Journal of Neuroscience Methods 293 (2018) 310–320

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth

Research article

Double fluorescent labelling of a bipolar epithelial cell *in vitro*: The outer hair cell

Csaba Harasztosi, Entcho Klenske, Susanne Badum, Emese Harasztosi, Anthony W. Gummer*,[1]

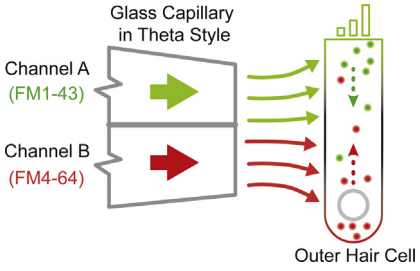
Section of Physiological Acoustics and Communication, Faculty of Medicine, Eberhard Karls University Tübingen, 72076 Tübingen, Germany

HIGHLIGHTS

- Double-barrel perfusion establishes laminar fluid flow for focused staining of a specific membrane region.
- The basal or apical pole of the OHC membrane was stained exclusively.
- The opposite poles of the OHC membrane were stained independently.
- Both apicobasal and basoapical vesicle traffic were visualized simultaneously in a single cell.

GRAPHICAL ABSTRACT

Double-barrel local perfusion system used to independently label the apical and the basal poles of outer hair cells isolated from the guinea-pig cochlea. Simultaneous perfusion from both barrels established laminar flow in front of the perfusor, ensuring that the opposite poles of this bipolar cell are labelled independently. The technique allows real time fluorescence imaging either 1) to investigate the apicobasal or the basoapical vesicle traffic along the entire length of the cell, or 2) to visualize simultaneously both apicobasal and basoapical vesicle traffic in the same cell.



ARTICLE INFO

Article history:
Received 17 May 2016
Received in revised form 16 October 2017
Accepted 16 October 2017
Available online 17 October 2017

Keywords:
Fluorescent membrane markers
Endocytosis
Transcytosis
Vesicle traffic
Cochlea
Retina

ABSTRACT

Background: Fluorescence membrane markers are efficient tools for visualizing the dynamics of membrane recycling processes in living cells. The outer hair cell (OHC) – a bipolar epithelial cell in the cochlea – possesses endocytic activity at both its apical and basal poles. The best visual overview of transcytosis in the OHC is achieved when the cell is isolated, so that both the apical and the basal poles are in the same focal plane to allow confocal imaging. Until now, fluorescent markers were applied to the extracellular environment of isolated OHCs without distinguishing the apical and basal poles. The drawback of that configuration is that apicobasal and basoapical vesicle traffic labelled at the opposite poles cannot be visualized independently because the same fluorescent marker has access to both poles.
New method: A double-barrel, capillary perfusion system was developed to independently stain either one pole or both the apical and the basal poles of isolated OHCs using different types of fluorescence membrane markers.
Results: Producing laminar fluid flow, the double-barrel perfusor allows investigation of the dynamics of apicobasal and basoapical vesicle traffic independently and/or simultaneously in the same OHC.

Abbreviations: A, apical centre; Ch, channel; DIC, differential interference contrast; DMSO, dimethylsulphoxide; FM1, -43, *N*-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide; FM4-64, *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium dibromide; HB, hair bundle; HBBS, Hanks' balanced salt solution; IC, infracuticular; IN, infranuclear; M, middle; MET, mechanoelectrical transduction; Mon, monastrol; OHC, outer hair cell; PAO, phenylarsine; PM, plasma membrane; PMA, apical plasma membrane; PMB, basal plasma membrane; ROI, region of interest; SN, supranuclear.

* Corresponding author at: Section of Physiological Acoustics and Communication, University of Tübingen, Elfriede-Aulhorn-Str. 5, 72076, Tübingen, Germany.
E-mail address: anthony.gummer@uni-tuebingen.de (A.W. Gummer).
[1] Homepage: www.cochlea.uni-tuebingen.de.

https://doi.org/10.1016/j.jneumeth.2017.10.014

Comparison with existing method: This method offers a unique option for investigating bidirectional vesicle traffic in bipolar epithelial cells, which is superior to other already established labelling techniques.
Conclusions: The double-barrel perfusion system, suitable for selectively staining a longitudinal section of the plasma membrane of an isolated bipolar epithelial cell, opens new possibilities for investigating cell labelling and intracellular vesicle traffic.

1. Introduction

Outer hair cells (OHCs), the electromechanically sensitive epithelial cells of the cochlea, are responsible for the extraordinary sensitivity, the high frequency discrimination, and the high dynamic range of hearing (Ashmore, 2008; Dallos, 2008). The basis of these properties is electromechanical force generated by the OHC soma (Dallos et al., 2008), which acts against intracochlear frictional forces (Dong and Olson, 2013). The fundamental electromechanical properties are based on mechanical (Ashmore, 1987; Brownell et al., 1985; Dallos et al., 1991; Frank et al., 1999) and electrical (Gale and Ashmore, 1997; Huang and Santos-Sacchi, 1993; Oliver et al., 2001; Santos-Sacchi, 1989) recordings from OHCs *isolated* from the organ of Corti. The electromechanical action can be modulated not only directly by its efferent inputs (Ryugo, 2011), but also indirectly by its afferent outputs (Froud et al., 2015). Since these processes are based on endocytic and exocytic activities, it is expected that, in general, both endocytosis and exocytosis are essential components of mechanisms for modulating the mechanics of the cochlear partition. To investigate the dynamics and properties of these processes at both poles of the OHC independently, an adequate dye-application system for fluorescence labelling is required.

Applying the membrane marker FM1-43 to the extracellular fluid of OHC *in vitro* (Kaneko et al., 2006; Meyer et al., 2001) and to the cuticular plate of OHC in endolymph *in situ* (Griesinger et al., 2004), it has been shown that OHCs possess rapid endocytic activity at their apical pole. It was also demonstrated that membrane particles endocytosed at the apical pole are transcytosed to different locations, namely into the basolateral membrane and along a central strand down to the nucleus (Griesinger et al., 2004; Kaneko et al., 2006).

Endocytic vesicles have been also demonstrated in the basal pole of the OHC using electron microscopy (Nadol, 1983). Moreover, using horseradish peroxidase staining, the presence of coated vesicles and small vacuoles in the synaptic region has been shown (Siegel and Brownell, 1986). However, the significance of the infranuclear endocytic activity has not been investigated, a shortcoming which is mostly due to the lack of suitable experimental settings. Therefore, it is of fundamental importance to establish a reliable labelling-perfusion method to investigate membrane internalization in isolated OHCs.

In the present study, a double-barrel capillary perfusion system was designed and demonstrated for its ability to distinguish endocytic uptake at the opposite poles of the OHC and to visualize and quantify bidirectional intracellular traffic in a single OHC. It is asserted that this new perfusion technique will contribute to investigating the dynamics of endocytosis and transcytosis, together with their associated physiological processes, and could be applicable not only to OHCs but also to other bipolar cells in certain other systems, such as the retina.

2. Material and methods

2.1. Ethical approval

The study was approved by the Animal Protection, Veterinary Service and Veterinary Medicine Department of the University of Tübingen and by the Regional Council Tübingen (Reference numbers: 11.06.2012, 11.03.2014, 16.03.2015, and 29.06.2016), complying with legal requirements of the European Communities Council Directive of 24 November 1986 (86/609/EEC) for the protection of animals used for experimental purposes.

2.2. Preparation of OHCs

OHCs were isolated as previously described (Harasztosi and Gummer, 2016) from the apical third of the cochlea of pigmented guinea pigs (N = 72, weight 400–900 g), using methods that were originally established in our laboratory for experiments characterizing high-frequency electromechanical properties of isolated OHCs (Frank et al., 1999; Ludwig et al., 2001). Cell lengths ranged from 55 to 83 μm.

Animals were bred in the animal facilities of the University of Tübingen (Einrichtung für Tierschutz, Tierärztlichen Dienst und Labortierkunde, directed by Dr. Franz Iglauer). Animals were anesthetized by intraperitoneal injection of a mixture of 100 mg/kg ketamine and 4 mg/kg xylazine and were killed by cervical dislocation.

Temporal bones were dissected and placed in ice-chilled Hanks' balanced salt solution (HBSS; Biochrom KG, Berlin, Germany), containing (in mM): 137 NaCl, 5.4 KCl, 1.25 $CaCl_2$, 4.2 $NaHCO_3$, 0.81 $MgSO_4 7H_2O$, 0.44 KH_2PO_4, 0.34 $Na_2HPO_4 2H_2O$, 5.0 glucose and 5 HEPES, with osmolarity of 310 mOsm/L adjusted with D-(+)-glucose; pH was 7.3. HBSS was used as extracellular fluid throughout the preparation steps and experiments. All chemicals were from Sigma-Aldrich (Taufkirchen, Germany), unless otherwise stated. HEPES was from MERCK (Darmstadt, Germany).

The bulla was opened and most of its wall was removed by a bone rongeur and placed in fresh HBSS. The majority of the cochlear wall was removed by a scalpel; the modiolus was separated from the cochlea and placed in fresh HBSS. Stria vascularis was removed by forceps, while the organ of Corti was separated from the modiolus by a sharpened stainless-steel fine needle. Pieces of organ of Corti originating from the apical third of the cochlea were placed in 200 μL HBSS containing 1 mg/mL collagenase IV (Collagenase IV from *Clostridium histolyticum*, Sigma-Aldrich Chemie GmbH, Munich, Germany) for 10 min using a 10 μL Eppendorf pipette. The enzymatic treatment served primarily to enhance the cell isolation process, and also to free the basal pole of the OHC of most of the nerve endings.

After replacing the HBSS with enzyme-free HBSS, the pieces of the organ of Corti were transferred into the experimental chamber containing 200 μL HBSS. OHCs were dissociated by gentle aspiration using a 100 μL Eppendorf pipette. After the OHCs settled down onto the coverslip, requiring ~10 min, the chamber was filled with ~2 mL HBSS. For the experiments with the single-barrel perfusor (Section 2.6.1), the coverslips were pre-coated with poly-L-lysine (0.01%). However, in the case of experiments with the double-barrel perfusor (Section 2.6.2), with its relatively small exit lumen, the coverslips were coated with the cell-and-tissue adhesive Cell-Tak™ (Corning Inc., Corning, NY, USA) to increase adhesion.

OHCs were used within two hours *post mortem*. Only cells possessing a typical cylindrical cell body, visibly without Brownian