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Immunolocalization of $P2Y_4$ and $P2Y_2$ Purinergic Receptors in Strial Marginal Cells and Vestibular Dark Cells

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Abstract. K^+ secretion by strial marginal cell and vestibular dark cell epithelia is regulated by UTP and ATP at both the apical and basolateral membranes, suggesting control by $P2Y_2$ and/or $P2Y_4$ purinergic receptors. Immunolocalization was used to determine the identity and distribution of these putative receptors. Membrane proteins from gerbil brain, gerbil vestibular labyrinth and gerbil stria vascularis were isolated and analyzed by Western blot. $P2Y_2$ antibody stained one band at 42 kDa for each tissue, whereas $P2Y_4$ antibody stained 3 bands on gerbil brain (75, 55 and 36 kDa), one band on gerbil stria vascularis (55 kDa) and two bands on vestibular labyrinth (42 and 56 kDa). All bands were absent when the antibodies were blocked with their respective antigenic peptide. $P2Y_4$ was immunolocalized by fluorescence confocal microscopy to only the apical membrane of strial marginal cells and vestibular dark cells and was similar to apical immunostaining of KCNE1 in the same cells. By contrast, $P2Y_2$ was observed on the basolateral but not the apical membrane of dark cells. Similarly, in the stria vascularis $P2Y_2$ was observed in the basolateral region but not the apical membrane of marginal cells. Additional staining was observed in the spiral ligament underlying the stria vascularis. These findings identify the molecular bases of the regulation of K^+ secretion by apical and basolateral UTP in the inner ear.

Key words: Purinergic receptors — Western blot — Immunocytochemistry — Vestibular dark cells — Strial marginal cells — Fluorescence confocal microscopy

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Introduction

It is known that extracellular nucleotides are involved in regulation of several processes in the inner ear (Housley, 1998) including secretion of K^+ (Liu, Kozakura & Marcus, 1995; Butlen et al., 1997). K^+ is maintained at a concentration of approximately 150 mM K^+ by strial marginal cells in the lateral wall of the cochlea and vestibular dark cells in the vestibular labyrinth. K^+ is the primary charge carrier for transduction of stimuli.

K^+ secretion by the strial marginal cells and vestibular dark cells is decreased by luminal purinergic agonists. This signaling was shown to occur via a G protein, phospholipase C, protein kinase C pathway acting on the apical KCNQ1/KCNE1 (also termed IsK (minK)/KvLQT1) K^+ channel (Marcus et al., 1997; Marcus et al., 1998). PKC downregulates K^+ secretion via phosphorylation of the KCNQ1/KCNE1 channel. K^+ secretion was also shown to be regulated by two basolateral purinergic receptors (Liu et al., 1995).

Two families of purinergic receptors have been described (North & Barnard, 1997; Hollopeter et al., 2001) and members of both families have been found in the inner ear (Housley, 1998; Xiang, Bo & Burnstock, 1999). The P2X family consists of ionotropic receptors or ligand-gated ion channels and the P2Y family consists of metabotropic G protein-coupled receptors. The P2X family consists of 7 subtypes ($P2X_1$–$P2X_7$), whereas the P2Y family consists of 6 subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$ and $P2Y_{12}$).

It was demonstrated that mRNA transcripts for $P2Y_2$ and $P2Y_4$ are present in both the stria vascularis (unpublished observation) and vestibular labyrinth (Marcus & Scofield, 2001). Further, K^+ secretion was controlled by UTP as well as by ATP at both the apical receptor and at one of the basolateral

receptors. This finding showed that the purinergic receptors coupled to ion transport were of the $P2Y_2$ and/or $P2Y_4$ subtypes. Unambiguous identification of these two subtypes of purinergic receptor is made difficult by a lack of specific potent antagonists and of highly specific agonist potency profiles (North & Barnard, 1997). Rat $P2Y_2$ and $P2Y_4$ receptors have similar agonist profiles and accept ATP and UTP with the same affinity, creating difficulty in the identification and localization of these two receptors (Webb et al., 1998; Bogdanov et al., 1998b).

The aim of the present study was to investigate the localization of $P2Y_2$ and $P2Y_4$, in the nonsensory cells of the vestibular labyrinth and in the lateral wall of the cochlea using immunohistochemistry techniques. Gerbil tissues were used to correlate with the previous electrophysiologic findings.

Materials and Methods

Animal and Tissue Preparation

Adult Sprague-Dawley rats and adult gerbils were used for this study. All animals were deeply anesthetized by injection of pentobarbital sodium (50 mg/kg body weight). Sacrifice and collection of tissues was performed according to a protocol approved by the Institutional Animal Care and Use Committee of Kansas State University.

Reagents

The affinity-purified $P2Y_4$ antibody was raised in rabbit against the purified peptide CHEES ISRWA DTHQD, corresponding to the C-terminus (amino-acid residues 337–350) of rat $P2Y_4$ (Webb et al., 1998; Bogdanov et al., 1998b) and was purchased from Alomone Lab (Jerusalem, Israel; cat# APR-006). The affinity-purified $P2Y_2$ antibody raised in rabbit against the purified peptide CSISS DDSRR TESTP AGSET KDIRL (Kishore et al., 2000) was a gift from B.K. Kishore. As control for the visualization of apical membrane immunostain, we used an antibody raised in rabbit against a fusion protein made with a part of the amino-acid sequence (67–129) of KCNE1 (Alomone Lab, cat# APC-008). The immunostain using this antibody will be referred to as the KCNE1 stain. The secondary antibody for Western blot was anti-rabbit labeled with horseradish peroxidase (Amersham Biosciences, Piscataway, NJ, Cat# RPN2108) and for immunohistochemistry was anti-rabbit fluorescence-labeled Alexa 488 (Molecular Probes, Eugene, OR Cat# A-11008).

Western Blot

Western Blotting Tissue Preparation

Membrane-enriched fractions of proteins were obtained from brain, stria vascularis and vestibular labyrinth. All animals were transcardially perfused with phosphate buffered saline (PBS; 1 mM KH_2PO_4, 3 mM Na_2HPO_4, 150 mM NaCl, pH 7.3). The tissues were collected immediately after perfusion and placed in lysis buffer (2 mM EDTA; 50 mM Tris-Base; 320 mM Sucrose and 2.3% SDS). The brain was first disrupted using an homogenizer. Brain homogenate and inner-ear tissues were sonicated for 1 hr at 4°C. Insoluble materials were removed by centrifugation at 15,000 × g for 10 min (this step was omitted with the inner-ear tissue). The protein was collected by ultracentrifugation (100,000 × g for 45 minutes), resuspended and quantified using Bio-Rad Laboratories' protein assay (cat# 500-0006). Inner-ear tissues from 16 ears were pooled.

Western Blot

25 µg of total protein from inner-ear tissues and/or brain tissues was used in each gel lane. The proteins were incubated for 10 min at 70°C in the presence of detergent (SDS) and reducing agents (β-mercapto-ethanol). The proteins were then loaded in a 4–15% Tris-HCl precast polyacrylamide gel (Bio-Rad, cat# 161-1104). The electrophoresis was run under standard conditions (120 V for 1.5 hr) and the proteins were electroblotted to a 0.45 µm nitrocellulose membrane (Bio-Rad, cat# 162-0145) using the Trans-Blot SD Semi-Dry Electrophoretic system (Bio-Rad) under standard conditions (15 V for 45 minutes using Towbin transfer buffer: 25 mM Tris-Base, 192 mM glycine, 20% methanol, pH 8.3). The proteins on the membrane were then blocked with a 5% nonfat dry milk in Tween 20 PBS (0.1% Tween 20 in PBS: TPBS) for 2 hr at room temperature. The primary antibodies were diluted in a solution of 1% nonfat dry milk in TPBS to a final concentration of 1.5 µg/ml for $P2Y_4$ and 0.37 µg/ml for $P2Y_2$. As control, the primary antibody was pre-absorbed with the antigenic peptide at a concentration of 1.5 µg/ml ($P2Y_4$) and 2 µg/ml ($P2Y_2$) over night at 4°C. Antigenic peptide for $P2Y_2$ was synthesized by the Molecular Biology Core Facility at Kansas State University. The membrane was incubated overnight at 4°C using a multi-screen apparatus (Bio-Rad, cat# 170-4017), which allowed incubation of the membrane with more than one solution, with the antibodies and the pre-absorbed antibodies. The membrane was then extensively washed with PBS and incubated with the secondary antibody conjugated to horseradish peroxidase, which was diluted in a 5% nonfat dry milk solution in TPBS to a final dilution of 1:3000 for 1 hr at room temperature. Finally, the membrane was extensively washed with PBS before producing chemiluminescence using the ECL+ Western blotting kit from Amersham Pharmacia. Films (Hyperfilm ECL, Amersham, cat# RPN1674K) were exposed for 10 min and developed.

We made a "double Western blot" with both antibodies to $P2Y_2$ and $P2Y_4$ combined with one or two antigenic peptide treatments as listed in Table 1 using identical procedures as above.

The apparent molecular weights of the bands were determined by constructing calibration curves from the standards using the Lorentzian curve-fitting function in OriginLab software, (version 6.1, Northampton, MA) and interpolating the weight of the unknown band. Each result is based on 3 replications for each of the inner-ear tissues and 2 to 6 replications for the brain tissues.

Immunocytochemistry

Tissue Preparation for Immunocytochemistry

All animals were transcardially perfused with PBS and then with 2% paraformaldehyde in PBS. The temporal bones were dissected out and then the stria vascularis and the vestibular labyrinth were removed and postfixed, by immersion, with a fresh solution of 2% paraformaldehyde in PBS for 3 hr.

Cryosections

Following postfixation, the tissues were washed with PBS for 5 min and incubated in 30% sucrose in PBS overnight at 4°C. The tissues

Table. Solution scheme for the double immunoblots of Fig. 2

Antibody against	Final Antibody Concentration (μg/ml)			
	Lane 1	Lane 2	Lane 3	Lane 4
P2Y$_2$	0.37	0.37		
P2Y$_2$, pre-absorbed with 2 μg/ml peptide			0.37	0.37
P2Y$_4$	1.5		1.5	
P2Y$_4$, pre-absorbed with 1.5 μg/ml peptide		1.5		1.5

were embedded in Tissue-Tek (Sakura Finetek USA, Torrance, CA), cryosectioned at 12 μm thickness using a cryostat (−20°C chamber, −18°C chuck) and mounted on ProbeOn Plus charged glass slides (Fisher, cat# 15-188-52).

The tissue sections were warmed for 15 min at 37°C and then rehydrated with PBS for 10 min. The tissues were then permeabilized and the nonspecific antigenic sites blocked using the blocking solution (0.3% Triton X100, 10% normal donkey serum in PBS). The sections were incubated overnight at 4°C with the primary antibody, diluted in 0.01% Triton X100 and 1% donkey serum in PBS to a final concentration of 1.5 μg/ml (P2Y$_4$) and 0.37 μg/ml (P2Y$_2$). The specificity of the immunocytochemical stain observed was controlled both by diluting the antibody in a solution containing 1.5 μg/ml or 2 μg/ml of antigenic peptide used to produce P2Y$_4$ or P2Y$_2$ antibodies, respectively, in 0.01% Triton X100 and 1% normal donkey serum in PBS and by omitting the primary antibodies. The sections were extensively washed with PBS, then incubated in the dark with the secondary antibody (anti-rabbit Alexa 488; Molecular Probes) diluted in 0.01% Triton X100 and 1% normal donkey serum in PBS to a final dilution of 1:1100 for 1 hr at room temperature. Finally, the sections were extensively washed with PBS and overlaid with 20 μl of Fluorsave (Calbiochem, La Jolla, CA, cat# 345789) and a cover glass. Sections were observed with a Zeiss 410 Confocal microscope using a 40× oil objective (numerical aperture 1.3) and a 100× oil objective (numerical aperture 1.3).

Vibratome Sections

Following postfixation, the tissues were washed with PBS for 5 min and embedded in 8% agarose in PBS, sectioned at 50 μm thickness using a vibratome and harvested in PBS.

The tissues were permeabilized and the nonspecific antigenic sites blocked using the blocking solution (0.3% Triton X100, 10% normal donkey serum in PBS). The sections were incubated 48 hr at 4°C with the primary antibody, diluted in 0.03% Triton X100 and 1% donkey serum in PBS to a final concentration of 1.5 μg/ml (P2Y$_4$) and 0.37 μg/ml (P2Y$_2$). The specificity of the immunocytochemical stain observed was controlled both by diluting the antibody in a solution containing 1.5 μg/ml (P2Y$_4$) or 2 μg/ml (P2Y$_2$) of antigenic peptide used to produce P2Y$_4$ or P2Y$_2$ antibodies in 0.03% Triton X100 and 1% normal donkey serum in PBS and by omitting the primary antibodies. The sections were extensively washed with PBS, then incubated in the dark with the secondary antibody (anti-rabbit Alexa 488; Molecular Probes) diluted in 0.03% Triton X100 and 1% normal donkey serum in PBS to a final dilution of 1:1100 for 1 hr at room temperature. Finally the sections were extensively washed with PBS, mounted on ProbeOn Plus charged glass slides (Fisher, cat# 15-188-52), overlaid with 20 μl of fluorsave (Calbiochem cat# 345789) and a cover glass. Sections were observed with a Zeiss 410 Confocal microscope using a 40× oil objective (numerical aperture 1.3) and a 100× oil objective (numerical aperture 1.3).

Results

Western Blot

P2Y$_4$ Antibody Characterization

Rat brain was used as a control to establish the characteristics of each antibody. The P2Y$_4$ antibody stained one band at approximately 75 kDa on the membrane-enriched rat brain protein (Fig. 1*A*, *rB* lane *1*). This band was not found when the antibody was pre-absorbed with the antigenic peptide used to make the antibody (Fig. 1*A*, *rB* lane *2*). The antibody was then used with membrane-enriched gerbil brain proteins in order to verify whether this antibody was also able to recognize the gerbil protein. Three bands were stained with a molecular weight of approximately 75 kDa, 55 kDa and 36 kDa (Fig. 1*A*, *gB* lane *1*). All bands disappeared when the antibody was pre-absorbed with the antigenic peptide (Fig. 1*A*, *gB* lane *2*).

Subsequently, the antibody was used on the target tissues stria vascularis and vestibular labyrinth. The P2Y$_4$ antibody was able to stain a single band at approximately 55 kDa on stria vascularis tissues and two bands at approximately 42 kDa and 56 kDa on the vestibular labyrinth (Fig. 1*A*, *gSV* lane *1* and *gVL* lane *1*). All bands disappeared when the antibody was pre-absorbed with the antigenic peptide (Fig. 1*A*, *gSV* lane *2* and *gVL* lane *2*). The finding that no bands were stained when the antibody was blocked with the peptide also demonstrates that there was no unspecific binding of the secondary antibody.

P2Y$_2$ Antibody Characterization

The P2Y$_2$ antibody was able to recognize one band on rat and gerbil brain with a molecular weight of approximately 42 kDa (Fig. 1*B*, *rB* and *gB*). Similarly, using stria vascularis and the vestibular labyrinth, the antibody also stained one band at approximately 42 kDa (Fig. 1*B*, *gSV* lane *1* and *gVL* lane *1*). These bands disappeared when the antibody was blocked with the antigenic peptide (Fig. 1*B*, *gSV* lane *2* and *gVL* lane *2*). The finding that no bands

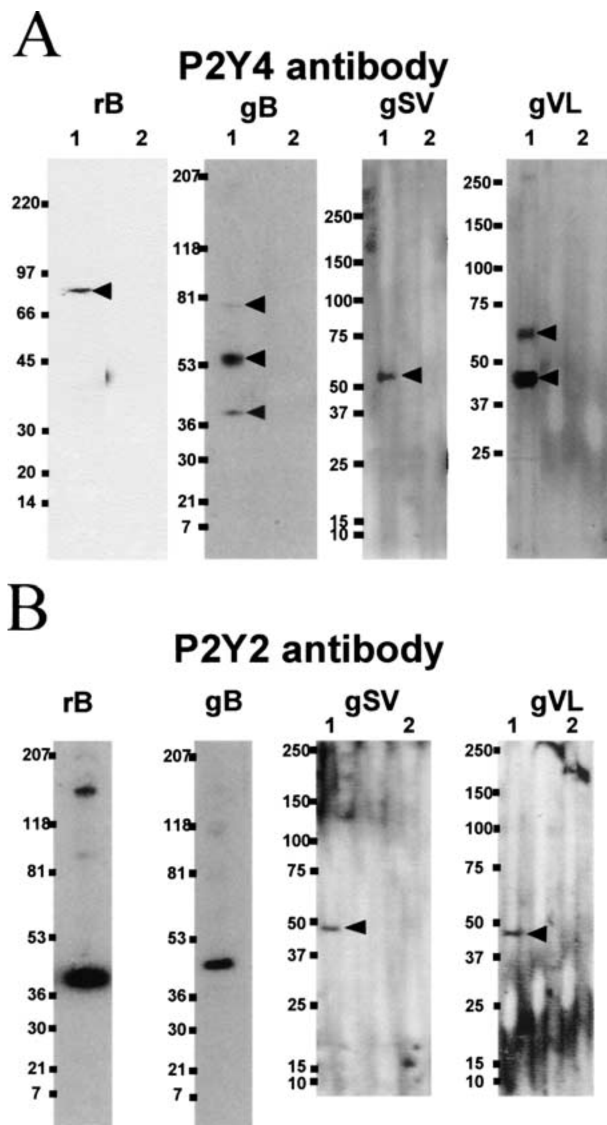

Fig. 1. Immunoblot analysis of membrane-enriched protein. 25 μg of membrane protein were separated on SDS-PAGE and blotted on nitrocellulose membrane (A) $P2Y_4$ characterization. 1:200 dilution of $P2Y_4$ antibody (lane *1*) and pre-absorbed $P2Y_4$ antibody (lane *2*). *rB*: rat brain. *gB*: gerbil brain. *gSV*: gerbil stria vascularis. *gVL*: gerbil vestibular labyrinth. (*B*) $P2Y_2$ characterization. 1:500 dilution of $P2Y_2$ antibody (lane *1*) and pre-absorbed $P2Y_2$ antibody (lane *2*). *rB*: rat brain. *gB:* gerbil brain. *GsV*: gerbil stria vascularis. *gVL*: gerbil vestibular labyrinth. Each picture is a representative experiment. Images of gel were cut along lanes and repositioned for clarity of comparison; dimensions and relative alignments were maintained.

were stained when the antibody was blocked with the peptide also demonstrates that there was no unspecific binding of the secondary antibody.

Double Western Blot

A double Western blot on each inner-ear tissue was made, in which both antibodies were simultaneously used and combinations of antigenic peptide were included to unambiguously determine the specificity of each of the antibodies, since the molecular weights of the $P2Y_2$ and $P2Y_4$ receptors appeared to be very close.

The $P2Y_2$ and $P2Y_4$ antibodies applied to Western blots of stria vascularis protein stained two bands with a molecular weight of 42 kDa and 55 kDa, respectively (Fig. 2*A*, lane *1*). When only one of these two antibodies was blocked with its respective peptide, only one band disappeared: the band at 55 kDa disappeared when the $P2Y_4$ antibody was blocked with its respective peptide but the band at 42 kDa remained (Fig. 2*A*, lane *2*). In contrast, the band at 42 kDa disappeared when the $P2Y_2$ antibody was blocked with its respective peptide and the band at 55 kDa remained (Fig. 2*A*, lane *3*). Finally, both of these bands (42 kDa and 55 kDa) disappeared when both antibodies were blocked with their respective peptide (Fig. 2*A*, lane *4*).

Similar results were found for the vestibular labyrinth (Fig. 2*B*). The $P2Y_2$ and $P2Y_4$ antibodies used simultaneously stained two bands: a thick band with a molecular weight between 41 and 44 kDa and a band at approximately 56 kDa. The first band is the result of superposition of the 42 kDa band stained with $P2Y_4$ antibody and the 42 kDa band stained with $P2Y_2$ antibody and the second band is the 56 kDa band found previously with $P2Y_4$ antibody (Fig. 2*B*, lane *1*). When $P2Y_4$ antibody was blocked with its antigenic peptide only one thin band at approximately 42 kDa remained (the thick band between 41 kDa and 44 kDa became a thin band at 42 kDa) (Fig. 2*B*, lane *2*). When $P2Y_2$ antibody was blocked with its antigenic peptide, two bands appeared at approximately 42 kDa (the thick band between 41 and 44 kDa became a thin band at 42 kDa) and at 56 kDa, respectively (Fig. 2*B*, lane *3*). Finally, all bands disappeared when both antibodies were blocked with their respective antigenic peptide (Fig. 2*B*, lane *4*). The finding that no extra bands were stained when the antibody was blocked with the peptide also demonstrates that there was no unspecific binding of the secondary antibody.

Immunocytochemistry

Immunolocalization was used to identify the cell types expressing $P2Y_2$ and $P2Y_4$ and to determine the specific membrane domain (apical or basolateral). The specific antibodies for $P2Y_2$ and $P2Y_4$ were used to stain sections of cochlear lateral wall and nonsensory cells of the ampulla of the semicircular canals in the vestibular labyrinth. Images of confocal fluorescence are shown and compared with brightfield images of the same sections. Antibody blocking with antigenic peptide and secondary antibody alone were used as controls for specificity. KCNE1, a protein known to be

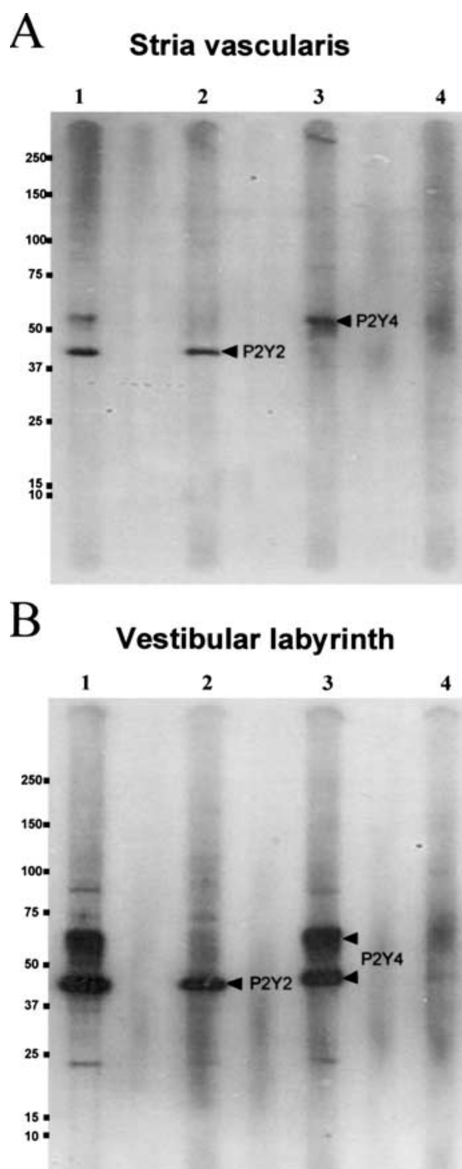

Fig. 2. Double immunoblot analysis of $P2Y_4$ and $P2Y_2$ receptors from membrane-enriched protein. 25 μg of membrane protein were separated on SDS-PAGE and blotted on nitrocellulose membrane. (*A*) Stria vascularis. (*B*) Vestibular labyrinth. 1:200 dilution of $P2Y_4$ antibody and 1:500 dilution of $P2Y_2$ antibody (lane *1*). 1:200 dilution of pre-absorbed $P2Y_4$ antibody and 1:500 dilution of $P2Y_2$ antibody (lane *2*). 1:200 dilution of $P2Y_4$ and 1:500 dilution of pre-absorbed $P2Y_2$ antibody (lane *3*). 1:200 dilution of pre-absorbed $P2Y_4$ and 1:500 dilution of pre-absorbed $P2Y_2$ antibody (lane *4*).

expressed only at the apical membrane of vestibular dark cells and stria marginal cells (Mori et al., 1993), was used as a point of reference for comparing the results with the purinergic receptors.

Localization of $P2Y_4$ Purinoceptor in the Lateral Wall of the Cochlea and the Vestibular Labyrinth

In both epithelia, stria vascularis and vestibular dark cells, $P2Y_4$ purinoceptor seemed to have an identical expression pattern. By comparison to the KCNE1 stain (Fig. 3), only the apical membrane of vestibular dark cells and strial marginal cells seemed to express the $P2Y_4$ protein (Fig. 4*A*, *C*, *E*, 5*A*, *C* and *E*). In no experiment was any signal observed when the primary antibody was either pre-absorbed with the antigenic peptide or omitted (Fig. 4*G* and *H*, 5*G* and *H*).

Localization of $P2Y_2$ Purinoceptor in the Lateral Wall of the Cochlea and the Vestibular Labyrinth

In contrast to $P2Y_4$, the $P2Y_2$ antibody clearly stained the basolateral membrane of vestibular dark cells (Fig. 6*A*, *C*, *E*). Similarly, $P2Y_2$ antibody stained a region that appeared to be predominantly the basolateral membrane of strial marginal cells (Fig. 7*A*, *C*, *E*) as judged by comparison with the basolateral stain obtained using ClC-K antibody as previously reported (Sage & Marcus, 2001). It is not possible from these images to unequivocally exclude the interpretation that other membranes within the stria were also stained due to the density of the strial structure. No signals were observed when the primary antibody was pre-absorbed with the antigenic peptide or omitted (Fig. 6*G*, H, 7*G*, *H*).

Discussion

This is the first report of localization of $P2Y_4$ and $P2Y_2$ G protein-coupled purinergic receptors in epithelia of the inner ear using immunohistochemical techniques. Previous identification of purinergic receptors in inner-ear epithelia was based on electrophysiologic/pharmacologic and molecular biologic methods. Transcripts for both $P2Y_2$ and $P2Y_4$ receptors were found in stria vascularis (Marcus & Scofield, unpublished observations) and vestibular labyrinth (Marcus & Scofield, 2001).

Electrophysiologic measurements of K^+ secretion as short-circuit current were made with a micro-Ussing chamber (aperture 80 μm), which allowed separate perfusion of the apical or basolateral membranes with selected agonists and antagonists. These studies showed a monotonic decrease in K^+ secretion in response to apical agonists, including ATP and UTP (Liu et al., 1995; Marcus & Scofield, 2001). The agonist-potency order was consistent with the action of $P2Y_2$ or $P2Y_4$ receptors. In contrast, the basolateral membrane responded to purinergic agonists with a transient increase followed by a sustained decrease in K^+ secretion. The increase had the agonist-potency order for $P2Y_1$ and the sustained decrease had an agonist potency order consistent with the presence of $P2Y_1$ and either $P2Y_2$ or $P2Y_4$ receptor (Liu et al., 1995). The uncertainty between $P2Y_2$ and $P2Y_4$ receptors arose because of recent findings that

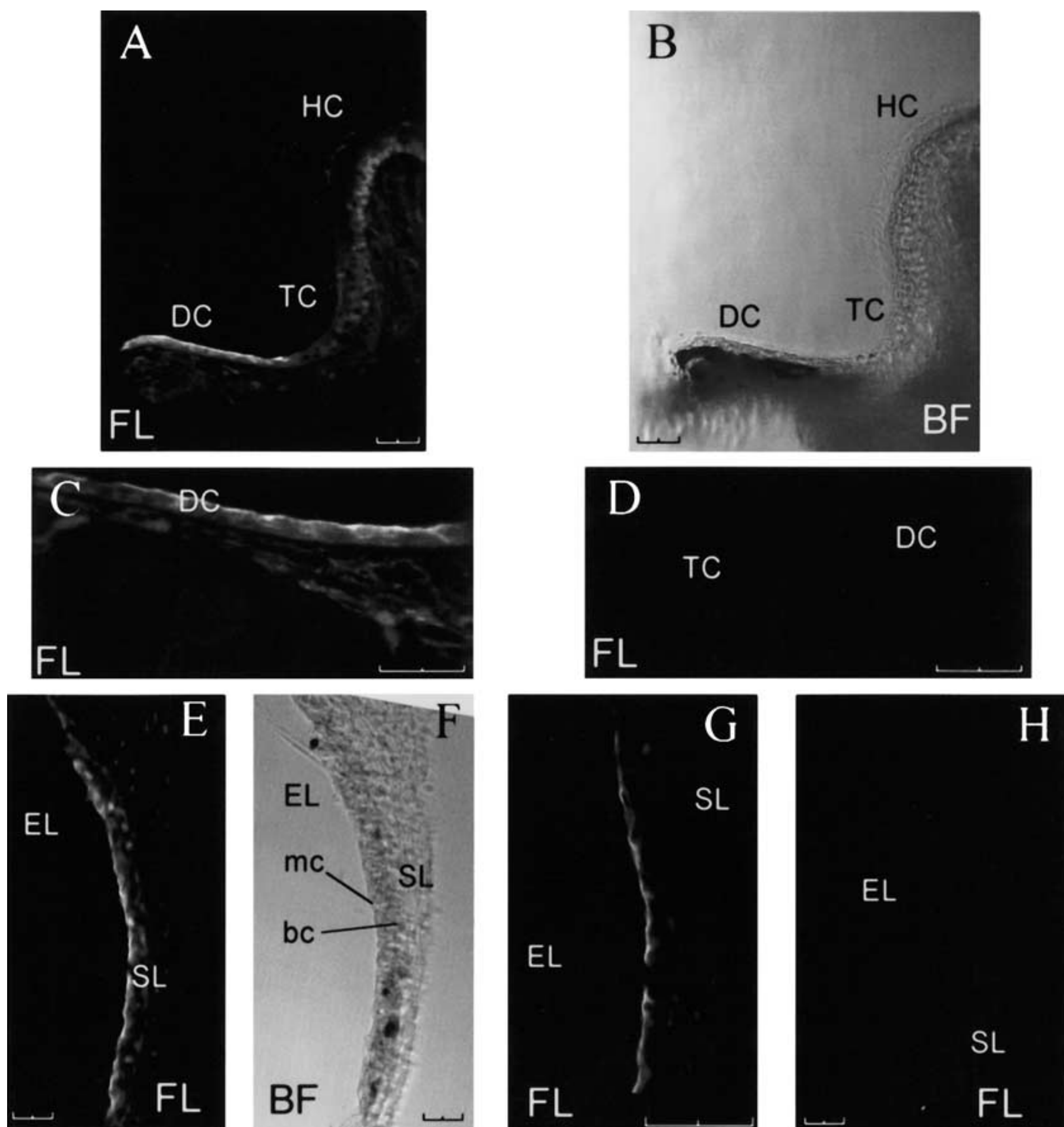

Fig. 3. Immunolocalization of KCNE1 proteins in the stria vascularis and vestibular labyrinth. *A*, *C*, *E*, and *G*: 1/200 dilution of the KCNE1 antibody. *D* and *H*: 1/200 dilution of pre-absorbed KCNE1 antibody (*D*: vestibular labyrinth; *H*: stria vascularis). *B* and *F*: brightfield image of *A* and *E*, respectively. *FL*, fluorescent image; *BF*, brightfield image; *HC*, hair cells; *TC*, transitional cells; *DC*, dark cell region localized by the melanin pigment beneath the epithelial layer; *mc*, location of marginal cell layer in stria vascularis; *bc*, location of basal cell layer in stria vascularis. Bar, total length: 25 μm.

rodent $P2Y_2$ and $P2Y_4$ receptors have the same agonist potency profile for both receptors (Webb et al., 1998; Bogdanov et al., 1998b) although it was earlier recognized that human $P2Y_2$ and $P2Y_4$ receptors have a distinct agonist profile with ATP only a partial agonist to human $P2Y_4$ (Communi et al., 1995). It was found that high concentrations of suramin, a purinergic receptor inhibitor, could be used to differentiate between the two subtypes (Robaye, Boeynaems & Communi, 1997; Otero et al., 2000). Electrophysiological studies with suramin were consistent with the interpretation that the apical receptors in vestibular dark cells (Marcus & Scofield, 2001) and strial marginal cells (Lee & Marcus, unpublished observations) are of the $P2Y_4$ subtype. Our results in the present study support that conclusion.

The antibody against $P2Y_2$ peptide recognized a single band at approximately 42 kDa for rat brain, gerbil brain, gerbil stria vascularis and gerbil vestibular labyrinth that corresponds to the predicted molecular weight of the $P2Y_2$ protein according to the amino-acid sequence (Rice et al., 1995). The band for stria vascularis and vestibular labyrinth disappeared when the antibody was blocked with its antigenic

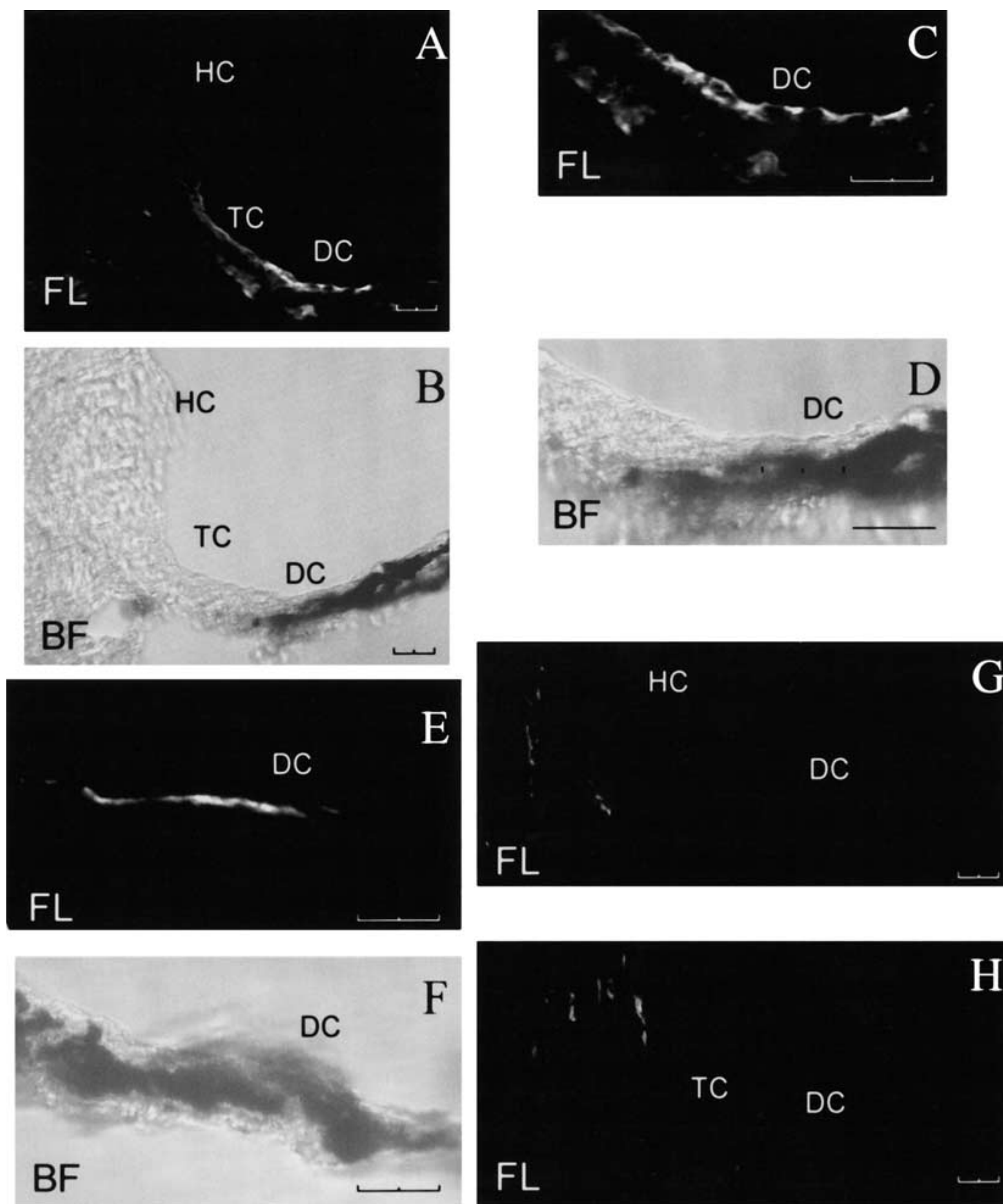

Fig. 4. Immunolocalization of $P2Y_4$ proteins in the vestibular labyrinth. *A*, *C* and *E*: 1/200 dilution of $P2Y_4$ antibody. *G*: 1/200 dilution of pre-absorbed $P2Y_4$ antibody. *H*: $P2Y_4$ antibody omitted. *B*, *D* and *F*: brightfield image of *A*, *C* and *E*, respectively. *FL*, fluorescent image; *BF*, brightfield image; *HC*, hair cells; *TC*, transitional cells; *DC*, dark cell region localized by the melanin pigment beneath the epithelial layer. Bar, total length: 25 μm.

peptide. A similar-sized protein was identified in rat kidney with the same antibody (Kishore et al., 2000).

In contrast, $P2Y_4$ antibody recognized in Western blots several bands of different molecular weights in the different tissues. One band was found at approximately 75 kDa for rat brain, three bands for gerbil brain at approximately 75 kDa, 55 kDa and 36 kDa, two bands for vestibular tissues with a molecular weight approximately 42 kDa and 56 kDa and one single band for stria vascularis at approximately 55 kDa. In spite of the apparent complexity, all of the observed bands were absent when the antibody was blocked with antigenic peptide.

The predicted molecular weight of $P2Y_4$ proteins according to the amino-acid sequence is 41 kDa (Webb et al., 1998; Bogdanov et al., 1998b). The apparent variability in the molecular weight of the bands among tissue types may be the result of several

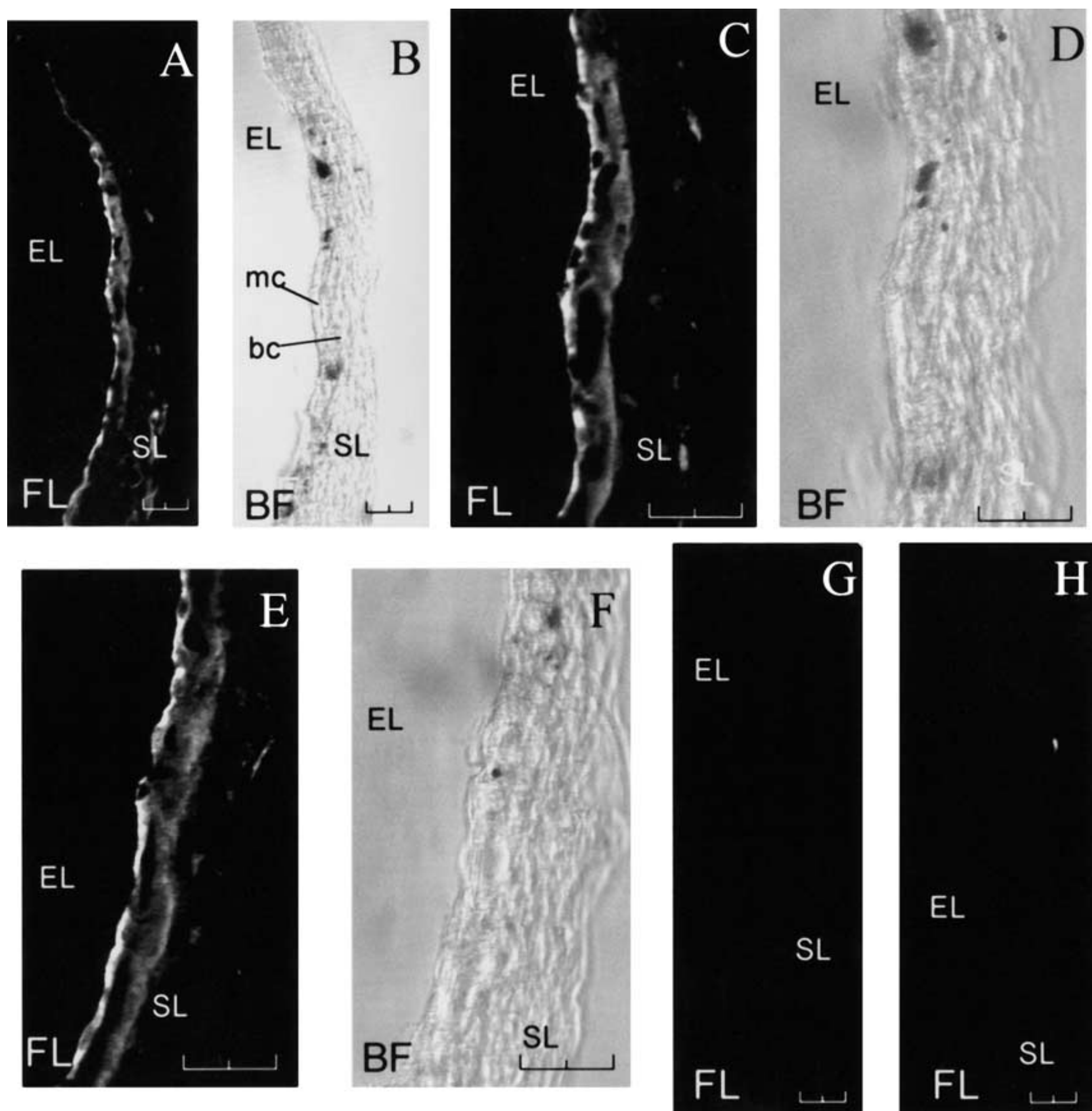

Fig. 5. Immunolocalization of $P2Y_4$ proteins in the stria vascularis. *A*, *C* and *E*: 1/200 dilution of $P2Y_4$ antibody. *G*: 1/200 dilution of pre-absorbed $P2Y_4$ antibody. *H*: $P2Y_4$ antibody omitted. *B*, *D* and *F*: brightfield image of *A*, *C* and *E*, respectively. *FL*, fluorescent image; *BF*, brightfield image; *mc*, location of marginal cell layer in stria vascularis; *bc*, location of basal cell layer in stria vascularis. Bar, total length: 25 μm.

factors. It was shown previously that during SDS-PAGE anomalous movements could occur for proteins that bound greater or lesser amounts of SDS than protein standards (Grefrath & Reynolds, 1974; Thornhill & Levinson, 1987). Differences could also be due to incomplete linearization. However, we performed several techniques of extraction and linearization and in all cases the molecular weight of the proteins in each tissue appeared to be unchanged even in the presence of protease inhibitors (*data not shown*). A more likely occurrence is that $P2Y_4$ may have had post-translational modifications that differed among tissues. The apparent difference in molecular weight between the 55/56 kDa bands and the predicted weight of 41 kDa falls within the range commonly found for glycosylated proteins (Dube & Flynn, 1998). There is one N-glycosylation site on $P2Y_4$ at amino acid 175 and one O-glycosylation site at amino acid 11 (NetOGlyc 2.0 and PROSITE (Bairoch, Bucher & Hofmman, 1997).

The antibody was raised against a small portion of the whole protein, so it is conceivable that this antibody could bind related but different proteins sharing the same epitope. However, the specificity of the $P2Y_4$ antibody was checked by making a BLASTP search in the NCBI GenBank using the whole sequence of the peptide against which the antibody was raised. The search showed under stringent conditions

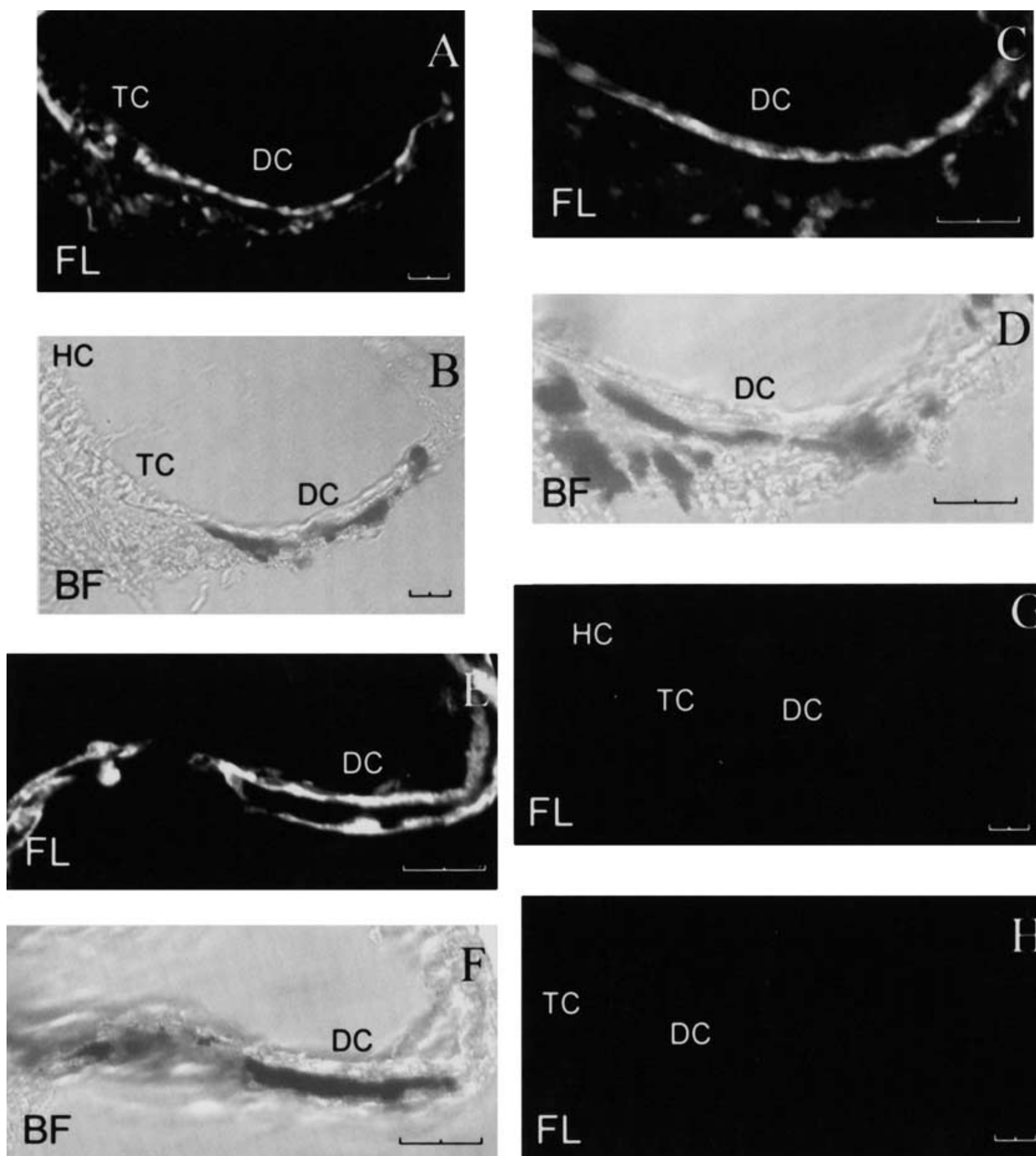

Fig. 6. Immunolocalization of P2Y$_2$ proteins in the vestibular labyrinth. *A*, *C* and *E*: 1/500 dilution of P2Y$_2$ antibody. *G*: 1/500 dilution of pre-absorbed P2Y$_2$ antibody. *H*: P2Y$_2$ antibody omitted. *B*, *D* and *F*: brightfield image of *A*, *C* and *E*, respectively. *FL*, fluorescent image; *BF*, brightfield image; *HC*, hair cells; *TC*, transitional cells; *DC*, dark cell region localized by the melanin pigment beneath the epithelial layer. Bar, total length: 25 μm.

(*e* value < 1) that the sequence was specific to P2Y$_4$ purinergic receptor (human, rat and mouse).

Immunolocalization of the purinergic receptors P2Y$_2$ and P2Y$_4$ in the vestibular labyrinth and the stria vascularis showed distinct expression patterns. P2Y$_4$ purinoceptor seemed to be expressed only at the apical membrane of the strial marginal cells and vestibular dark cells. We confirmed the localization of P2Y$_4$ at the apical membrane of strial marginal cells and vestibular dark cells by comparison to the stain for KCNE1 protein. In strial marginal cells and vestibular dark cells, KCNE1 protein is only expressed at the apical membrane (Mori et al., 1993; Nicolas et al., 2001).

In contrast, we found P2Y$_2$ localized exclusively in the basolateral membrane of vestibular dark cells and also in a region of the stria vascularis that is likely the basolateral membrane of the strial marginal cells. The interpretation of the basolateral localization of P2Y$_2$ receptors in the strial marginal cells and in the vestibular dark cells was based on comparison with the immunolocalization of ClC-K proteins in the same epithelia (Sage & Marcus, 2001). However,

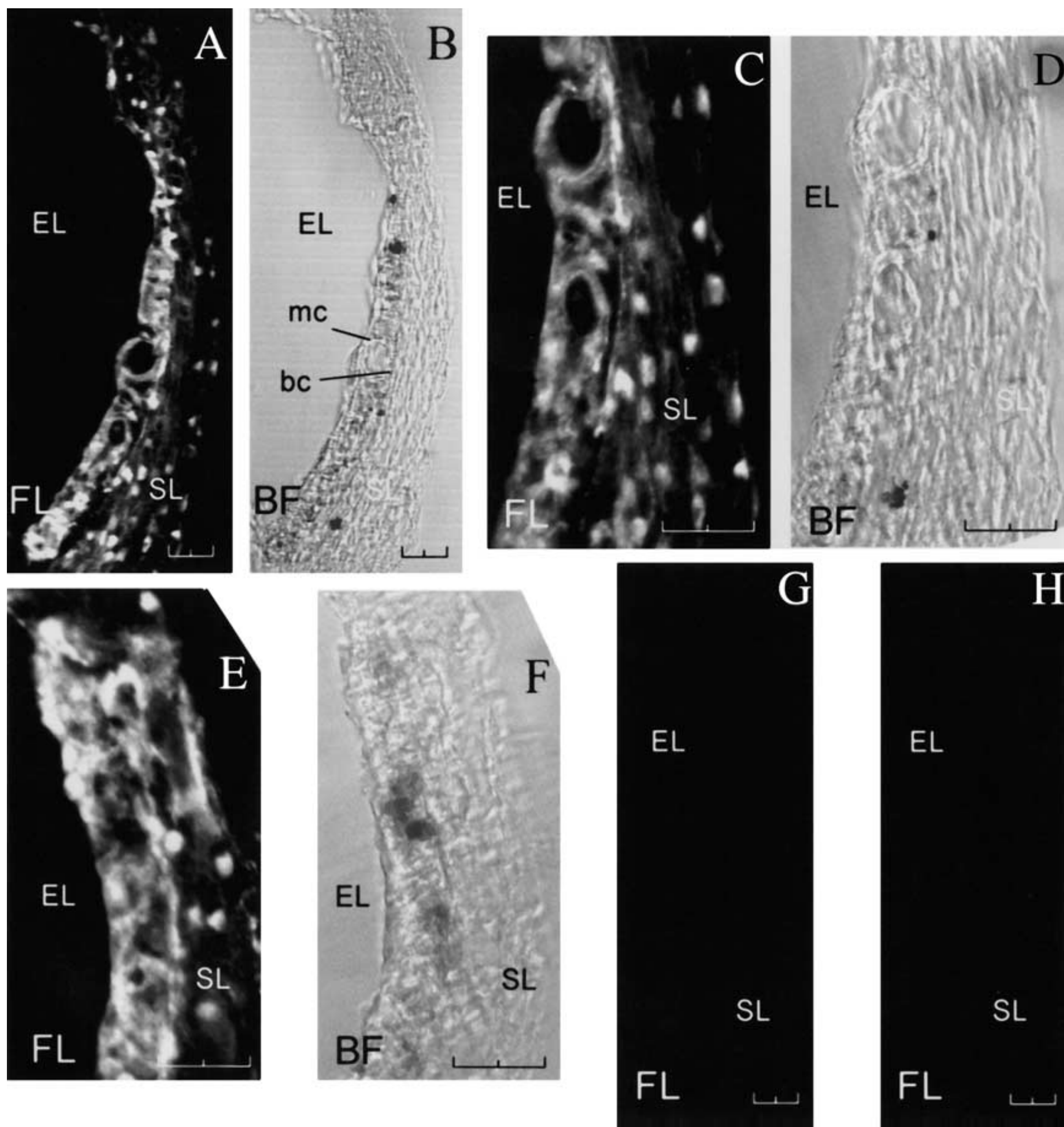

Fig. 7. Immunolocalization of $P2Y_2$ proteins in the stria vascularis. *A*, *C* and *E*: 1/500 dilution of $P2Y_2$ antibody. *G*: 1/500 dilution of pre-absorbed $P2Y_2$ antibody. *H*: $P2Y_2$ antibody omitted. *B*, *D* and *F*: brightfield image of *A*, *C* and *E*, respectively. *FL*, fluorescent image; *BF*, brightfield image; *mc*, location of marginal cell layer in stria vascularis; *bc*, location of basal cell layer in stria vascularis. Bar, total length: 25 μm.

we cannot exclude the possibility that intermediate cells or capillary cells may also express $P2Y_2$ receptors since there is extensive interdigitation and connection among these. Considering the identical responses of strial marginal cells' and vestibular dark cells' short-circuit current to basolateral perfusion of purinergic agonists (Liu et al., 1995), it is highly likely that the staining occurred at least at the basolateral membrane of strial marginal cells but may also occur in addition in other cells. The immunolocalization of $P2Y_2$ is therefore consistent with the interpretation that K^+ secretion is regulated by a basolateral $P2Y_2$ receptor.

$P2Y_2$ and $P2Y_4$ receptors have been identified in other tissues using molecular biological techniques. In fact, expression of multiple purinergic receptors has been shown in the apical and basolateral membranes of pancreatic duct epithelial cells (Hede et al., 1999; Luo et al., 1999). Previous studies found mRNA transcripts for $P2Y_4$ in the central nervous system (Webb & Barnard, 1999), peripheral nervous system (Liu et al., 2000), heart (Webb, Boluyt & Barnard, 1996), fetal human heart (Bogdanov, Rubino & Burnstock, 1998a), kidney (proximal tubule, outer medullary collecting duct and thin ascending

limb of Henle) (Bailey et al., 2000), haematopoietic cells (Jin et al., 1998), tracheal gland cells (Merten et al., 1998), human lung cell lines (Communi et al., 1999) and human hepatocytes (Schofl et al., 1999). Furthermore, it was demonstrated that expression levels of $P2Y_4$-receptor transcripts decreased during the development in the heart (Webb et al., 1996).

It was shown that the $P2Y_2$ receptor is expressed in a wide variety of cells and tissues including astrocytes, several types of blood cells, endothelial cells and epithelial cells (Ralevic & Burnstock, 1998). In particular, mRNA transcripts for $P2Y_2$ were found to be expressed in several epithelia such as human keratinocytes (Dixon et al., 1999), human osteoblasts (Bowler et al., 1995), murine thymic epithelial cells (Bisaggio et al., 2001) and the central and peripheral nervous system (Filippov et al., 1998). Further, functional expression of $P2Y_2$ was demonstrated in mesenteric arteries and veins of rats by the response of contractions to a series of purinergic agonists and by its sensitivity to suramin (Galligan et al., 2001).

Purinergic receptors of the P2Y family are coupled to a G protein intracellular signaling pathway that involves activation of phospholipase C, production of inositol 3,4,5-trisphosphate (IP3) with subsequent elevation of intracellular Ca^{2+}, activation of protein kinase C (PKC) and the final cellular effects due to phosphorylation by PKC or due to elevated Ca^{2+}. Activation of these receptors can be detected by measuring activation of any of these cellular indicators. Elevation of inositol phosphates was found in the lateral wall of the cochlea (Ogawa & Schacht, 1995) and ampulla of the semicircular canal (Butlen et al., 1997) after stimulation by purinergic agonists. An increase of intracellular free Ca^{2+} was seen in both native and cultured strial marginal cells in response to ATP, which was independent of extracellular Ca^{2+} (Ikeda et al., 1995; Suzuki et al., 1995). PKC mediates the action of the apical $P2Y_4$ receptor (Marcus et al., 1997) and several isoforms of PKC were shown to be expressed in the stria vascularis (Agrup, Bagger-Sjoback & Fryckstedt, 1997).

The role of purinergic receptors in the K^+-secreting epithelial cells of the inner ear is not yet established although several mechanisms have been proposed. ATP and/or analogs have been found in both the endolymphatic (luminal) and perilymphatic (basolateral) compartments (Munoz et al., 1995). Although both $P2Y_2$ and $P2Y_4$ receptors were found to desensitize in other systems upon prolonged exposure to agonist (Robaye et al., 1997; Otero et al., 2000), we found that the apical $P2Y_4$ receptor in vestibular dark cells (Marcus & Scofield, 2001) and strial marginal cells (Marcus & Liu, unpublished observations) are resistant to desensitization for more than 15 minutes. Static or slowly changing levels of agonist in endolymph may therefore influence the rate of K^+ secretion.

Changes in endolymphatic agonist concentration near the strial marginal cells and vestibular dark cells are thought to be produced by release of sub-apical vesicles containing concentrated ATP (White et al., 1995). This would result in autocrine signaling and could overcome the lack of communication among these cells because of the absence of gap junctions, an unusual constellation for epithelial cells.

The $P2X_2$ ionotropic purinergic receptor has been identified in several epithelial cell types of the adult cochlear duct and has been shown to contribute to the homeostasis of the cation composition of endolymph. $P2X_2$ receptors were shown to be expressed in hair cells (outer and inner), supporting cells (Deiters and Hensens), outer and inner sulcus cells, Claudius cells and Reissner's membrane (Housley, 1998; Housley et al., 1999). Specific immunolocalization demonstrated that $P2X_2$ is expressed at the apical aspect of the stereocilia of the inner and outer hair cells, at the endolymphatic surface of Hensen's cells (Housley et al., 1999; Lagostena et al., 2001), over the majority of the surface of the Deiter's cells (Housley, 2000), and in the epithelial cells that line the endolymphatic surface of Reissner's membrane (King et al., 1998). $P2X_2$ receptors were immunolocalized at a very high expression level to the stria vascularis by one group without any specific cellular localization (Xiang, Bo & Burnstock, 1999) but were found to be negligible by another group (Housley et al., 1999).

It would be expected for those receptors expressed in the apical cell membranes that the $P2X_2$ receptors would contribute to the homeostasis of cations in endolymph. In fact, it was shown that $P2X_2$ receptors in cochlear outer sulcus cells and vestibular transitional cells functionally produce transepithelial currents in response to purinergic agonists. These currents had the pharmacologic agonist- and antagonist-profiles of the $P2X_2$ receptor (Lee, Chiba & Marcus, 2001).

In summary, diverse purinergic receptor subtypes (including $P2Y_2$, $P2Y_4$ and $P2X_2$) contribute to the homeostasis of both cochlear and vestibular endolymph. $P2Y_2$ and $P2Y_4$ receptors were both found in strial marginal cells and in vestibular dark cells. $P2Y_4$ is expressed only at the apical membrane of both cells and accounts for the non-desensitizing decrease in K^+ secretion upon apical perfusion of UTP. $P2Y_2$ is expressed in the basolateral membrane of both cells and accounts for the sustained decrease in K^+ secretion upon basolateral perfusion of UTP. The physiological advantage to inner-ear function of trafficking receptors of identical agonist potency profiles to separate membrane domains is not clear. However, this finding could conceivably lead to development of therapeutic strategies to control inner-ear K^+ secretion by gene therapy under pathological conditions such as Meniere's Disease.

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References

Agrup, C., Bagger-Sjoback, D., Fryckstedt, J. 1997. Protein kinase and protein phosphatase presence in the stria vascularis. *Pfluegers Arch* **433:**603–608

Bailey, M.A., Imbert-Teboul, M., Turner, C., Marsy, S., Srai, K., Burnstock, G., Unwin, R.J. 2000. Axial distribution and characterization of basolateral P2Y receptors along the rat renal tubule. *Kidney lnt*. **58:**1893–1901

Bairoch, A., Bucher, P., Hofmann, K. 1997. The PROSITE database, its status in 1997. *Nucleic Acids Res*. **25:**217–221

Bisaggio, R.D., Nihei, O.K., Persechini, P.M., Savino, W., Alves, L.A. 2001. Characterization of P2 receptors in thymic epithelial cells. *Cell Mol. Biol.* (*Noisy.-le-grand*) **47:**19–31

Bogdanov, Y., Rubino, A., Burnstock, G. 1998a. Characterisation of subtypes of the P2X and P2Y families of ATP receptors in the foetal human heart. *Life Sci.* **62:**697–703

Bogdanov, Y.D., Wildman, S.S., Clements, M.P., King, B.F., Burnstock, G. 1998b. Molecular cloning and characterization of rat P2Y4 nucleotide receptor. *Br. J. Pharmacol.* **124**:428–430

Bowler, W.B., Birch, M.A., Gallagher, J.A., Bilbe, G. 1995. Identification and cloning of human P2U purinoceptor present in osteoclastoma, bone, and osteoblasts. *J. Bone Miner. Res.* **10:**1137–1145

Butlen, D., Bernard, C., Ammar, A., Ferrary, E. 1997. Purine and pyrimidine nucleotide-sensitive phosphoinositidase C in ampulla from frog semicircular canal. *Am. J. Physiol.* **272:**R51–R58

Communi, D., Paindavoine, P., Place, G.A., Parmentier, M., Boeynaems, J.M. 1999. Expression of P2Y receptors in cell lines derived from the human lung. *Br. J. Pharmacol.* **127:**562–568

Communi, D., Pirotton, S., Parmentier, M., Boeynaems, J.M. 1995. Cloning and functional expression of a human uridine nucleotide receptor. *J. Biol. Chem.* **270:**30849–30852

Dixon, C.J., Bowler, W.B., Littlewood-Evans, A., Dillon, J.P., Bilbe, G., Sharpe, G.R., Gallagher, J.A. 1999. Regulation of epidermal homeostasis through P2Y2 receptors. *Br. J. Pharmacol.* **127:**1680–1686

Dube, S., Flynn, E. 1998. Estimating protein molecular weights using SDS-PAGE. FOCUS (*Invitrogen*) **20:**24–25

Filippov, A.K., Webb, T.E., Barnard, E.A., Brown, D.A. 1998. P2Y2 nucleotide receptors expressed heterologously in sympathetic neurons inhibit both N-type Ca^{2+} and M-type K^+ currents. *J. Neurosci.* **18:**5170–5179

Galligan, J.J., Hess, M.C., Miller, S.B., Fink, G.D. 2001. Differential localization of P2 receptor subtypes in mesenteric arteries and veins of normotensive and hypertensive rats. *J. Pharmacol. Exp. Ther.* **296:**478–485

Grefrath, S.P., Reynolds, J.A. 1974. The molecular weight of the major glycoprotein from the human erythrocyte membrane. *Proc. Natl. Acad. Sci.* U.S.A **71:**3913–3916

Hede, S.E., Amstrup, J., Christoffersen, B.C., Novak, I. 1999. Purinoceptors evoke different electrophysiological responses in pancreatic ducts. P2Y inhibits K^+ conductance, and P2X stimulates cation conductance. *J. Biol. Chem.* **274:**31784–31791

Hollopeter, G., Jantzen, H.M., Vincent, D., Li, G., England, L., Ramakrishnan, V., Yang, R.B., Nurden, P., Nurden, A., Julius, D., Conley, P.B. 2001. Identification of the platelet M ADP receptor targeted by antithrombotic drugs. *Nature* **409:**202–207

Housley, G.D. 1998. Extracellular nucleotide signaling in the inner ear. *Mol. Neurobiol.* **16:**21–48

Housley, G.D. 2000. Physiological effects of extracellular nucleotides in the inner ear. *Clin. Exp. Pharmacol. Physiol.* **27:**575–580

Housley, G.D., Kanjhan, R., Raybould, N.P., Greenwood, D., Salih, S.G., Jarlebark, L., Burton, L.D., Setz, V.C., Cannell, M.B., Soeller, C., Christie, D.L., Usami, S., Matsubara, A., Yoshie, H., Ryan, A.F., Thorne, P.R. 1999. Expression of the $P2X_2$ receptor subunit of the ATP-gated ion channel in the cochlea: implications for sound transduction and auditory neurotransmission. *J. Neurosci.* **19:**8377–8388

Ikeda, K., Suzuki, M., Furukawa, M., Takasaka, T. 1995. Calcium mobilization and entry induced by extracellular ATP in the non-sensory epithelial cell of the cochlear lateral wall. *Cell Calcium* **18:**89–99

Jin, J., Dasari, V.R., Sistare, F.D., Kunapuli, S.P. 1998. Distribution of P2Y receptor subtypes on haematopoietic cells. *Br. J. Pharmacol.* **123:**789–794

King, M., Housley, G.D., Raybould, N.P., Greenwood, D., Salih, S.G. 1998. Expression of ATP-gated ion channels by Reissner's membrane epithelial cells. *Neuroreport* **9:**2467–2474

Kishore, B.K., Ginns, S.M., Krane, C.M., Nielsen, S., Knepper, M.A. 2000. Cellular localization of P2Y(2) purinoceptor in rat renal inner medulla and lung. *Am. J. Physiol.* **278:**F43–F51

Lagostena, L., Ashmore, J.F., Kachar, B., Mammano, F. 2001. Purinergic control of intercellular communication between Hensen's cells of the guinea-pig cochlea. *J. Physiol.* **531:**693–706

Lee, J.H., Chiba, T., Marcus, D.C. 2001. $P2X_2$ receptor mediates stimulation of parasensory cation absorption by cochlear outer sulcus cells and vestibular transitional cells. *J. Neurosci.* **21:**9168–9174

Liu, D.M., Katnik, C., Stafford, M., Adams, D.J. 2000. P2Y purinoceptor activation mobilizes intracellular Ca^{2+} and induces a membrane current in rat intracardiac neurones. *J. Physiol.* **526 Pt 2:**287–298

Liu, J., Kozakura K, Marcus, D.C. 1995. Evidence for purinergic Receptors in Vestibular Dark cell and Strial Marginal cell epithelia of Gerbil. *Auditory Neuroscience* **1**:331–340

Luo, X., Zheng, W., Yan, M., Lee, M.G., Muallem, S. 1999. Multiple functional P2X and P2Y receptors in the luminal and basolateral membranes of pancreatic duct cells. *Am. J. Physiol* **277:**C205–C215

Marcus, D.C., Scofield, M.A. 2001. Apical $P2Y_4$ purinergic receptor controls K^+ secretion by vestibular dark cell epithelium. *Am. J. Physiol.* **281:**C282–C289

Marcus, D.C., Sunose, H., Liu, J., Bennett, T., Shen, Z., Scofield, M.A., Ryan, A.F. 1998. Protein kinase C mediates P2U purinergic receptor inhibition of K^+ channel in apical membrane of strial marginal cells. *Hear. Res.* **115:**82–92

Marcus, D.C., Sunose, H., Liu, J., Shen, Z., Scofield, M.A. 1997. P2U purinergic receptor inhibits apical lsK/KvLQT1 channel via protein kinase C in vestibular dark cells. *Am. J. Physiol.* **273**:C2022–C2029

Merten, M.D., Saleh, A., Kammouni, W., Marchand, S., Figarella, C. 1998. Characterization of two distinct P2Y receptors in human tracheal gland cells. *Eur. J. Biochem.* **251:**19–24

Mori, N., Sakagami, M., Fukazawa, K., Matsunaga, T. 1993. An immunohistochemical and electrophysiological study on lsk protein in the stria vascularis of the guinea pig. *Eur. Arch. Otorhinolaryngol.* **250:**186–189

Munoz, D.J., Thorne, P.R., Housley, G.D., Billett, T.E. 1995. Adenosine 5′-triphosphate (ATP) concentrations in the endolymph and perilymph of the guinea-pig cochlea. *Hear. Res.* **90:**119–125

Nicolas, M., Dememes, D., Martin, A., Kupershmidt, S., Barhanin, J. 2001. KCNQ1/KCNE1 potassium channels in mammalian vestibular dark cells. *Hear. Res.* **153:**132–145

North, R.A., Barnard, E.A. 1997. Nucleotide receptors. *Curr. Opin. Neurobiol.* **7:**346–357

Ogawa, K., Schacht, J. 1995. P2y purinergic receptors coupled to phosphoinositide hydrolysis in tissues of the cochlear lateral wall. *Neuroreport* **6:**1538–1540

Otero, M., Garrad, R.C., Velazquez, B., Hernandez-Perez, M.G., Camden, J.M., Erb, L., Clarke, L.L., Turner, J.T., Weisman, G.A., Gonzalez, F.A. 2000. Mechanisms of agonist-dependent and -independent desensitization of a recombinant P2Y2 nucleotide receptor. *Mol. Cell Biochem.* **205:**115–123

Ralevic, V., Burnstock, G. 1998. Receptors for purines and pyrimidines. *Pharmacol. Rev.* **50:**413–492

Rice, W.R., Burton, F.M., Fiedeldey, D.T. 1995. Cloning and expression of the alveolar type II cell P2u-purinergic receptor. *Am. J. Respir. CelMol. Biol.* **12:**27–32

Robaye, B., Boeynaems, J.M., Communi, D. 1997. Slow desensitization of the human P2Y6 receptor. *Eur. J. Pharmacol.* **329:**231–236

Sage, C.L., Marcus, D.C. 2001. Immunolocalization of CIC-K chloride channel in strial marginal cells and vestibular dark cells. *Hear. Res.* **160:**1–9

Schofl, C., Ponczek, M., Mader, T., Waring, M., Benecke, H., von zur Muhlen, A., Mix, H., Cornberg, M., Boker, K.H., Manns, M.P., Wagner, S. 1999. Regulation of cytosolic free calcium concentration by extracellular nucleotides in human hepatocytes. *Am. J. Physicol.* **76:**G164–G172

Suzuki, M., Ikeda, K., Sunose, H., Hozawa, K., Kusakari, C., Katori, Y., Takasaka, T. 1995. ATP-induced increase in intracellular Ca^{2+} concentration in the cultured marginal cell of the stria vascularis of guinea-pigs. *Hear. Res.* **86:**68–76

Thornhill, W.B., Levinson, S.R. 1987. Biosynthesis of electroplax sodium channels in *Electrophorus* electrocytes and *Xenopus* oocytes. *Biochemistry* **26:**4381–4388

Webb, T.E., Barnard, E.A. 1999. Molecular biology of P2Y receptors expressed in the nervous system. Prog. Brain *Res.* **120:**23–31

Webb, T.E., Boluyt, M.O., Barnard, E.A. 1996. Molecular biology of P2Y purinoceptors: expression in rat heart. *J. Auton. Pharmacol.* **16:**303–307

Webb, T.E., Henderson, D.J., Roberts, J.A., Barnard, E.A. 1998. Molecular cloning and characterization of the rat P2Y4 receptor. *J. Neurochem.* **71:**1348–1357

White, P.N., Thorne, P.R., Housley, G.D., Mockett, B., Billett, T.E., Burnstock, G. 1995. Quinacrine staining of marginal cells in the stria vascularis of the guinea-pig cochlea: a possible source of extracellular ATP? *Hear. Res.* **90:**97–105

Xiang, Z., Bo, X., Burnstock, G. 1999. P2X receptor immunoreactivity in the rat cochlea, vestibular ganglion and cochlear nucleus. *Hear. Res.* **128:**190–196