Differential endocytic traffic of a novel $P2Y_{12}$ purinoreceptor mutant

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Background: ADP plays a key role in platelet function by activation of the G protein-coupled $P2Y_1$ and $P2Y_{12}$ purinergic receptor, the regulation of which is critical for controlling haemostasis and thrombosis (Nisar et al., 2011). Both the $P2Y_1$ and $P2Y_{12}$ receptors possess a type 1 PDZ ligand in their C-terminal tail. Following a screen of patients with a mild bleeding disorder, a patient carrying a heterozygous mutation in the PDZ binding motif of $P2Y_{12}$ (P341A) was identified (Nisar et al., 2011). PDZ-motifs facilitate the trafficking of other GPCRs and in the case of $P2Y_{12}$ required for effective receptor internalisation (Nisar et al., submitted). Analysis of $P2Y_{12}$ traffic in platelets from the P341A expressing patient revealed that $P2Y_{12}$ recycling following agonist-induced internalization was significantly compromised. Here we investigated the intricate endocytic sorting pathways involved in $P2Y_{12}$ traffic in order to understand dysregulation of the $P2Y_{12}$-P341A receptor mutant.

Methods: HA-tagged wild type (WT)-$P2Y_{12}$ and $P2Y_{12}$-P341A receptors expressed in HEK293 cells were used in these studies. For receptor internalization, cells were stimulated with ADP (10µM) followed by the addition of apyrase (0.2 U/ml) to remove ADP and promote receptor recycling. Receptor internalization and recycling was visualised using live cell confocal microscopy and quantified using surface ELISA. The role of RabGTPase family members in receptor traffic were explored using targeted siRNA and dominant negative approaches. Protein interactions were investigated by co-immunoprecipitation. Protein degradation was assessed by immunoprecipitation of receptor levels before and after treatment with cycloheximide (50 µg/ml) and ADP (10 µM) for 6 hours.

Results: Treatment with ADP resulted in delayed Rab5-dependent internalization of $P2Y_{12}$-P341A when compared with WT-$P2Y_{12}$. Following ADP removal WT-$P2Y_{12}$ rapidly recycled back to the membrane via Rab4 and Rab11 recycling pathways. We observed limited P341A receptor recycling and interaction with Rab11, but not Rab4, although this was not observed until 90 mins post-ADP treatment, suggesting an alternative endocytic sorting mechanism from WT-$P2Y_{12}$. Interestingly despite $P2Y_{12}$-P341A and Rab7 co-localisation in late endosomes, receptor degradation was negligible. Prolonged ADP treatment resulted in intracellular compartmentalisation of $P2Y_{12}$-P341A in the *trans*-Golgi network (TGN). Rab7 activity is known to regulate the recruitment of retromer complex proteins to endosomes in order to transport cargo to the TGN. Here we identified that $P2Y_{12}$-P341A, not WT-$P2Y_{12}$, co-localized with the retromer cargo-recognition complex, depletion of which blocked the limited receptor recycling and promoted degradation of $P2Y_{12}$-P341A.

Conclusions: In this study we have identified critical points of divergence in endocytic traffic following disruption of the PDZ motif of the $P2Y_{12}$. Given that these pathways are retained in human platelets this research may help explain the compromised receptor function in the platelets of the $P2Y_{12}$-P341A expressing patient.

References:

Nisar S, Daly ME, et al. (2011). "An intact PDZ motif is essential for correct $P2Y_{12}$ purinoceptor traffic in human platelets" ***Blood*** **118** (20): 5641-5651.

Nisar S, Cunningham MR et al., (2012). 'Arrestin scaffolds NHERF1 to the $P2Y_{12}$ receptor to regulate receptor internalisation' Submitted JBC.