PKCγ Phosphorylates Connexin50 on Serine-430.

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Abstract

Purpose: The purpose of this study is to determine the PKCγ phosphorylation site on Cx50 in lens epithelial cells and subsequent functional results of phosphorylation.

Methods: Mutation (S430A) was introduced into the wild type Cx50:EGFP by site-directed mutagenesis. Wild-type and mutated (S430A) Cx50 were transfected into 80% confluent N/N lens epithelial cells, and stably transfected cells were selected in DMEM media. PKCγ was activated by phorbol-12 myristate 13 acetate (TPA, 200nM). Expression and localization of wild type and mutated Cx50-EGFP fusion proteins before and after TPA treatment were measured by confocal microscopy. Cell surface Cx50 gap junction plaques were immuno-labeled and counted by confocal microscopy. Co-localization of Cx50 and PKCγ was determined by co-immunoprecipitation. Functional effects were measured by gap junction plaque assembly-disassembly.

Results: Cx50S430A localization was similar to the wild type and had punctate membrane localization at cell/cell contacts. PKCγ activation by TPA resulted in disassembly of wild type Cx50 plaques, but this had no effect on the Cx50S430A mutant. PKCγ was co-localized with both wild type Cx50 and the mutant Cx50S430A demonstrating similar interactions in both Cx50s.

Conclusions: Cx50 is phosphorylated at S430 by PKCγ and this results in Cx50 gap junction disassembly.

Introduction

PKCγ is a classic isoform of Protein Kinase C (PKC) found in the eye and is responsible for control of gap junctions in lens. Gap junctions allow the passage of ions and small molecules between adjacent cells and are composed of a family of proteins known as connexins. Cx50 (Connexin 50) is a member of this family of proteins involved in channel or pore transporter activity. In the lens and retina there are ample Cx50 involved in gap junction activity. PKCγ has been found to regulate gap junction activity by phosphorylating connexin proteins. The purpose of this study is to determine the PKCγ phosphorylation site on Cx50.

Methods: Mutation S430A in the wild type Cx50:EGFP was introduced by site directed mutagenesis. 80% confluent N/N1003A lens epithelial cells were transfected with wild type and mutated Cx50 :EGFP fusion proteins and stably transfected cells were selected in DMEM media. Cells were treated with 200 nM TPA for 20 minutes to activate PKCγ on glass cover slips in six well plates.

Cx50 Mutations

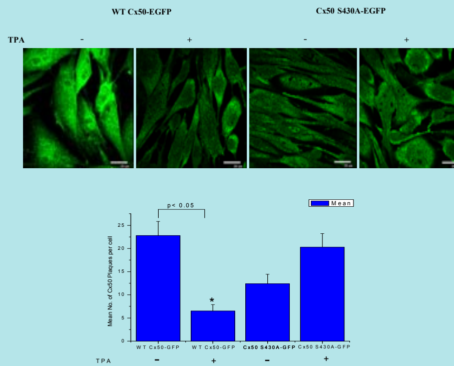

Fig 1: Cell surface Cx50 gap junction plaques were revealed by immunolabeling. Representative figures are shown. Data was recorded on a Nikon confocal microscope. Plaque number was counted and graphed.

Results: TPA activation of PKCγ caused decreases in the cell surface Cx50 plaques in the cells transfected with WT CX50-EGFP whereas the TPA activation of PKCγ had no effect on the cell surface Cx50 plaques in the cells transfected with Mutant Cx50S430A-GFP. The decrease in the number of cell surface Cx50 plaques may be because of the phosphorylation of Cx50 at S430 and disassembly of gap junction plaques. And the lack of S430 in the mutant Cx50S430A escapes the phosphorylation and plaque disassembly step.

Immunoprecipitation

Fig 2: N/N1003A cells transfected with the WT Cx50-EGFP and Mutant Cx50S430A-EGFP were treated with 200nM TPA for 20 minutes. Cells were harvested in lysis buffer and centrifuged at 13,000 rpm for 10 minutes. Supernatant was incubated with anti-GFP antibodies overnight. Protein G-Agarose was added as recommended. The precipitate was immunoblotted with anti-PKCγ antibody.

Results: The results reveal that PKCγ co-localizes with both the WT Cx50-EGFP and the mutant Cx50S430A-GFP. However, this did not increase after TPA treatment with the mutant.

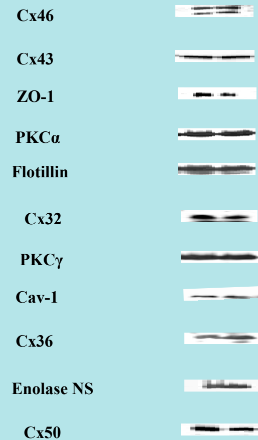

Fig 3: R28 Cell Line Protein Profile (SDS-PAGE and Western blotting)

Dye transfer / Scrape loading

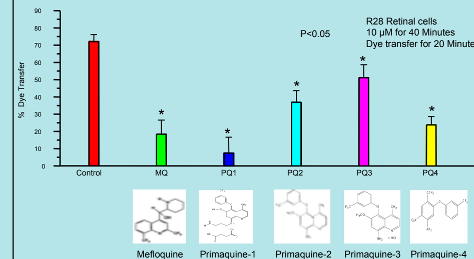

Fig 4: R28 cells were grown to 90% confluency on coverslips. They were treated with different antimalarial drugs and drug analogs @ 10μM for 40 minutes. A mixture of lucifer yellow (LY) and rhodamine dextran (RD) was added to the cells at the center of the coverslip. Two cuts across the coverslip were made to form transient tear in the plasma membranes of the cells to permit dye transfer through gap junctions. Cells were incubated with the dye for 20 minutes, then fixed and examined by fluorescent microscopy. For quantitative analysis the extent of dye transfer was estimated by counting the number of LY and RD labeled cells in the microscopic field and graphed.

Cx50-Primaquine Analog Studies

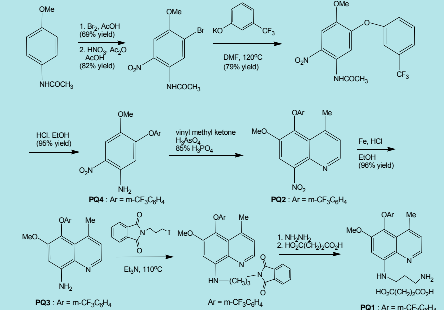

Fig 5: The synthetic procedure of the Primaquine analogs is outlined in the scheme above.

Results: All the primaquine (PQ) analogs and mefloquine (MQ) blocked dye transfer significantly in comparison to the untreated control cells. However, PQ1 and MQ were the most potent blocker of gap junction passage of the lucifer yellow dye as there was hardly any passage of the dye in cells treated with these two anti-malarial drugs.

Conclusions

Cx50 Mutation Studies:

- PKCγ activation by TPA results in phosphorylation of Cx50 which is not observed in the Cx50 S430A mutant.
- PKCγ association with Cx50 increases after TPA activation but not with the Cx50 S430A mutant.
- S430 is a PKCγ phosphorylation site on Cx50 which is required for TPA-induced Cx50 plaque disassembly.

Mefloquine and Primaquine Analog Studies:

- We have synthesized 4 novel primaquine derivatives which show varying abilities to inhibit dye transfer at the concentration 10 μM.
- Since mefloquine acts mainly on Cx50 these drugs may be useful as specific Cx50-targeted drugs.

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