Purkinje Cells Neurodegeneration in the PKCγ H101Y Transgenic Mouse

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Abstract

Spinocerebellar ataxia type 14 (SCA14) is an autosomal, dominant neurodegenerative disorder caused by mutations in protein kinase C gamma (PKCγ), with onset ages of three to thirty years in humans. PKCγ is a neuronal specific PKC. Activated PKCγ phosphorylates and inhibits gap junctions such as Cx57, a process that is controlled by the oxidative state of the cell. We have previously demonstrated that PKCγ SCA14 mutations lead to endoplasmic reticulum (ER) stress and apoptosis through "out of control" gap junctions in HT22 cells in culture. The objective of this study is to determine effects of H101Y mutation in transgenic mice in vivo on Purkinje cells.

Methods

Generation of transgenic animal: HA-tagged PKCγ mice were generated under a CMV promoter in a C57BL/6J background at the University of Missouri-Columbia Transgenic animal facility.

Mouse cerebellar slices: Cerebella from 4 week old mice were sectioned at 250µm, cultured in BME medium supplemented with 2.5 mM glutamine, 5mM glucose and 5 ng/mL nerve growth factor. Samples treated with H2O2 were incubated at 37˚C for up to 1hr, then homogenized.

Immunohistochemical confocal microscopy: Cerebellar tissue was fixed using 2% paraformaldehyde and sagittally sectioned at 20µm thickness using a cryostat. Primary antisera (PKCγ, PKCγ H101Y, and Cx57) were applied overnight. After washing, secondary antisera with Alexa568 or Alexa488 conjugation for 2 hours at room temperature.

Light microscopy: Cerebellar tissue was fixed and sagittally sectioned at 1µm thickness. Samples were stained with 1% toluidine blue.

Tail suspension photography: Wild type and transgenic mice were suspended vertically for 1min by attaching tape to the surface of the tail and to a fixed lever.

Cx57 gap junction plaques: Murine Hippocampal HT22 cells were treated with 200nM 12_O-tetradecanoylphorbol-13-acetate (TPA), 100uM H2O2, or phosphate-buffered solution (PBS) for 30min. Cells were incubated in anti-Cx57 antisera overnight at 4˚C. After washing, secondary antisera conjugated with Alexa568 was applied for 2hours. Graphs are compilations of 10 points per slide and 3 slides per treatment.

Western blot analysis: Cells were collected and lysed on ice with cell lysis buffer, then homogenized and sonicated. Cells lysate was centrifuged at 12,000 X g for 20 min and supernatant was seperated.on 7.5% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and probed with antibodies for target proteins. Protein bands were visualized with chemiluminescence and digitized for quantification.

Enzyme Activity: The activity of PKCγ was measured using a Peptag Kit, after immunoprecipitation. Equal amounts of cerebellar protein extracts were taken from wild type and transgenic mice then immunoprecipited with anti-body containing agarose beads. Recovered wild-type and mutant PKCγ was incubated in PepTag reaction mixture as per the manufacturer's instructions. The reaction was stopped by heating the mixture to 100˚C for 10 min. The PepTag Kit (Promega) uses a flourescent peptide that is highly specific for PKC. Phosphorylation of the substrate peptide allows for separation on a 0.8% agarose gel. Excised bands of phosphorylated peptide were quantified by spectrophotometry at 570nm.

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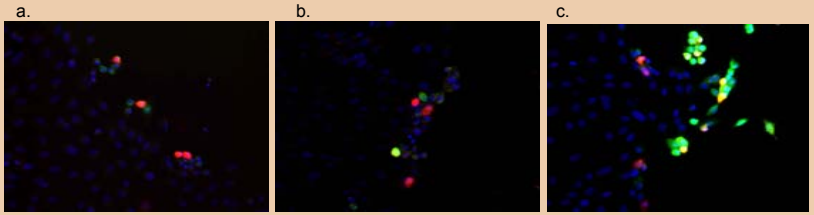


Figure 2. Pictures of Dye transfer showing that TPA completely inhibits gap junction activity (a), as does C1B1(b). In the third picture, non-treated cells show extensive gap junction activity (c).

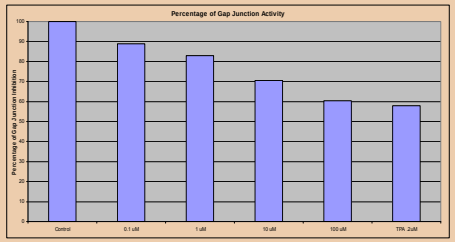


Figure 3. The effects of increasing concentrations of C1B1 peptide on dye transfer in NN1003 cells.

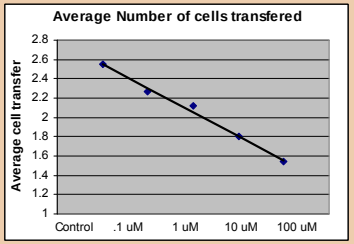


Figure 4. Linear interpretation of the results seen in Figure 3.

Results: The results show a linear relationship between the concentration of C1B1 Peptide and the amount of dye transfer prevented. The higher the concentration of C1B1, the less dye transfer noted. TPA is used as an example of total PKC activation and therefore represents a positive control. C1B1 at 100uM and TPA have a similar effect on dye transfer.

NMR Studies

NMR Methods: 1D & 2d ^{1}H -^{1}H NMR experiments were performed on a 500 MHz Varian UNITYplus spectrometer at 10 °C in H_2O: TFE-d_3 (1:1 v/v). TOCSY and NOESY experiments were used to collect structural information. Sparky software (Goddard & Kneller, 2004) was used for data analysis. Proton resonance assignments were made using 2D TOCSY for intra-residue spin systems and NOESY spectra for inter-residue connectivities by standard techniques. Distance constraints were used in the program Crystallography & NMR System (CNS) version 1.1 (Brunger et al., 1998) for structure calculation. CNS uses both a simulated annealing protocol and molecular dynamics to produce low energy structures with the minimum distance and geometry violations. A total of 200 conformers were simulated from an extended conformation and we chose 20 structures with minimal NOE violations. These 20 conformations are in good agreement with the experimental data, with no distance violations larger than 0.3 Å and no angle violations of more than 5°. The lowest total energy and then took the average of these to represent the three-dimensional NMR structure.

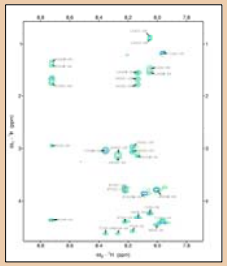


Figure 5. Wild type fingerprint region of TOCSY spectrum at 10 °C, obtained with mixing time of 100 ms.

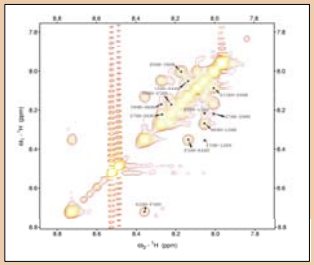


Figure 7. Wild type NH-NH region of NOESY spectra at 10 °C, obtained with mixing time of 300 ms.

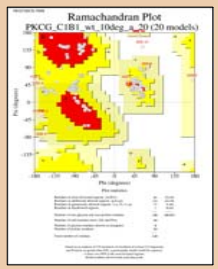
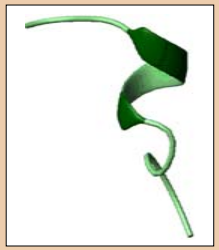


Figure 6. Ramachandran Plot and statistics of twenty lowest energy structures of wild type PKCγ C1B1 domain.

Energy	(kcal/mol)
Total	13.34±1.02
Bonds	0.32±0.06
Angles	10.19±0.25
Improper	0.33±0.05
van der Waals	2.66±0.70
NOE	0.22±0.18
Cdih	0.01±0.02
Root-Mean-Square Deviations (Å)	
Pairwise RMSD	6.554±1.475
RMSD from the mean structure	
residues 1-12	17.343±1.144
residues 6-9	1.32 ±0.44

Table 1. Structural statistics for 20 conformations used to represent the solution structure of the C1B1 peptide



Figures 7 & 8. Left: Averaged structure of wild type PKCγ C1B1 domain from twenty accepted conformations. Right: Structure overlay of the twenty lowest energy structures from 200 generated conformations of wild type PKCγ C1B1 domain.

Conclusions:

1. C1B1 peptide associates with 14-3-3 releasing PKCγ. PKCγ then becomes activated. Once activated it can phosphorylate Connexins thereby decreasing Gap Junction activity. The EC50 for the C1B1peptide is 1 uM.
2. The tertiary structure of the C1B1 peptide takes a helical conformation in the Ser6-Ser9 region, while its N- and C- termini are flexible. The flexibility may facilitate the binding mechanism of C1B1 peptide to 14-3-3.
3. It can be postulated that the flexible structure of the peptide in its 12 amino acid form allows it to associate with 14-3-3 in a non hindered manner and releases PKCγ from its docked position in the cytosol allowing it to phosphorylate Connexins.

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