitor staurosporine (Frassetto et al., 2006), which normally is used to induce apoptosis. Differentiation with staurosporine causes RGC-5 cells to stop dividing, express some ion channels, and assume a neuronal morphology. The somas become round and elevated, and neurites are extended (Frassetto et al., 2006). These neurites immunostain for microtubule-associated protein 2, tau, and growth-associated protein 43, and axon-like and dendrite-like processes can be distinguished (Lieven et al., 2007). Given that differentiated RGC-5 cells more closely resemble primary RGCs in morphology and physiology, they serve as a more relevant model cell population for primary RGCs, than undifferentiated RGC-5 cells. The use of differentiated RGC-5 cells also has advantages over the use of primary RGCs, namely the comparative ease of maintaining a population of RGC-5 cells in culture, and the homogeneity of the population of cells obtained.

Differentiation with staurosporine is surprising, because this kinase inhibitor is typically used as a potent inducer of apoptosis. RGC-5 cells can also be differentiated by inhibiting histone deacetylase with trichostatin A (Schwechter et al., 2007). However, this mechanism of differentiation differs from that seen with staurosporine in that the extension of neurites induced by HDAC inhibition requires RNA transcription and the cells produced are neurotrophic factor-dependent (Schwechter et al., 2007).

The presumed target(s) of kinase inhibition mediating staurosporine-induced differentiation are unknown. Treatment of RGC-5 cells with a variety of specific kinase inhibitors alone or in combination does not induce similar phenotypic changes (Frassetto et al., 2006). Treatment of RGC-5 cells with H-1152, a Rho-kinase

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inhibitor, or H-89, a non-specific protein kinase A inhibitor, results in some process formation, but not the somal rounding seen with staurosporine-induced differentiation (Frassetto et al., 2006). Although phosphorylation state changes in RGC-5 cells treated with staurosporine can be studied, it is not clear which phosphorylation targets signal differentiation and which are secondary or unrelated to differentiation (Frassetto et al., 2006). Yet staurosporine has been shown to differentiate other neuronal precursor cell lines: PC-12 (Hashimoto and Hagino, 1989), NB-1 (Morioka et al., 1985), SK-N-SH (Lombet et al., 2001), and SH-SY5Y (Shea and Beermann, 1991).

Given the difficulty in understanding the specificity and molecular mechanism of staurosporine differentiation of neuronal precursors into neurons via a functional (kinase-based) approach, we focused on the molecular structure of staurosporine. We examined the morphological effects on RGC-5 cells of the structurally related molecules K252a, K252b, K252c, UCN-01, and PKC-412, and quantitatively and qualitatively correlated differentiation with the presence of specific chemical groups on those molecules. To examine how specific the differentiation process was to RGC-5 cells, we also examined differentiation of two other neuron precursor cell lines (PC-12 and PC6-3) and the non-neuronal 3T3 line. PC-12 and PC6-3 are pheochromocytoma cell lines which when differentiated with NGF have features of sympathetic neurons. PC6-3 cells are a subline of PC-12 cells, and are significantly more dependent on nerve growth factor for survival (Pittman et al., 1993). 3T3 is a fibroblast cell line derived from mouse embryonic tissue (Todaro and Green, 1963). We found that the presence of specific structural elements found in some of the kinase inhibitors tested were associated with cellular differentiation to a neuron-like phenotype.

1. Experimental procedures

1.1. Materials

Staurosporine (isolated from *Streptomyces staurosporeus*) was obtained from Alexis Biochemical (San Diego, CA). Fetal bovine serum was obtained from Gemini Bio-products (West Sacramento, CA). Other cell culture reagents were obtained from Mediatech (Herndon, VA) unless otherwise noted. Dimethyl sulfoxide (DMSO), K252a, K252b, K252c (staurosporine aglycone), cycloheximide, and actinomycin D were obtained from Sigma–Aldrich (St. Louis, MO). UCN-01 (7-hydroxystaurosporine) was obtained from Calbiochem (San Diego, CA). PKC-412 (midostaurin, 4′-N-benzoyl-staurosporine) was obtained from LC Laboratories (Woburn, MA). Paraformaldehyde (16% solution) was obtained from Electron Microscopy Sciences (Hatfield, PA). All other materials were obtained from Fisher Scientific (Pittsburgh, PA).

1.2. Cell culture

RGC-5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 1 g/L glucose and L-glutamine, supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. 3T3 cells were grown in DMEM with 4.5 g/L glucose and L-glutamine, supplemented with 10% calf serum. PC-12 cells were cultured in DMEM with 1 g/L glucose and L-glutamine, supplemented with 10% horse serum, 5% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. PC6-3 cells were cultured in RPMI-1640 with L-glutamine, supplemented with 10% horse serum, 5% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells to be treated with kinase inhibitors were plated onto 12 mm round cover glass slips in 24-well plates, at a density of approximately 8000 cells in 450 μL growth media per well. All cells were incubated at 37 °C in humidified 5% CO_2.

1.3. Treatment with kinase inhibitors

All kinase inhibitors were stored as 1 mM stock solutions in dimethyl sulfoxide (DMSO). Stock solutions were then diluted in growth media and added to cultures to final concentrations of 0 nM, 100 nM, 316 nM, or 1 μM. In some conditions transcription and translation were inhibited with actinomycin D (4 μM) or cycloheximide (100 μM).

After 24 h in culture, pharmacological agents or additional media were added to a total volume of 500 μL per well. After 24 h of treatment, cells were fixed in 4% paraformaldehyde for 10 min and ice-cold methanol for 5 min. Coverslips were then mounted on slides and sealed. All conditions were tested in duplicate.

1.4. Cell morphology

Digital photomicrographs of the mounted cells were taken at 400× total magnification on a Zeiss Axiophot microscope and stored as TIFF images. Photomicrographs were analyzed and the number of primary neurites per cell counted by an observer masked to the experimental group. Primary neurites were classified as projections that originated at the cell soma and were at least as long as the soma was long or wide. Neurite counts were averaged for each condition. Student's *t*-tests were used to compare neurite counts.

The NeuronJ plug-in for NIH ImageJ software was used to assess neurite length and branching. Neurites were traced, and labeled as primary, secondary, tertiary, or quaternary. Percentages of each type of neurite were tabulated for each condition. Length measurements from individual neurites were converted to microns, and the value for total neurite field length for each cell was calculated.

1.5. Cell viability

Cells were grown in a 96-well tissue culture plate at a density of approximately 2,000 cells/well, and treated in triplicate. Viability was assessed 24 h after treatment. Growth media was removed, and replaced with calcein-AM (10 μM) and propidium iodide (1.5 μM) in phosphate-buffered saline. Cells were then incubated for a further 30 min at room temperature in the dark. Digital photomicrographs of the wells were taken at a total magnification of 200× on a Zeiss Axiovert inverted microscope under epifluorescence. ImageJ software was used to analyze the number of cells stained with calcein (living) and propidium iodide (dead).

1.6. Statistical analysis

Comparisons between 2 groups was by Student's *t*-test. Comparisons across 2 or more groups with a control group was by ANOVA followed by Bonferroni correction for multiple comparisons. All procedures were run using the Data Analysis add-in in Microsoft Excel 2004.

2. Results

2.1. Morphologic changes in RGC-5 cells

RGC-5 cells treated with staurosporine for 24 h had a morphology similar to that of primary RGCs, including somal rounding and extension of neurites, as previously reported (Frassetto et al., 2006). When treated with 316 nM staurosporine, RGC-5 cells had 3.3 ± 0.2 primary neurites per cell, compared to 0.19 ± 0.1 primary neurites per cell seen in cells treated with vehicle control ($p < 0.001$). Twenty-four hours of treatment with 1 μM UCN-01 also induced morphological differentiation, which was not seen at lower concentrations. Treatment with UCN-01 caused both the extension of neurites (2.5 ± 0.3 at 1 μM vs. 0.19 ± 0.1 with vehicle; $p = 0.012$) and somal rounding. Staurosporine-induced extension of a greater number of neurites than UCN-01 when 316 nM treatments of the compounds were observed (3.3 ± 0.2 vs. 0.5 ± 0.2, respectively, $p < 0.001$). However, when 1 μM concentrations of staurosporine and UCN-01 were compared, the cells treated with UCN-01 extended a greater number of neurites (1.0 ± 0.2 average neurites per cell with 1 μM staurosporine, 2.5 ± 0.3 with UCN-01, $p < 0.001$) (Fig. 1B).

Treatment of RGC-5 cells with K252a resulted in a subset of the morphological changes seen with staurosporine or UCN-01. Cells treated with K252a extended neurites, but the somas did not become round and elevated. Neurites appeared broader than those seen with staurosporine (Fig. 1A). Cells treated with K252a extended fewer neurites than cells treated with staurosporine (1.4 ± 0.3 with 1 μM K252a vs. 3.3 ± 0.2 with 316 nM staurosporine; $p < 0.001$).

Further analysis of cell photomicrograhs with NeuronJ software showed that neurites induced by staurosporine and UCN-01 were morphologically similar, while neurites induced by K252a were slightly shorter and significantly less branched (Fig. 1C and D). The neurites compared were those induced by treatment of cells with each kinase inhibitor at the concentration at which it most effectively induced morphological changes (316 nM staurosporine, 1 μM K252a, and 1 μM UCN-01). RGC-5 cells treated with K252a