Acetylcholine-Evoked Calcium Increases in Deiters’ Cells of the Guinea Pig Cochlea Suggest α9-Like Receptors

Takeshi Matsunobu,1,2 Jong Woo Chung,1,3 and Jochen Schacht1*
1Kresge Hearing Research Institute, University of Michigan, Ann Arbor, Michigan
2Department of Otolaryngology, School of Medicine, Keio University, Tokyo, Japan
3Department of Otolaryngology, Asan Medical Center, University of Ulsan, Seoul, Korea

The medial efferent system innervates outer hair cells in the organ of Corti. Neurotransmission at this synapse is mediated by acetylcholine (ACh) acting on nicotinic ACh receptors containing the α9 subunit. In addition to the sensory cells, the supporting cells of the mammalian cochlea also receive efferent innervation but the neurotransmitter(s) at these synapses are not known. We show slow transient increases of intracellular calcium evoked by ACh in isolated Deiters’ cells of the guinea pig cochlea. The antagonists atropine, d-tubocurarine and strychnine blocked the ACh-effect. Nicotine was an ineffective agonist. The pharmacologic profile and the kinetics of the calcium response suggest an α9-like ACh receptor on Deiters’ cells similar but not identical to that on the outer hair cells. J. Neurosci. Res. 63:252–256, 2001. © 2001 Wiley-Liss, Inc.

Key words: cholinergic receptors; inner ear; supporting cells; α9 subunit

The major neurotransmitter of the medial olivocochlear efferent system is acetylcholine (ACh; Bledsoe et al., 1988; Eybalin, 1993; Puel, 1995; Bobbin, 1996). The medial efferent neurons originate in the superior olivary complex (for review see Warr, 1992) and terminate primarily on outer hair cells in the cochlea. The ACh receptors on these sensory cells contain the α9 subunit, a subtype with unique pharmacologic properties (Erostegui et al., 1994; Elgoyhen et al., 1994). These properties include a characteristic block by atropine, curare and strychnine, and a high permeability to calcium (Katz et al., 2000) that is also seen in isolated outer hair cells (Doi and Ohmori, 1993).

Deiters’ cells of the organ of Corti are in direct contact with the outer hair cells. They contain microfilaments and microtubules and have primarily been considered a structural reinforcement of the neurosensory epithelium. More recent speculation, however, links these cells to dynamic structural changes (Dulon, 1994; Flock et al., 1999) and calcium-based regulatory processes in the inner ear (Matsunobu and Schacht, 2000). Deiters’ cells are innervated (Nadol and Burgess, 1994; Burgess et al., 1997; Fechner et al., 1998) yet the transmitters and receptors at these synapses are not known. Intracellular free calcium \([\text{Ca}^{2+}]_i\) in Deiters’ cells is regulated by ATP (Dulon et al., 1993) and nitric oxide (Matsunobu and Schacht, 2000). In the present study, we investigate the effect of ACh on \([\text{Ca}^{2+}]_i\) in isolated Deiters’ cells of the guinea pig cochlea and characterize the ACh effect pharmacologically.

MATERIALS AND METHODS

Materials and Animals

Except where noted, all chemicals were purchased from Sigma (St. Louis, MO). Pigmented guinea pigs initially weighing 250–300 g were obtained from Murphy’s Breeding Laboratories (Plainfield, NJ). The experimental protocols are in compliance with guidelines of the National Institutes of Health and Declarations of Helsinki and were approved by the University of Michigan’s Committee on Use and Care of Animals. Animal care was under supervision of the University of Michigan’s Unit for Laboratory Animal Medicine.

Cell Preparation and Calcium Imaging

Guinea pigs were anesthetized with CO2 and decapitated, the temporal bones were quickly removed and the bony wall of the cochlea was immediately opened. After detaching the stria vascularis and the tectorial membrane, the organ of Corti (mostly from the apical and middle turns) was microdissected from the spiral lamina. The dissected strips were incubated for 20 min in Hank’s balanced salt solution (HBSS, Life Technologies, Grand Island, NY) additionally buffered with 5.0 mM sodium HEPES to pH 7.4 and its osmolality adjusted at 300 ± 2 mOsm with NaCl) containing collagenase (Sigma type IV; final concentration, 1 mg/ml). The strips were then transferred into a 50 μl droplet of HBSS on a glass coverslip sealed in the middle of a perforated plastic Petri dish. The isolation procedure was com-
pleted by gentle influx and efflux of the tissue pieces through a micropipette. All experiments were conducted at room temperature.

One-mM stock solutions of the cell-permeant acetoxy methyl ester derivative of fluo-3, fluo-3/AM (Molecular Probes Inc., Eugene, OR) were prepared in dry dimethyl sulfoxide (DMSO) and stored below 0°C. Deiters’ cells were incubated for 30 min with 2 μM fluo-3/AM in HBSS in a saturated humid chamber. Cells were finally carefully rinsed with HBSS.

Cells loaded with fluo-3 were observed with an inverted microscope (Leitz Fluovert FS; Leitz, Wetzlar, Germany) fitted with an epifluorescence system (ArcLamp HBO 100 W, a 450–490 nm band pass exciter filter, 510 nm dichroic mirror, and a 520 nm barrier filter for excitation and emission, respectively) and Nikon objectives. The excitation irradiance was reduced by neutral density filters to prevent photobleaching.

Cell fluorescence was monitored via a Silicon Intensified Target camera (SIT 66, Dage-MTI Inc., Michigan City, IN), and the recorded images were analyzed with Axon Workbench 2.1 software (Axon Instruments, Inc., Foster City, CA). Sixteen to 32 sequential frames were averaged and fluorescence intensity of the images was quantified by averaging the pixel radiance value of the entire cell.

Changes in [Ca2+]i were calculated by a single-excitation wavelength ratio method (Neher and Augustine, 1992). For each cell, the pre-stimulus fluorescence, F0, and the fluorescence at a given time after the stimulus, Fpre, were measured. The response is expressed as the ratio F/F0 or as Fmax/F0 for the maximal response.

**Application of Drugs**

The agonists ACh or nicotine were dissolved in HBSS and applied to the cells for 60 sec via a glass pipette (Sterile Femtotips, Eppendorf Scientific, Westbury, NY). The pipette (opening, 0.5 μm) was held about 50 μm away from the base of the cell. Preliminary experiments with fluorescent dyes showed that the pressure-ejected bolus (in the nL range) reached the cell within 1 sec. Concentrations of ACh or nicotine are stated as concentrations in the delivery pipette.

Atropine, d-tubocurarine or strychnine, dissolved in HBSS, pH 7.4, was applied to the bath 10 min before the injection of ACh. Five μL of a stock solution were added with a micropipette to the 50-μL droplet containing the cells.

**Data Analysis**

Changes in [Ca2+]i were calculated by measuring the peak of the response above basal level. All values are presented as means ± SEM. Differences between mean values were evaluated using ANOVA followed by Newman–Keuls as a post hoc test. A P value of <0.05 was considered significant.

**RESULTS**

**Agonist-Induced Increase of Intracellular Calcium**

In isolates of cochlear cells, Deiters’ cells were easily identified by their phalangeal processes (Fig. 1, arrow). They retained morphological integrity for approximately 4 hr post mortem as judged by their light microscopic appearance. All experiments reported here were conducted within 1 hr after cell isolation. Fluo-3 fluorescence was stable in resting cells, and an estimate of [Ca2+]i was made from a combined in vivo and in situ calibration as described previously (Dulon et al., 1990). [Ca2+]i at rest was approximately 55 nM. A puff of HBSS did not cause any significant changes in [Ca2+]i.

The injection of a bolus of ACh (1 mM solution of ACh in the delivery pipette) produced an increase of fluorescence in 44% (20/46) of the cells with a delay ranging from 20–100 sec (Fig. 2). After the maximum, fluorescence decreased with a half-life of approximately 60 sec, indicating calcium buffering or sequestering by the cell. A second application of ACh elicited a much attenuated response or none at all.

The amplitude of the calcium response depended on the ACh concentration (Fig. 3). Fmax/F0 increased up to 1.68 ± 0.10 in responding cells with an EC50 of 30 μM. Because the bolus of ACh was diluted by the 50 μL bath, the effective ACh concentrations at the cell can be estimated to be in the low micromolar range or less. The dose-response curve could be fitted well (r2 = 0.97) to a Hill plot yielding a Hill coefficient of 0.92 consistent with a 1:1 ratio of ACh receptor binding.

When 1 mM nicotine was injected into the bath in the same manner (Fig. 4), only one of six cells responded (Fmax/F0 = 1.28) although 11/25 (44%) reacted to 1 mM acetylcholine in this experimental set. At 10 mM nicotine, only two of ten cells responded and did so with a lower increase than seen with 0.1 or 1 mM ACh (Fmax/F0 = 1.43 ± 0.02).

**Effects of Antagonists**

The pharmacology of the cholinergic response was further characterized by applying several antagonists (Fig. 4).
5). Atropine, a classical antagonist of muscarinic receptors, attenuated the ACh-evoked transient increase of \([\text{Ca}^{2+}]_i\). At \(10\ \mu\text{M}\) atropine, the peak was significantly decreased to \(F_{\text{max}}/F_0 = 1.17 \pm 0.04\) (\(n = 5\), \(P < 0.05\)) with 31\% (5/16) of the cells responding. Only one cell (1/19) responded to ACh after pre-incubation with 50 \(\mu\text{M}\) atropine.

The nicotinic antagonist d-tubocurarine (1 \(\mu\text{M}\)) also suppressed the ACh-evoked calcium response. Thirty-three percent (5/15) of the cells responded but the magnitude of the increase was significantly smaller (\(F_{\text{max}}/F_0 = 1.11 \pm 0.03\); \(n = 5\), \(P < 0.05\)).

The alkaloid strychnine, classically used as a blocker of glycine-gated chloride channels, also acted as a dose-dependent antagonist. After pretreatment with 1 \(\mu\text{M}\) strychnine, 38\% (7/18) of the cells still responded to ACh with an increase of calcium to \(F_{\text{max}}/F_0 = 1.54 \pm 0.15\) (\(n = 7\); n.s.). Ten \(\mu\text{M}\) strychnine significantly reduced the ACh response to an amplitude of \(F_{\text{max}}/F_0 = 1.25 \pm 0.10\) (\(n = 5\), \(P < 0.05\)), observed in 36\% (5/14) of the cells.
DISCUSSION

The results suggest the presence of an α9-like receptor on Deiters’ cells of the guinea pig cochlea. Although ACh is the major neurotransmitter released from efferent fibers in the cochlea (Bledsoe et al., 1988; Eybalin, 1993; Puel, 1995; Bobbin, 1996), this site of action is unexpected. It is the outer hair cells in the neurosensory epithelium of the organ of Corti that are considered the targets of the medial efferent innervation (Kimura and Wersäll, 1962; Spoendlin, 1966; Warr, 1992). Consistent with this view, outer hair cells possess ACh receptors that have been characterized pharmacologically, immunocytochemically and by in situ hybridization as containing the α9 subunit (Elgoyhen et al., 1994; Erostegui et al., 1994; Blanchet et al., 1996; Park et al., 1997; Luo et al., 1998; Morley et al., 1998; Vetter et al., 1999).

On the other hand, supporting cells in the organ of Corti (Deiters’ and Hensen’s cells) do receive efferent (Nadol and Burgess, 1994; Burgess et al., 1997) and possibly afferent innervation (Fechner et al., 1998). Deiters’ cells contain purinergic P2Y and P2X receptors (Dulon et al., 1993; Dulon, 1995; Housley et al., 1999), and the nitric oxide/cyclic GMP pathway attenuates the ATP-evoked increases of intracellular calcium (Matsunobu and Schacht, 2000). The demonstration of cholinergic responses makes ACh an additional transmitter candidate. Interestingly, the slow response to ACh contrasts to the fast response of these cells to ATP (Dulon et al., 1993; Matsunobu and Schacht, 2000) and is more reminiscent of the action of ACh in outer hair cells where calcium peaks 40 sec after the onset of the stimulus (Doi and Ohmori, 1993). The response rate is also similar in both cell types with 38% of outer hair cells responding compared to 44% of Deiters’ cells in our study.

The α9 subunit is part of an ionotropic ACh receptor that includes a calcium channel and shows specific pharmacologic features (Elgoyhen et al., 1994; Vetter et al., 1999; Rothlin et al., 1999; Katz et al., 2000). Important for a characterization of an α9 receptor are its block by both classical muscarinic and nicotinic agonists, atropine and d-tubocurarine, respectively. In addition, the α9 receptor can be inhibited by strychnine (Elgoyhen et al., 1994; Rothlin et al., 1999) that is also a potent antagonist of the olivo-cochlear efferent system (Kujawa et al., 1992, 1994). All three antagonists attenuate the ACh-evoked calcium increases in Deiters’ cells at concentrations comparable to those that attenuate ACh-evoked currents in isolated outer hair cells of the guinea pig (Doi and Ohmori, 1993; Erostegui et al., 1994). The correlation is, however, not strictly quantitative because d-tubocurare seems more effective in blocking calcium-responses at Deiters’ cells than at outer hair cells (Doi and Ohmori, 1993).

This pharmacologic profile has to be interpreted with some caution because α7-subunits of the acetylcholine receptor may respond similarly, particularly because strychnine can block calcium channels non-specifically (Oyama et al., 1988). Both α7 and α9 subunits are expressed in the organ of Corti of the rat (Morley et al., 1998). In the case of α7-subunits, however, nicotine should be a more potent agonist than ACh (Seguela et al., 1993; Gopalakrishnan et al., 1995) that it clearly is not at Deiters’ cells. Therefore, the efficacy of agonists and antagonists suggests that Deiters’ cells possess an α9-like ACh receptor. Although similar to that of outer hair cells, the slower kinetics of the response and the greater effectiveness of d-tubocurare at the Deiters’ cells suggest that the receptors are not identical. Such a notion is also in agreement with the finding that Deiters’ cells of the 4-day-old rat test negative for the α9 ACh receptor subunit (Glowatzki et al., 1995). It should be kept in mind, however, that the auditory periphery of the 4-day-old rat is a developing organ although our study uses the mature guinea pig.

The efferent system and ACh have well-defined actions on evoked cochlear potentials and are considered an important feedback on peripheral auditory processing (Mountain, 1980; Siegel and Kim, 1982; Kujawa et al., 1992, 1994). These effects are believed to be mediated through outer hair cells, the primary targets of medial efferent innervation. Deiters’ cells are closely associated with outer hair cells and provide a scaffolding for the sensory neuroepithelium (Slepecky, 1996). Their phalangeal process attaches to the apex of an outer hair cell at the level of the reticular lamina although their basal end extends to the base of another outer hair cell. Calcium influx induces a small movement of the head of the phalangeal process and an increase of its stiffness (Dulon et al., 1993; Dulon, 1995), and overstimulation by sound can cause a contraction of the organ of Corti including the supporting cells (Friedberger et al., 1998). Neurotransmitter-induced changes of intracellular calcium in Deiters’ cells may therefore contribute mechanical forces that influence the basilar membrane and its vibration.

ACKNOWLEDGMENTS

The authors thank Drs. Sanford Bledsoe, Colleen LePrell and David Dolan for helpful comments. This research was supported by program project grant DC-02982 from the National Institutes of Deafness and Other Communication Disorders, National Institutes of Health.

REFERENCES


