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Identification of an intracellular microdomain of the $P2X_7$ receptor that is crucial in basolateral membrane targeting in epithelial cells

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ABSTRACT

We investigated membrane targeting of the $P2X_7$ receptor ($P2X_7R$) in polarized epithelial cells using immunofluorescent confocal imaging. The wild-type receptor was targeted to the basolateral membrane, independently of adaptor protein μ1B. Deletion of the majority of the intracellular C-terminus, or the last 26 residues (P570-Y595), conferred targeting of the protein to the apical membrane. Alanine substitution in the microdomain P582-Q587 caused similar apical membrane targeting without major effect on the receptor function and surface expression. Our results show basolateral membrane targeting of the $P2X_7R$ in epithelial cells and that the intracellular C-terminal microdomain P582-Q587 is crucial in this process.

Structured summary:
MINT-8055849:*Beta-catenin* (uniprotkb:B6V8E6) and *P2X7R* (uniprotkb:Q64663) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)

1. Introduction

The $P2X_7$ receptor ($P2X_7R$) for extracellular ATP is abundantly expressed in immune cells and plays a crucial role in release of inflammatory cytokines [1–3]. In normal kidney, $P2X_7R$ expression level is usually low or undetectable but increases dramatically in rodent models of renal pathologies such as polycystic kidney disease [4,5]. Up-regulated $P2X_7R$ expression has also been reported in patients suffering from autosomal recessive polycystic kidney disease and inflammatory bowel disease [5,6]. These findings constitute compelling evidence supporting a role of the $P2X_7R$ in the pathogenesis of some epithelial diseases. The plasma membranes of epithelial cells are asymmetric or polarized, and are composed of basolateral and apical domains that are separated by tight junctions and which possess distinctive membrane protein and lipid compositions. There has been significant progress in deciphering the molecular machineries targeting proteins to the apical and basolateral membranes and particularly in identifying basolateral targeting motifs within proteins [7,8]. Immunostaining studies demonstrate that the $P2X_7R$ is present in the apical membrane of tubular cells in the cortex and medulla, and also intracellularly in some proximal tubules [3,4]. The molecular basis governing $P2X_7R$ targeting in polarized epithelial cells is unknown. Here, using immunofluorescent confocal imaging, we studied localization of the recombinant $P2X_7R$ in polarized epithelial cells. Our results show basolateral membrane targeting of the $P2X_7R$ and identify an intracellular C-terminal microdomain P582-Q587 that is crucial in this process.

2. Materials and methods

2.1. Chemicals and clones

Chemicals were purchased from Sigma unless indicated otherwise. The plasmids encoding wild-type (WT) and truncation mutant rat $P2X_7Rs$ with a C-terminal EE (EYMPME) epitope were from previous studies [9,11]. Alanine substitutions were introduced by site-directed mutagenesis and confirmed by sequencing.

Abbreviations: MDCK, Madin-Darby canine kidney epithelial cells; LLC-PK1, porcine kidney epithelial cells; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; eGFP, enhanced green fluorescent protein; WT, wild-type

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2.2. Cell culture and transfection

Madin-Darby canine kidney epithelial cells (MDCK strain II), porcine kidney epithelial cells (LLC-PK1), and human embryonic kidney (HEK293) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (Invitrogen) at 37 °C in a humidified 5% CO_2 incubator. For electrophysiological studies, HEK293 or MDCK cells in a 35 mm petridish were co-transfected with 1 μg of $P2X_7R$ plasmid and 0.1 μg of enhanced green fluorescent protein (GFP) plasmid. Cells were dispersed on coverslips after transfection and used within 24–48 h. HEK293 cells for biotin-labelling and immunostaining were transfected in the same way, except for omission of GFP plasmid for immunostaining experiments. Confluent MDCK and LLC-PK1 cells grown on 24.5 mm-Transwell filters (0.4 μm pore size; Costar) were transfected with 1–2 μg of $P2X_7R$ plasmid and used 24 h later. Transfection was performed using lipofectamine2000 according to the manufacturer's instructions (Invitrogen). Based on the GFP-positive cells, the transfection efficiency for MDCK cells (5–10%) was much lower than that of HEK293 cells (>50%).

2.3. Electrophysiological recording

Whole-cell inward currents were recorded using an Axopatch 200B amplifier as described previously [10]. Extracellular solution contained (concentrations in mM) 147 NaCl, 2 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES and 13 glucose, pH 7.3, and intracellular solution contained 145 NaCl, 10 EGTA and 10 HEPES, pH 7.3. ATP was applied via a RSC160 rapid solution changer (BioLogic Science Instrument).

2.4. Immunofluorescent confocal imaging

Immunostaining of HEK293 cells was performed using primary rabbit anti-EE antibody (1:1000; Bethyl) and secondary Alexa 488-conjugated goat anti-rabbit IgG antibody (1:1000–1:2000; Molecular Probes) as detailed previously [11]. Immunostaining of MDCK and LLP-PK1 cells, monolayers were fixed with 4% paraformaldehyde in PBS containing 5% goat serum (PBS/GS) (or bovine serum albumin in some experiments), washed thrice in PBS/GS, then permeabilized with 0.1% Triton X-100 in PBS/GS for 10 min. Cells were then incubated in PBS/GS for 1 h before incubating with primary rabbit anti-EE antibody (1:1000; Bethyl) and mouse anti-β-catenin (1:500; BD Sciences) in PBS/GS for 24 h at 4 °C, washed in PBS, and then incubated with secondary Alexa 488-conjugated goat anti-rabbit IgG (1:1000, Molecular Probes), and Cy5-conjugated goat anti-mouse IgG (1:250; Jackson) antibody for 1 h at room temperature. Finally, monolayers were incubated with lectins (tetramethyl rhodamine iso-thiocynate-conjugated peanut agglutinin and wheat germ agglutinin: 1:500; Vector Labs) in PBS/GS for 30 min. The filters were removed and mounted using Vectashield (Vector Labs). Images were captured using a Zeiss Meta 510 confocal microscope and processed using Zeiss LSM image browser software.

2.5. Biotin-labelling and Western blotting

Biotin-labelling was carried out by modifying the protocols described in our previous studies [12]. In brief, transfected HEK293 cells were labeled with sulfo-NHS-LC-biotin (Pierce) for 30 min at 4 °C before they were lysed in lysis buffer. Total protein concentrations in lysate were determined using the bicinchoninic acid protein assay (Thermo Scientific). The biotin-labeled proteins were purified from total 200 μg protein by incubating the lysate with EZ view red streptavidin affinity beads (Sigma) overnight at 4 °C, and were eluted in 50 μL protein electrophoresis buffer (6% SDS, 10% glycerol, 50 mM Tris–HCl pH 6.8, 2 mM ETDA, 0.05% bromophenol blue and 10% β-mercaptoethanol). Whole cell lysate (10 μg) or biotin-labeled samples (30 μL) were separated on 12% SDS–PAGE gels. Proteins were detected using primary rabbit anti-EE antibody (1:5000; Bethyl) or mouse anti-GFP (1:2000; Santa Cruz) antibody and secondary horseradish peroxidase-conjugated anti-rabbit (1:5000) or anti-mouse IgG (1:2000) antibodies (Santa Cruz). LLC-PK1 cells were lysed in lysis buffer at 4 °C for 30 min, and proteins in the cell lysates were detected using primary rabbit polyclonal anti-μ1B antibody (1:1000) and secondary horseradish peroxidase-conjugated anti-mouse IgG antibody (1:1000; Dako).

2.6. Data analysis

All data, where appropriate, are presented as mean ± S.E.M. The EC_{50} for ATP was estimated by fitting the data from individual cells to the Hill equation: $I = 100/[1 + (EC_{50}/[ATP])^n]$, where I is the current at a given [ATP] expressed as percentage of maximum, and n is the Hill coefficient. Curve fitting was performed using Origin and statistical analysis using Student's *t*-test.

3. Results

3.1. Functional expression of $P2X_7R$ in non-polarized HEK293 and MDCK cells

It is well-established that transfection of HEK293 cells with $P2X_7R$ plasmids results in robust functional $P2X_7R$ expression [e.g., 9–11]. We therefore determined whether this was achievable in MDCK cells. Fig. 1A shows currents evoked by 3 mM ATP, a maximal concentration for the $rP2X_7R$ [9]. Such currents were not observed in non-transfected cells. These results show functional $P2X_7Rs$ were expressed in MDCK cells although the maximal ATP-induced currents were relatively lower than in HEK293 cells (Fig. 1A).

3.2. Basolateral targeting of $P2X_7R$ in MDCK and LLC-PK1 cells

We determined the localization of $P2X_7Rs$ in polarized MDCK and LLC-PK1 as well as HEK293 cells. As observed in previous studies [10–12], the $P2X_7R$ immunoreactivity in HEK293 cells was uniformly concentrated in or close to the plasma membrane (Fig. 1B), and such immunoreactivity was completely absent in non-transfected cells (data not shown). In polarized MDCK cells, the $P2X_7R$ immunostaining was observed in transfected cells, and strongly co-localized with β-catenin, the basolateral membrane marker, but not with lectins, the apical membrane markers (Fig. 1C). To examine whether such basolateral localization was MDCK cell-specific and involved the adaptor protein μ1B that is endogenously expressed in MDCK cells [7], we repeated these experiments in LLC-PK1 cells devoid of (WT) or stably expressing μ1B [13]. The μ1B expression was verified by Western blotting (Fig. 1D). In both cell lines, the $P2X_7R$ was targeted to the basolateral membrane (Fig. 1E), suggesting that the basolateral targeting of $P2X_7R$ is not cell specific and is independent of μ1B.

3.3. Apical localization of $P2X_7Rs$ with deletion of intracellular C-terminus

In contrast with the $P2X_7R$, the $P2X_2R$ was targeted to the apical membrane (Fig. 1F). The $P2X_4R$ was also targeted to the apical domain but mainly resided intracellularly (Fig. 1G). Both the $P2X_2R$ and $P2X_4R$ possess shorter C-termini than the $P2X_7R$ [1], suggesting that the $P2X_7R$ may contain a dominant basolateral targeting motif within its extended C-terminus. We thus examined two truncated receptors, $P2X_7R$-Δ418 which lacks the majority of the