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P2X ANTAGONISTS INHIBIT STYRYL DYE ENTRY INTO HAIR CELLS

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Abstract—The styryl pyridinium dyes, FM1-43 and AM1-43, are fluorescent molecules that can permeate the mechanotransduction channels of hair cells, the sensory receptors of the inner ear. When these dyes are applied to hair cells, they enter the cytoplasm rapidly, resulting in a readily detectable intracellular fluorescence that is often used as a molecular indication of mechanotransduction channel activity. However, such dyes can also permeate the ATP receptor, $P2X_2$. Therefore, we explored the contribution of P2X receptors to the loading of hair cells with AM1-43. The chick inner ear was found to express P2X receptors and to release ATP, similar to the inner ear of mammals, allowing for the endogenous stimulation of P2X receptors. The involvement of these receptors was evaluated pharmacologically, by exposing the sensory epithelium of the chick inner ear to 5 μM AM1-43 under different experimental conditions and measuring the fluorescence in hair cells after fixation of the tissue. Pre-exposure of the tissue to 5 mM EGTA for 15 min, which should eliminate most of the gating "tip links" of the mechanotransduction channels, deceased fluorescence by only 44%. In contrast, P2X receptor antagonists (pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid [PPADS], suramin, 2′,3′-O-(2,4,6-trinitrophenyl) ATP [TNP-ATP], and *d*-tubocurarine) had greater effects on dye loading. PPADS, suramin, and TNP-ATP all decreased intracellular AM1-43 fluorescence in hair cells by at least 69% when applied at a concentration of 100 μM. The difference between *d*-tubocurarine-treated and control fluorescence was statistically insignificant when *d*-tubocurarine was applied at a concentration that blocks the mechanotransduction channel (200 μM). At a concentration that also blocks $P2X_2$ receptors (2 mM), *d*-tubocurarine decreased dye loading by 72%. From these experiments, it appears that AM1-43 can enter hair cells through endogenously activated P2X receptors. Thus, the contribution of P2X receptors to dye entry should be considered when using styryl pyridinium dyes to detect hair cell mechanotransduction channel activity, especially in the absence of explicit mechanical stimulation of stereocilia.

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Abbreviations: AP, artificial perilymph; $[ATP]_o$, bulk concentration of ATP resulting from ATP release; CBX, carbenoxolone; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid; LCHBSS, low-calcium Hanks' balanced salt solution; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; ROIs, regions of interest; TNP-ATP, 2′,3′-O-(2,4,6-Trinitrophenyl) adenosine 5′-triphosphate.

Key words: mechanotransduction, basilar papilla, auditory, AM1-43, FM1-43, pannexin.

Styryl pyridinium ("styryl") dyes, such as FM1-43, have been used classically to study the dynamics of endo- and exocytosis at synapses (Betz and Bewick, 1992; Betz et al., 1992; Choi et al., 2005) but are becoming increasingly popular as molecular markers of mechanotransduction channel function in hair cells, the sensory cells of the auditory and vestibular systems. These dyes are amphipathic molecules that can incorporate into vesicles and their membranes during the vesicle cycle (Betz et al., 1992). For examining vesicle dynamics, endocytosis is ideally the only means by which the dyes enter a cell. However, when applied to hair cells, the dyes enter the intracellular compartment not only in endocytosed vesicles (Meyer et al., 2001; Griesinger et al., 2002, 2004; Kaneko et al., 2006), but also through a faster, channel-mediated mechanism (Nishikawa and Sasaki, 1996; Gale et al., 2001; Meyers et al., 2003). There is compelling evidence that the fast dye entry can occur via the hair cell transduction channels, which are located at the tips of the stereocilia (Gale et al., 2001; Meyers et al., 2003). Because of this, styryl dyes have been used as molecular markers of mechanotransduction channel activity in inner ear tissue (Geleoc and Holt, 2003; Si et al., 2003; Cheatham et al., 2004; Stepanyan et al., 2006; Taura et al., 2006; Spencer et al., 2008) and in experiments on the manipulation of tissue to produce hair cells (Doyle et al., 2007; Hu and Corwin, 2007). However, rapid dye entry may not be a reliable indicator of transduction channel function. For example, differences in *in vitro* environment and species-specific factors could confound the ability to associate rapid dye uptake specifically with transduction channel function. This idea is supported by the disparity of dye loading mechanisms between guinea-pig cochlear hair cells, where dye enters seemingly through endocytosis alone (Griesinger et al., 2002, 2004; Kaneko et al., 2006), and mouse, frog, chick, and fish hair cells, which show rapid, presumably channel mediated, dye entry (Nishikawa and Sasaki, 1996; Geleoc and Holt, 2003; Meyers et al., 2003; Si et al., 2003). Moreover, styryl dyes enter other types of sensory cells, apparently through non-specific cation channels that are distinct from that of the hair cell's transduction apparatus (Meyers et al., 2003). Since hair cells express many types of non-specific cation channels, fast dye loading may not only indicate transduction channel activity but also the action of these other channels.

P2X receptors are extracellularly activated, ATP-gated, non-specific cation channels found in many cell types, including hair cells, where they are localized to the

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apical surfaces and stereocilia (Housley et al., 1992, 1998, 1999; Jarlebark et al., 2000). When these receptors are expressed in a cell line, FM1-43 rapidly enters the cells upon stimulation with ATP (Meyers et al., 2003). Likewise, stimulation of hair cells with ATP enhances their FM1-43 uptake (PhD thesis, MacDonald, 2002). In the cochlea, there is a baseline presence of nanomolar concentrations of ATP in cochlear fluids. ATP is released in the cochlea during development (Tritsch et al., 2007), in response to intense noise exposure (Munoz et al., 2001), and by hypoxia (Munoz et al., 1995). Physical perturbation of the organ of Corti, as might occur during a dissection, is a robust means of inducing ATP release from supporting cells (Zhao et al., 2005). In light of the subcellular location of P2X receptors in hair cells and the endogenous presence of extracellular ATP in cochlear tissue, P2X receptors could partially mediate rapid styryl dye entry into hair cells, producing a spatial pattern similar to a transduction-mediated mechanism. Lending further support to this idea, stimulation of P2X receptors in some cells confers permeability to large dye molecules (e.g., ethidium bromide and YO-PRO-1), thought to be due to dilation of the channel pore (Virginio et al., 1999; Chaumont and Khakh, 2008; Yan et al., 2008) or secondary activation of pannexin hemichannels (Pelegrin and Surprenant, 2006). Here, by using molecular and pharmacological techniques, we test the hypothesis that rapid styryl dye entry into hair cells can occur via a P2X-mediated mechanism.

EXPERIMENTAL PROCEDURES

Tissue preparation for dye experiment

White Leghorn chickens (*G. gallus*) were hatched in-house from embryonated eggs obtained from the Michigan State University Poultry Teaching and Research Center (Lansing, MI, USA). The use of animals was approved by the University of Michigan's University Committee on Use and Care of Animals. Care was taken to minimize the number of animals used and their suffering. Chicks, 5–10 days post-hatch, were anesthetized with ketamine and xylazine (approximately 40 and 10 mg/kg, respectively, via i.m. injection) and decapitated. The temporal bone was opened, the partition between the round and oval windows was breached, and additional bone was dissected away so that the cochlea could be pulled out of its bony encasement. Cochleae were briefly treated (1 min) with 0.01% protease dissolved in an artificial perilymph (AP) saline (in mM: 154 NaCl, 6 KCl, 5 $CaCl_2$, 2 $MgCl_2$, 5 Hepes, at pH 7.4) in order to facilitate further microdissection. After this treatment, the cochleae were transferred to a low-calcium Hanks' balanced salt solution (LCHBSS: GIBCO 14175 HBSS (Invitrogen, Carlsbad, CA, USA) with 50 μM $CaCl_2$ added as 0.1 M $CaCl_2$). To improve reagent access to sensory hair cells, the tegmentum vasculosum and tectorial membrane were removed.

Dye treatment

Cochleae were kept in LCHBSS (control) or put in LCHBSS containing pharmacological agents before exposure to AM1-43 (pretreatment). In one set of experiments, a 15 min pretreatment with 5 mM EGTA (Sigma-Aldrich, St. Louis, MO, USA) was used to disrupt tip links in order to inhibit dye entry through mechanotransduction channels. In experiments with P2X antagonists, a 5 min pretreatment was used. The antagonists were pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS, Tocris Bioscience, Bristol, UK), suramin (Sigma-Aldrich), 2′,3′-O-(2,4,6-trinitrophenyl) ATP (TNP-ATP, Sigma-Aldrich) and *d*-tubocurarine (Sigma-Aldrich). Additionally, carbenoxolone (CBX, Sigma-Aldrich), an efficient blocker of gap junctions and related hemichannels, was used to test for dye entry through pannexin hemichannels using a 1 h pretreatment. After pretreatment with a pharmacological agent or control saline, cochleae were exposed to LCHBSS with 5 μM AM1-43 (Biotium, Hayward, CA, USA), a fixable analogue of FM1-43. The working dye solution was made from a concentrated stock solution daily. Except in the case of EGTA, the pharmacological agent used in the pretreatment was also present during the exposure to dye. The EGTA pretreatment solution was washed away with LCHBSS before exposing the tissue to dye. Exposure to the dye was for 1 min, and it was immediately followed by three 30-s washes in LCHBSS containing 500 μM Advasep-7 (CyDex Pharmaceuticals, Lenexa, KS, USA) to remove stray AM1-43. After washing, cochleae were fixed (4% paraformaldehyde, 1 h), washed in 0.12 M phosphate buffer, and mounted on slides for fluorescence microscopy and digital imaging. The corresponding control tissues were handled similarly to the treated tissues, except for the omission of the pharmacological agent that was being tested.

Imaging and image analysis

The mounted tissue was viewed on a Leica DM LB fluorescence microscope (Leica Microsystems, Wetzlar, Germany). Images were acquired through a GFP filter with a cooled-CCD color digital camera (MicroPublisher, QImaging, Surrey, BC, Canada) using the same exposure settings for comparisons between drug and the corresponding non-drug control. AM1-43 fluorescence intensity in hair cells was quantified using MetaMorph image analysis software (Molecular Devices, Downingtown, PA, USA). Elliptical regions of interest (ROIs) that corresponded closely to the perimeter of individual hair cells were defined in the raw fluorescence images, and the fluorescence intensity within the ROIs was quantified in arbitrary units (Fig. 1). When fluorescence intensity was below visual detection levels, ROIs were defined from transmitted light images. For each piece of tissue examined, the intensity of signal in 4 to 20 ROIs was averaged to give a value for that piece of tissue. This value was then averaged across all pieces of tissue subjected to a given experimental condition to give a mean$\pm$ standard error of the mean (SEM). The condition means were compared using an unpaired Student's *t*-test, and $P<0.05$ was the criterion for statistical significance. For the images of AM1-43 loading, the only manipulations performed on the raw images were cropping, the addition of a scale bar and ROI labels, conversion to CMYK color space, and resizing.

ATP measurement

Cochleae were obtained as described above using the AP saline in place of LCHBSS. Papillae were dissected, transferred to a microcentrifuge tube (two papillae per tube), and incubated for 5 min in 200 μl of fresh AP. After this time period elapsed, the entire 200 μl of saline was collected and stored on ice. Using fresh AP, the procedure was repeated to collect saline from two more 5-min incubations of the same papillae. Care was taken to perform solution exchanges without disturbing the tissue samples. Sterile reaction vessels and certified ATP-free plasticware were used throughout the procedures.

The bulk concentration of ATP resulting from ATP release ($[ATP]_o$) was measured with a single-tube photometer (Model 20 Photometer, Turner Designs, Mountain View, CA, USA) using a luciferin–luciferase ATP Assay Mix (FLAAM, Sigma-Aldrich). The assay mix was diluted 10-fold with dilution buffer. For each 5-min fluid sample, 100 μl of the diluted assay mix was added to a reaction vessel and allowed to sit at room temperature for 30 min