The effect of proteolytic enzymes on the α9-nicotinic receptor-mediated response in isolated frog vestibular hair cells

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Abstract

In frog vestibular organs, efferent neurons exclusively innervate type II hair cells. Acetylcholine, the predominant efferent transmitter, acting on acetylcholine receptors of these hair cells ultimately inhibits and/or facilitates vestibular afferent firing. A coupling between α9-nicotinic acetylcholine receptors (α9nAChR) and apamin-sensitive, small-conductance, calcium-dependent potassium channels (SK) is thought to drive the inhibition by hyperpolarizing hair cells thereby decreasing their release of transmitter onto afferents. The presence of α9nAChR in these cells was demonstrated using pharmacological, immunocytochemical, and molecular biological techniques. However, fewer than 10% of saccular hair cells dissociated using protease VIII, protease XXIV, or papain responded to acetylcholine during perforated-patch clamp recordings. When present, these responses were invariably transient, small in amplitude, and difficult to characterize. In contrast, the majority of saccular hair cells (~90%) dissociated using trypsin consistently responded to acetylcholine with an increase in outward current and concomitant hyperpolarization. In agreement with α9nAChR pharmacology obtained in other hair cells, the acetylcholine response in saccular hair cells was reversibly antagonized by strychnine, curare, tetraethylammonium, and apamin. Brief perfusions with either protease or papain permanently abolished the α9-nicotinic response in isolated saccular hair cells. These enzymes when inactivated became completely ineffective at abolishing the α9-nicotinic response, suggesting an enzymatic interaction with the α9nAChR and/or downstream effector. The mechanism by which these enzymes render saccular hair cells unresponsive to acetylcholine remains unknown, but it most likely involves proteolysis of α9nAChR, SK, or both.

Key words: Acetylcholine; α9-Nicotinic receptor; Papain; Protease; Hair cells; Patch clamp

1. Introduction

The impetus for investigating acetylcholine (ACh) and its receptors among inner ear sensory cells began over 40 years ago with the demonstration of acetylcholinesterase staining in cochlear and vestibular efferent fibers (Churchill et al., 1956; Dohlmann et al., 1958). A preponderance of evidence has since substantiated that ACh is indeed the major inner ear efferent transmitter among vertebrates (Eybalin, 1993; Guth et al., 1998). The frog offers a distinct advantage for studying the cholinergic function of vestibular efferents in that efferent fibers, after an extensive ramification at the level of Scarpa’s ganglion, are thought to innervate type II vestibular hair cells exclusively (Hightstein, 1991; Precht, 1976; Lysakowski, 1996). Therefore, the effects elicited by efferent stimulation or ACh application on frog vestibular organs are most likely the result of ACh interacting with its receptors found on those
hair cells. In frog vestibular organs, ACh has been shown to produce both facilitation and inhibition of afferent firing (Guth et al., 1986; Norris et al., 1988; Guth et al., 1994; Perin et al., 1998). The inhibition is thought to be mediated through the activation of α9-containing nicotinic receptors (α9nAChR) present on vestibular hair cells (Sugai et al., 1992; Elgoyhen et al., 1994; Yoshida et al., 1994; Hiel et al., 1996; Anderson et al., 1997; Athas et al., 1997a,b; Gupta et al., 1997). Similar pharmacology has also been identified in auditory hair cells from many species (Housley and Ashmore, 1991; Shigemoto and Ohmori, 1991; Fuchs and Murrow, 1992a,b; Ero steregui et al., 1994; McNiven et al., 1996; Nenov et al., 1996a). To date, all ACh-induced inhibition of afferent responses from both auditory and vestibular organs has been attributed to α9-containing nAChR activation and as such, the consequences of this activation also seem well conserved. Calcium influx via these receptors subsequently activates apamin-sensitive, small-conductance, calcium-dependent potassium channels (SK) resulting in an efflux of potassium, which subsequently hyperpolarizes the hair cell. In frog vestibular organs, this translates into a decrease in the release of transmitter by the hair cell with an ensuing reduction of afferent firing. To better examine the mechanisms underlying the α9nAChR-mediated effects on the level of vestibular hair cells, these cells can be enzymatically dissociated from select vestibular organs and examined individually using patch clamping methodology.

Cell dissociation techniques frequently involve enzymes in order to isolate individual cells from their respective tissues (Freshney, 1994). In this regard, sensory cells (hair cells) of the inner ear are no exception. The arsenal of enzymes commonly used to isolate vestibular hair cells has included papain and several serine proteases (Lewis and Hudspeth, 1983; Steinacker and Rojas, 1988; Housley et al., 1990; Holt and Eatock, 1995; Chabbert, 1997). Collagenase has been successfully used to isolate cells from the cochlea (Ero steregui et al., 1994; Nenov et al., 1996a; Sugasawa et al., 1996; Nenov et al., 1998); but, it is rather ineffective in isolating frog vestibular hair cells. Initially, in this laboratory, a combination of papain and protease (VIII or XXIV) was successfully used for dissociating hair cells from frog vestibular organs. However, despite the detection of the α9nAChR subunit messenger RNA (mRNA) in isolated vestibular hair cells, we were unable to demonstrate ACh responses consistently in these cells. Failure to demonstrate routine ACh responses led to speculation that the α9nAChRs might undergo proteolysis during the isolation process and become non-functional. Indeed, a previous study had suggested that papain treatment might attenuate similar ACh responses in isolated hair cells from the turtle basilar papilla (Art and Goodman, 1996). Armstrong and Roberts (1998) have also recently shown that papain alters particular ion channels in frog saccular hair cells including SK, however, the exclusion of papain from our dissociation protocol did not significantly increase the number of ACh-responsive cells. It was therefore hypothesized that the remaining proteases (VIII or XXIV) might also participate in the degradation of components involved in the α9nAChR response in these cells.

Trypsin is also commonly used to isolate an assortment of different cell types. In many of these cells, receptors of interest have been shown to continue functioning following trypsinization (Freshney, 1994). Furthermore, trypsin has also been reported to affect membrane permeability and conductance only minimally (Narahashi and Tobias, 1964; Narahashi, 1974). When trypsin was substituted for either protease VIII or XXIV in the isolation protocol (papain excluded), the α9-nicotinic response to ACh was reliably and consistently observed in most (~90%) of the saccular hair cells examined. In addition, ACh-produced responses were also identified in several solitary semicircular canal (SCC) and utricular hair cells isolated with trypsin. The pharmacology of the ACh response observed in isolated saccular hair cells was consistent with observations made in the intact organ as assessed by multunit afferent firing recordings (reviewed in Guth et al., 1998). Moreover, trypsinized saccular hair cells demonstrated high intensity immunofluorescence when stained with anti-α9nAChR subunit antibody, providing an additional evidence for the presence of α9 subunit protein in these cells. A reliable response to ACh in trypsinized saccular hair cells provided the necessary means by which to investigate the effects of other enzymatic treatments on this response.

We report in this paper that both papain and two bacterial serine proteases (VIII, XXIV), enzymes commonly used to isolate vestibular hair cells, abolish the response to ACh mediated by α9-containing nicotinic receptors in these same cells. The α9-nicotinic ACh response, in both the intact organ and trypsinized saccular hair cells, was routinely and permanently eliminated by brief perfusions of either papain, protease VIII, or protease XXIV. The concentrations and exposure times mirror those used in previous isolation protocols. These same enzymes were without effect when inactivated by boiling before application. The abolition of the α9-nicotinic response in saccular hair cells is therefore most likely a function of the enzyme’s proteolytic activity. Similar perfusions with trypsin, collagenase, dispase, hyaluronidase, or elastase did not attenuate the α9-nicotinic response in frog saccular hair cells.

As evidenced here, when acutely isolated cells fail to behave as they do in situ, one of the problems may lie
in the means used to obtain those cells. These observations explain the difficulty in demonstrating α9-nicotinic responses in solitary frog vestibular hair cells isolated with either papain, protease VIII, or protease XXIV. The novelty of this work is not necessarily that α9nAChRs are present in the frog saccule or that they underlie the inhibition of afferent discharge following efferent stimulation or exogenous ACh application. Instead, the contribution of this study is that the proteolytic enzymes that have been commonly used to isolate vestibular hair cells may alter or degrade some of the components that one may be studying. This is not an attempt to reevaluate previous work but to raise awareness about the deleterious effects that these enzymes may produce. Although receptor protein and its corresponding mRNA may be apparently preserved, the functional properties of the membrane’s molecules could be unpredictably changed following enzymatic treatment. The deleterious effects of these enzymes may have similar consequences for other isolation/dissociation protocols particularly using cells that might express similar membrane proteins including receptors and ion channels. Some of the results presented here have previously appeared in abstract form (Holt and Guth, 1999).

2. Materials and methods

2.1. Multiunit afferent recordings

The method for recording the rate of saccular multiunit afferent firing was performed as previously described (Guth et al., 1994; Perin et al., 1998). The whole labyrinth bath (∼15 ml) was continuously superfused with artificial perilymph (AP) (in mM: 105 NaCl, 2.5 KCl, 0.81 MgCl2-6H2O, 1.8 CaCl2-2H2O, 3.4 NaHCO3, 0.5 NaH2PO4·H2O, 2.5 Na2HPO4, 4 glucose) at a flow rate of 3–5 ml/min. Drugs were applied either by bath substitution or by close injection (15–45 s at 50 μl/min) through a multibarrel perfusion pipette linked to a mechanical syringe pump. This drug delivery pipette was positioned within approximately 0.5 mm of the neuroepithelium. When antagonists were applied by local injection, they were typically given alone as a control and also with the agonist of interest.

2.2. Isolation of hair cells

Leopard frogs (Rana pipiens) were chilled, pithed, and decapitated. The superior portion of the head was sectioned sagittally and placed into AP. The inner ear was exposed by removing the ventral portions of the otic capsule whereby the whole labyrinth was then removed and placed into a low-calcium dissociation medium (in mM: 105 NaCl, 2.5 KCl, 2 MgCl2·6H2O, 0.1 CaCl2·2H2O, 3.4 NaHCO3, 0.5 NaH2PO4·H2O, 2.5 Na2HPO4, 1 ascorbate, 4 glucose, 5 pyruvate). The saccular macula or canal crista was dissected free from the whole labyrinth and trimmed to remove any excess tissue or membranes. The macula (or crista) was subsequently transferred to a dish with Hanks balanced salt solution (Gibco, Life Technologies, Grand Island, NY, USA) nominally devoid of calcium and magnesium containing 0.05% trypsin/EDTA (0.53 mM), for ∼10 min. After trypsinization, the macula (or crista) was then transferred into dissociation solution containing 10% fetal bovine serum (FBS; Gibco, Life Technologies, Grand Island, NY, USA) for 30–60 s, and finally into dissociation solution containing 500 μg/ml bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) for 5–10 min. FBS contains protease inhibitors that will stop the trypsin digestion, and the BSA is believed to act as a sacrificial substrate for any additional enzymatic components, which may be found as minute contaminants among trypsin formulations.

The macula (or crista) was then removed from the BSA-containing dissociation solution and held in place with forceps on the glass bottom of a 700 μl recording chamber containing only dissociation solution. Under the dissecting scope, a thin glass wisp was used to loosen the hair cells from the macula or crista through an alternating series of gentle mechanical agitations and longitudinal fluid motions. Following the isolation of hair cells, the dish was allowed to sit for 3–5 min so that suspended hair cells would settle and attach to the bottom of the dish. Two mg/ml concanavalin A (Sigma, St. Louis, MO, USA) was used to coat the recording chamber to promote hair cell adhesion to the glass bottom. Firn attachment to the glass prevented a number of problems during electrophysiological recording including mechanical artifacts (e.g. activation of transduction currents) or seal disruption induced by drug perfusion during patch clamp procedures.

2.3. Patch clamp recordings from isolated vestibular hair cells

The recording chamber containing the isolated vestibular hair cells was clamped onto the movable stage of a Nikon Diaphot TMD inverted microscope (Nikon; Garden City, NY, USA) equipped with Nomarski differential interference contrast optics. The dish was then superfused with the external solution (containing in mM): 105 NaCl, 2.5 KCl, 0.81 MgCl2·6H2O, 1.8 CaCl2·2H2O, 3.4 NaHCO3, 0.5 NaH2PO4·H2O, 2.5 Na2HPO4, 1 ascorbate, 4 glucose, 5 pyruvate) at a rate of 1 ml/min for the remainder of the experiment. Tight GΩ seals (1–15 GΩ) were obtained using recording pipettes constructed from 1.5 mm OD borosilicate.
were typically superfused onto the cell for several seconds utilizing a 12-bit digital-to-analog converter and data were digitally sampled every 50-300 μs utilizing a 12-bit analog-to-digital converter (Labmaster DMA; Scientific Solutions, Solon, OH, USA) coupled to a microcomputer (Gateway 233 MHz Pentium II, Gateway, North Sioux City, SD, USA). Data were stored on hard media for off-line analysis (pCLAMP, ver. 6.0.4, Axon Instruments, Foster City, CA, USA). The tip potential between the pipette’s internal solution and the bath was nullsed before seal formation. The junction potential, based on the constituents of our solutions was calculated to be approximately −7 mV using the junction potential calculator included with Clampex 7 (Axon Instruments, Foster City, CA, USA). Series resistance was partially compensated (60–80%). Series resistance and cell capacitance measurements were constantly monitored and updated throughout all experiments. The average values obtained during perforated-patch recordings (n > 150 cells) were as follows: zero holding potential (range −40 to −78 mV, avg. −58.8 ± 8.7 mV), series resistance (range 4.8−27 MΩ, avg. 14.9 ± 3.9 MΩ), and whole cell capacitance (range 7.1−17.3 pF, avg. 12.3 ± 2.1 pF). As most of the voltage protocols typically produced small currents (<2 nA), the voltage errors due to series resistance (avg. = ~15 MΩ) were generally less than 5 mV. Therefore, most of the data were not corrected for voltage errors due to series resistance. Experiments were performed at room temperature (20−22°C).

All the data shown here were recorded using the perforated-patch variant of patch clamping (Hamill et al., 1981; Akaike and Harata, 1994). Three mg of the polyclene antibiotic, amphotericin B, was dissolved in 60 μl of dimethylsulfoxide (DMSO), vortexed, and homogeneously suspended in 5 ml of normal internal solution (in mM: 75 KCl, 2 MgCl₂·6H₂O, 30 K₂SO₄, 6 glucose, 10 HEPES) which was used to back-fill the patch pipette.

Drug solutions were made up in external solution and applied by a gravity-driven microperfusion pipette at a rate of ~3.6 μl/s. This pipette was placed approximately 300–600 μm from the cell. ACh antagonists were typically superfused onto the cell for several seconds prior to co-application of the given antagonist and ACh. This allowed for observation of any effects that the antagonist alone might produce. Additionally, it permitted a standing concentration of the antagonist before administration of ACh and thus, a better assessment of the antagonist’s potency. Other studies have demonstrated that this may be an optimal way to apply the antagonist (Kehoe and McIntosh, 1998). All the enzymes used in this study were also locally superfused onto trypsinized vestibular hair cells to determine their effect, if any, on the ACh response. As a negative control in several experiments, protease VIII, protease XXIV, and papain were heat-inactivated by several minutes of boiling and superfused onto the cell prior to exposure to the intact, native protease.

2.4. Data analysis

The patch clamp data contained herein were analyzed and plotted using pCLAMP6 Clampfit (Axon Instruments, Foster City, CA, USA), Excel 7.0 (Microsoft Corp., Redmond, WA, USA), and Origin 5.0 (Microcal Software, Inc., Northampton, MA, USA).

2.5. Amplified RNA (aRNA) methodology

The aRNA™ procedure, used to amplify RNA from a single cell, combines reverse transcription of the cellular mRNA population into complementary DNA (cDNA) and reamplification of RNA from the cDNA template (Van Gelder et al., 1990; Eberwine et al., 1992). This process was made possible by use of an oligomeric deoxythymidine-T7 (oligo-dT/T7) primer. The oligo-dT/T7 primer consists of oligo-dT residues extended at their 5’ end with the DNA sequence containing the promoter site for T7 RNA polymerase. The oligo-dT/T7 primer was used to synthesize single-stranded cDNA from the cell’s mRNA population using reverse transcriptase (RT); thus the T7 sequence becomes part of the cDNA. During second strand cDNA synthesis, the T7 promoter was also made double-stranded creating a functional T7 promoter site and the means necessary to reamplify RNA.

Vestibular hair cells were isolated and electrophysiological recordings were made as already described. The micropipette (containing only RNase-free internal solution with DMSO/amphotericin) remained attached to the cell for between 5 and 35 min for electrophysiological recordings. The cellular contents were then aspirated and placed in a microfuge tube containing internal buffer (pH 8.3), 2.5 mM each dNTP, 5 ng/ml oligo-dT/T7 primer, and 10 U Avian Myeloblastosis Virus RT (Seikagaku America, Inc., Falmouth, MA, USA). First strand cDNA synthesis followed at 37°C for 1 h. This was followed by second strand synthesis, S1 nucle-
ase treatment to remove any hair-pin loops formed during the cDNA synthesis, blunting of the double-stranded cDNA with the Klenow fragment, and drop dialysis to remove excess salt using standard methodologies (Sambrook et al., 1989). RNA was made from one-fifth of the recovered volume from dialysis. Using 2000 U T7 RNA polymerase (Epicentre Technologies, Madison, WI, USA), aRNA was made off the T7 promoter binding site which had been incorporated into the cDNA using NTPs and radioactive $^{[32P]}$CTP. The aRNA product was phenol/chloroform-extracted and ethanol-precipitated and then reamplified through a second round of cDNA synthesis followed by aRNA amplification. Size distribution of aRNA transcripts was checked by autoradiography following denaturing agarose gel electrophoresis. Using trichloroacetic acid precipitation, the level of incorporation of radioactivity was used to determine the amount of aRNA made. Equivalent amounts of aRNA were added to slot blots containing linearized plasmid clones to generate an expression profile for each vestibular cell.

2.6. Expression profiling from individual hair cells

Expression profiling uses the radiolabeled aRNA population from a single cell as a probe to hybridize to cloned cDNAs of various candidate genes which have been immobilized on nitrocellulose via a slot blot apparatus. The relative levels of the corresponding mRNA are reflected by the intensity of the hybridization signal of each of the blotted cDNAs. These plasmid cDNA clones are in vectors of similar sizes, i.e. pUC-based vectors, so equal amounts of each clone (1 μg) were added to each slot well. Pertinent plasmid clones used in this study were: α ATCC 63103 GenBank # X03986; α3 ATCC 68278 GenBank # M86383; α9 (Elhoyhen et al., 1994); β ATCC 63104 GenBank # M14537; β2 ATCC 68277 GenBank # X53179; ε ATCC 63107 GenBank # X55718; γ ATCC 63105 GenBank # X03818.

Plasmid clones (all RNase-free) were first linearized with an appropriate restriction enzyme, denatured, and added to nitrocellulose filters in slot wells (BIO-DOT SF, Bio-Rad, Bio-Rad Laboratories, Hercules, CA, USA) at 1 μg/200 μl of 10×sodium chloride/sodium citrate buffer (SSC). The cDNA clones were fixed to the nitrocellulose using a Bio-Rad GS Gene Linker UV Chamber (Bio-Rad Laboratories, Hercules, CA, USA). Prehybridization and hybridization solutions consisted of 50% formamide, 5×SSC, 5×Denhardt’s solution, 1 mM sodium pyrophosphate and 100 mg/ml salmon sperm DNA and were made with diethylylpyrocarbonate-treated water. Hybridization proceeded for 48 h at 42°C in a Bellco microhybridization oven (Bellco Glass, Inc., Vineland, NJ, USA). Washes were also done under RNase-free conditions and consisted of two low stringency washes (2×SSC/0.1% sodium dodecyl sulfate (SDS) at 42°C×20 min×2) and one high stringency wash (0.1×SSC/0.1% SDS at 50°C×1). After washing, filters were exposed either to film (X-OMAT AR5, Kodak) or to a Fuji Imaging Plate for use in a BAS 1000 Bio Imaging Analyzer (Fuji Photo Film Co., Ltd., Japan).

2.7. Single-cell immunocytochemistry

Immunoreactivity for the ε9nACHR subunit was detected in hair cells by means of immunofluorescent staining. Briefly, frog saccular hair cells were dissociated (as described previously) into a 300 μl drop of the external solution placed within a probe-clip press-seal incubation chamber (Sigma, St. Louis, MO, USA) previously coated with concanavalin A (2 mg/ml) to facilitate adhesion. Within this chamber, the cells were then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 20 min, rinsed in PBS, and preincubated with 1% BSA in 0.3% Triton X-100 PBS for 1 h. The cells were incubated overnight (18–24 h) with one of two primary antibodies, both at a dilution of 1:1000. Antibodies T54 (made to a cytoplasmic loop of the rat ε9nACHR) and T56 (made to the N-terminus of the rat ε9nACHR) were developed in rabbits. These primary antibodies were previously characterized and kindly provided by Dr. Robert J. Wenthold (Park et al., 1997). Following the primary incubation with either T54 or T56, ε9nACHR subunit immunoreactivity was visualized using fluorescein isothiocyanate-tagged goat anti-rabbit secondary antibodies (1:170). The images were analyzed under an Olympus IX70 immunofluorescence microscope, transferred with the help of a spot camera, and printed by high resolution dye sublimation printer. As a control for non-specific binding, incubation with the primary antibody was excluded in several preparations. Under these conditions, the saccular hair cells failed to fluoresce. Incubation with the primary antibody following neutralization with fusion proteins to which they were developed or replacement of the primary antibody with the pre-immune serum were not performed due to availability issues.

2.8. Materials

Unless noted otherwise, the drugs used in this study were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Papain was obtained from Calbiochem (La Jolla, CA, USA) and apamin was from Molecular Probes (Eugene, OR, USA). Sources of molecular reagents are indicated in the text. These experimental protocols were carried out in ac-
cordance with the National Institute of Health Guide for the care and use of laboratory animals (NIH Publications No. 80-23).

3. Results

3.1. Multunit afferent recordings

The application of 1 mM ACh routinely produces a robust inhibition of multunit afferent firing as recorded from the intact frog saccule (Fig. 1A–D). This inhibition was potently blocked by strychnine (Fig. 1A) and attenuated by the classical nicotinic antagonists curare (Fig. 1B) and tetraethylammonium (Fig. 1C). Previous studies have demonstrated that strychnine (IC$_{50} =$ 100 nM) was more potent than curare (IC$_{50} =$ 400 nM) at blocking the α9nAChR-mediated response in the frog saccule (Guth et al., 1994). An index of this difference in affinity of the two antagonists can be inferred from the length of time required for the amplitude of the ACh response to return to control values following the single application of each antagonist at the same concentration (strychnine (Fig. 1A), >2 h; curare...

Fig. 1. The effect of ACh on the rate of resting multunit afferent firing from the intact frog saccule. ACh (1 mM) routinely produced an inhibition of afferent firing. This inhibition is potently antagonized by strychnine (A), curare (B), tetraethylammonium, TEA (C), and apamin (D). Both strychnine and TEA were coinjected with ACh whereas curare and apamin were given by bath substitution. Breaks in trace represent truncation of the interval between ACh application to allow visualization of many responses under control conditions. Time bars represent length of time between responses. Ringers injection was without effect (data not shown). None of the antagonists used produced effects on resting activity by themselves.
The inhibition of afferent firing is thought to be produced through the activation of presynaptic (i.e. hair cell) $\kappa_9$-containing nicotinic receptors whose calcium influx activates juxtaposed apamin-sensitive SK which in turn hyperpolarize the hair cell. This hyperpolarization decreases the release of afferent transmitter, presumably glutamate, onto afferents. In Fig. 1D, note that the inhibition produced by ACh was sensitive to the apamin, an SK antagonist (specifically SK2 and SK3) (Ishii et al., 1997; Bond et al., 1999). The blockade of SK prevents development of the $\kappa_9$-nicotinic subunit-mediated response.

### 3.2. aRNA\textsuperscript{®} expression profiling

Although anatomical studies of frog efferents suggest that ACh receptors will be most likely found on hair cells, exogenous application of ACh onto the whole labyrinth preparations could potentially activate ACh receptors on many cell types. Because the pharmacology suggests $\alpha_9$AChRs, both molecular biological and immunocytochemical techniques were used to determine if the $\alpha_9$AChRs could indeed be localized to hair cells. An expression profile, generated using the amplified mRNA from a solitary frog saccular hair cell, is shown in Fig. 2. As compared to both positive (Fra1, Fra2) and negative controls (blank, pUC18), the intensity of the hybridization signal in the blot containing the $\alpha_9$AChR cDNA would suggest that the $\alpha_9$AChR mRNA was present in the original cellular extract. Utilizing the aRNA\textsuperscript{®} technique, comparable positive $\alpha_9$AChR hybridization signals have been consistently identified in extracts from both frog saccular hair cells ($n=12$) and canal hair cells ($n=4$, data not shown) as well as from guinea pig outer hair cells (Athas et al., 1997a,b). Faint hybridization signals observed in several other slots are considered to be negative when compared to negative controls.

### 3.3. Single-cell immunocytochemistry

In addition to identifying the presence of $\alpha_9$AChR mRNA in hair cells, expression of the $\alpha_9$AChR subunit protein was confirmed through fluorescent immunocytochemistry (Fig. 3A–C2). Saccular hair cells stained with either T54 (cytoplasmic loop) or T56 (N-terminus) antibodies showed a similar pattern of immunoreactivity which was consistently distributed in the basal and, in some cases, in the apical parts of the cells (Fig. 3A,B). Comparable observations, using these anti-
bodies, have also been made in guinea pig outer hair cells (Park et al., 1997). Intense fluorescence at basal poles of cells may represent regions of efferent contact and/or area of possible α9nAChR sequestration (i.e. sites of synthesis or degradation). Receptor and/or membrane sequestration may be a source for apical fluorescence as well (Cunningham et al., 2000).

In short, Figs. 2 and 3 demonstrate that both the α9nAChR subunit mRNA and protein are found in saccular hair cells. This evidence supports the prediction that the inhibitory effect on the rate of saccular afferent firing produced by exogenous application of ACh (see Fig. 1) is most likely mediated by the activation of presynaptic (i.e. hair cell) α9-containing nAChR.

3.4. The effects of enzymes on the ACh-induced inhibition on the rate of saccular afferent firing

Despite functional, molecular biological, and immunocytochemical evidence for hair cell α9nAChRs, saccular hair cells dissociated and isolated using a combi-

![Fig. 3. The immunocytochemical detection of the α9 subunit protein.](image)

![Fig. 4. The effect of protease treatment on the ACh-induced inhibition of saccular multiunit afferent firing.](image)
ation of papain and protease (VIII or XXIV) failed to respond routinely to application of ACh. Perfusions of 50–100 μM ACh seldom produced consistent, repeatable electrical changes in saccular hair cells under perforated-patch clamp conditions. Elimination of papain from the dissociation protocol did not significantly change the outcome (data not shown). It was then hypothesized that the remaining proteolytic enzymes (protease VIII or XXIV) were disrupting the α9nAChR-mediated response in these cells. Indeed, perfusion of the whole organ preparation with protease VIII abolished the response to 1 mM ACh (Fig. 4). Under control conditions, 1 mM ACh consistently decreased the rate of saccular afferent firing. Multiple applications of ACh were given over a 3 h period to demonstrate the hardy nature of this preparation and that the ACh responses remained relatively unchanged under control conditions. Shortly following the onset of protease VIII treatment, the ACh-induced inhibition of afferent firing was quickly abolished and no longer observed. Only a small facilitation was noted (n = 5). This facilitation could represent the activation of some other cholinergic receptor (e.g. muscarinic) unmasked following elimination of the α9 ACh response (Guth et al., 1994). Baseline afferent firing was also affected by protease VIII perfusion which might suggest an alteration in presynaptic glutamate release or a loss of postsynaptic glutamate receptor sensitivity (e.g. degradation of afferent NMDA receptors). Both effects appear to be permanent as long periods of washing with AP failed to restore either parameter (data not shown).

Fig. 5. Example of the ACh ‘nicotinic’ response in isolated saccular hair cells. (A) In the voltage-clamp mode, a single 60 ms voltage step, from a holding potential of −60 mV to −10 mV (below trace), was applied every 2–3 s. External was perfused onto the cell through the drug delivery system during control and wash conditions. After several control traces were made, 50 μM ACh was applied resulting in a pronounced increase of outward current. (B) Under zero current conditions in the current-clamp mode, the zero current potential of the same isolated saccular hair cell (Fig. 4A) was monitored before and after the application of 50 μM ACh. Under these conditions, ACh was capable of producing a pronounced hyperpolarization (>20 mV). (C) Outward currents induced by 50 μM ACh in a trypsinized hair cell isolated from the frog posterior semicircular canal. (D) Dose–response curves generated for the effect of ACh on the rate of saccular multiunit afferent (○) and the increase in outward currents in trypsinized saccular hair cells (■). In isolated hair cells, the current induced by varying doses of ACh in each cell was normalized to those currents produced by 100 μM ACh. The points were best fit using Microcal Origin 5.0. The dose–response curve for the effect of ACh on saccular afferent firing was fit in a similar fashion. Each ACh response was normalized to the response to 1 mM ACh. Parallel curves suggest that the receptors which underlie each response are probably the same. EC50 (hair cell) = ~19 μM; EC50 (afferent firing) = ~360 μM.
3.5. The ACh response in isolated vestibular hair cells

As opposed to isolations with papain and protease, most saccular hair cells (~90%; 156 of 173) isolated with trypsin (500 μg/ml) routinely responded to ACh under perforated-patch clamp conditions. In the voltage-clamp mode, application of 50 μM ACh produced an increase in the amplitude of outward currents (Fig. 5) both at the holding potential (−60 mV; avg. = 196 pA, range = 25–1189 pA) and step potential (−10 mV; avg. = 729 pA, range = 78–3386 pA). These observations would be consistent with activation of a potassium current, presumably SK. As SK is relatively voltage-independent (Ishii et al., 1997; Bond et al., 1999), the smaller amplitude change at −60 mV as compared to the amplitude difference at −10 mV is probably a function of the driving force for potassium. This response was repeatable and reversible (‘control’ and ‘wash’ traces are superimposable).

In the current-clamp mode, the same hair cell (depicted in Fig. 5A) responded to 50 μM ACh with a pronounced hyperpolarization (Fig. 5B) which was also repeatable and reversible. Hyperpolarization of saccular hair cells would shift the cell to a potential along the voltage-dependent calcium channels’ (VDCC) current–voltage (I–V) curve at which little or no calcium influx occurs. As a consequence, calcium-dependent transmitter release from the hair cell and subsequent afferent firing would be reduced or abolished during this hyperpolarization. Hence, the decrease in saccular multiunit afferent firing observed in the intact organ following the addition of ACh is ultimately a function of α9nAChR activation and hair cell hyperpolarization. Most cells responded with a 10–30 mV hyperpolarization, values comparable to in situ preparations and cells isolated by non-enzymatic means (Art et al., 1984, 1985; Shigemoto and Ohmori, 1991; Sugai et al., 1992; Yoshida et al., 1994).

Fig. 6. Pharmacology of ACh responses in isolated saccular hair cells. (A–D) A–C illustrate the effect of four different antagonists on the response to 50 μM ACh in isolated saccular hair cells. Responses are altered comparably to those seen in the whole organ (rate of multiunit afferent firing, Fig. 1). A: 1 μM strychnine; B: 1 μM curare; C: 50 μM TEA; D: 1 μM apamin.

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Qualitatively, similar ACh responses were also identified in posterior canal (Fig. 5C) and utricular (not shown) hair cells isolated with trypsin. Under previous dissociation conditions using papain and protease, these responses were never observed in canal or utricular hair cells in our experiments. Unlike saccular hair cells, the amplitude of outward currents induced by ACh in canal hair cells was typically small ( < 200 pA). After proteolytic insult, the α9nAChR-mediated response would become very small or non-existent. The use of papain in an earlier study by Housley et al. (1990) as well as the use of both papain and protease (VIII or XXIV) in our initial work may explain the difficulty in observing appreciable α9nAChR-mediated responses in isolated vestibular hair cells.

Additional support that the ACh receptors underlying responses in the intact organ and on isolated hair cells are probably the same may be suggested by parallel ACh dose–response curves from each preparation (Fig. 5D). The ACh dose–response curve for isolated cells (EC50 = 19 μM) is positioned to the left (in a perfectly parallel fashion) of the dose–response curve for the effect of ACh on saccular afferent firing (EC50 = 360 μM). This leftward placement is most likely a product of the absence of diffusional barriers (e.g. synaptic accessibility, perfusion distance) and acetylcholinesterase activity normally associated with the intact whole organ preparation. In fact, the dose–response curve for the effect of ACh on saccular afferent firing is shifted to the left following replacement of normal AP with AP containing the anti-cholinesterase physostigmine (data not shown). The EC50 for ACh in trypsinized frog saccular hair cells agrees well with other reported hair cell α9nAChR EC50 values (chick short hair cells: 19–20 μM (Shigemoto and Ohmori, 1991; McNiven et al., 1996; guinea pig outer hair cells: 13.5 μM (Housley and Ashmore, 1991)).

## 3.6. Pharmacology of the hair cell ACh response

The pharmacology of the ACh response in trypsinized saccular hair cells was also consistent with the responses seen in the multiunit recordings from the intact saccular preparation. The increase in outward current induced by 50 μM ACh was potently and completely antagonized by 1 μM strychnine (data not shown) (Fig. 6A; n = 30), less potently and less completely by 1 μM curare (Fig. 6B; n = 3), and even less by tetraethyl ammonium (TEA) (Fig. 6C; n = 3). Note that 50 μM TEA was needed in order to produce a substantial block of the ACh response. The ACh response was also antagonized by 1 μM apamin (Fig. 6D; n = 8) suggesting that this response was a product of SK activation. The ACh-induced outward current seen in canal hair cells, speculated to be also driven by the α9nAChR/SK complex, was also completely inhibited by 1 μM strychnine (data not shown).

Taken together, these data suggest that the ACh response identified in isolated, trypsinized frog vestibular hair cells and the response observed at the level of the whole labyrinth preparation are likely to be mediated by the same receptor, namely an α9-containing nAChR.

## 3.7. The effects of enzymes on the α9nAChR response in isolated saccular hair cells

The α9nAChR-mediated response on saccular afferent firing in the intact organ was eliminated following exposure to both protease VIII and protease XXIV. Recent evidence had also suggested that papain via its alteration of SK in frog saccular hair cells would also affect the α9nAChR-mediated response (Armstrong and Roberts, 1998). Figs. 7 and 8 demonstrate the effects of protease VIII (n = 9), protease XXIV (n = 8), and papain (n = 5) on the α9nAChR-mediated response in saccular hair cells isolated with trypsin. Brief exposures (~3 min) to these enzymes completely and irreversibly abolished the response to 50 μM ACh (Figs. 7A and 8A). Higher concentrations of ACh were also without effect. The effect of the proteolytic enzymes on the α9nAChR-mediated response was not due to a shift or alteration of the ACh I–V relationship. Following enzyme perfusion, ramp protocols (25–400 ms, −110–80 mV) or voltage steps to various potentials (−100–100 mV) failed to demonstrate any ACh-mediated responses. In further support, the effect of protease VIII or XXIV cannot be washed out. If anything, the effect is more complete following a 5 min washout period in which both the bath and perfusion bath are going which should be more than enough time to wash away the enzyme. It appears that the enzyme may bind to the cell membrane and continue to degrade various proteins (including those involved in the ACh response) despite adequate superfusion with AP. In fact, ACh-responsive cells will completely lose their ACh response within 10 min (data not shown) even when only exposed to very brief perfusions of protease (≤1 min). For these reasons, each time the recording dish was exposed to protease VIII or XXIV, it was rinsed with boiling water and thoroughly cleaned before introduction of new, protease-naive cells. Previous dissociation protocols in our laboratory employed up to 5 min of incubation with papain and protease, thereby allowing enough time for these enzymatic changes to occur contributing to the lack of adequate α9nAChR-mediated responses in isolated saccular hair cells.

The effect of the enzymes was most likely a product of enzymatic alteration of proteins (α9nAChR, SK) central to the ACh response. Perfusions of protease VIII (n = 3), protease XXIV (n = 3), or papain (n = 4),
first placed in boiling water for 5–10 min to inactivate the enzymes (as evidenced by precipitation), failed to abolish the ACh response in trypsinized saccular hair cells (Figs. 7B and 8B). However, subsequent application of the native enzymes on the same cells quickly eliminated any further response to ACh.

Control currents were also affected by protease VIII, protease XXIV, and papain exposure. In most cells, the apparent amplitude of the outward current produced in response only to the depolarizing step to $-10$ mV was larger following treatment with papain, protease VIII, or protease XXIV (see control traces in Figs. 7 and 8) whereas the current at rest ($-60$ mV) appeared unaffected. The effects of papain on ionic currents and electrical tuning in frog saccular hair cells have previously been investigated (Armstrong and Roberts, 1998). In

Fig. 7. The effect of protease (VIII or XXIV) treatment on the ACh nicotinic response in isolated saccular hair cells. (A) The ‘control’ traces represent the currents produced during the 50 mV depolarizing step before and after the application of 50 μM ACh. These currents were generated in a protease-naive, trypsinized saccular hair cell and thus serve as a point of reference for subsequent exposure to either protease VIII or XXIV. Once control parameters were established, the cell was perfused with either protease VIII or XXIV (120 μg/ml) for intervals of 60 s. Following enzyme application, the cell was ‘washed’ thoroughly (perfused w/AP) before reapplying 50 μM ACh. With each additional minute of enzyme perfusion, the response to ACh was further attenuated, and finally abolished after 3 min of perfusion. Note also the change in control currents (in the absence of ACh). Neither effect appears to be reversible even following a 10–15 min wash. (B) The effect of heat-inactivated versus native protease on the ACh ‘nicotinic’ response in isolated saccular hair cells. In saccular hair cells, perfusion (120 s) of either protease (VIII or XXIV), heat-inactivated by boiling, did not affect control currents or the response to 50 μM ACh. However, when this same cell is exposed for 120 s to the native protease, the ACh response was nearly completely eliminated.
In their study, the average amplitude of outward currents in papain-treated cells was almost twice that of the untreated cells. Perhaps both protease VIII and XXIV affect control currents in a manner similar to papain. In agreement with Armstrong and Roberts, note that the amplitude of the currents (‘control’) produced in response to the depolarizing step to $-10$ mV increases with prolonged exposure to these enzymes (see Figs. 7A and 8A). Although the ‘enhancement’ of outward currents and abolition of the $\alpha 9$-containing AChR response appear contemporaneous in Figs. 7 and 8, these two effects typically occurred on different time scales. In many cells (particularly those treated with protease VII or XXIV), the ACh response was abolished before the amplitude of outward currents was grossly affected. In fact, the effect of these enzymes on the ACh response is
Fig. 9. Effects of other enzymes on the ACh response and control currents in isolated saccular hair cells. Although as few as 3 min of exposure to either protease VIII, XXIV, or papain can result in an apparent irreversible abolition of the ACh response in isolated saccular hair cells, comparable perfusions (≥3 min) of collagenase, dispase, elastase, hyaluronidase, and trypsin at the concentrations shown failed to demonstrate any significant alterations of the ACh response.

4. Discussion

We have demonstrated that the proteolytic enzymes papain, protease VIII, and protease XXIV permanently abolish the α9nAChR-mediated response in isolated frog saccular hair cells. It is suggested by the data that the complete and irreversible attenuation of the ACh response in these cells was the result of direct enzymatic alteration of components essential to the α9nAChR/SK mechanism.

There are several instances in the literature where enzymes have been shown to alter physiological responses. Trypsin, applied externally to *Aplysia* and *He-
It is not surprising that both papain and the bacterial proteases (VIII, XXIV) eliminate the ACh response in isolated saccular hair cells. An investigation into their cleavage sites reveals that both of these enzymes display broad selectivities for peptide bonds (Ottesen and Svendsen, 1970; Perlman and Laszlo, 1970). They are relatively indiscriminate about which peptide bonds they hydrolyze. This ‘promiscuity’ undoubtedly contributed to the initial success of isolating vestibular hair cells with them; however, it is precisely this broad substrate selectivity that allows these enzymes to affect proteins which participate in tuning (Armstrong and Roberts, 1998) and in the \( \alpha_9 \)nAChR-mediated response. Trypsin, on the other hand, preferentially cleaves only on the C-terminal side of lysine and arginine. These proteolytic sites are impaired by neighboring acidic residues (e.g. glutamate, aspartate) and are prohibited when proline is on the carboxyl side of the cleavage site. With one of the most restricted selectivities among endopeptidases, trypsin is widely used for protein sequencing and peptide mapping (Walsh, 1970; Perlman and Laszlo, 1970; Sinclair, 1999). It are these characteristics which have most likely permitted the observation of the \( \alpha_9 \)nAChR-mediated response in saccular hair cells isolated using trypsin.

Although trypsin may also alter proteins within the membrane of the hair cell, the \( \alpha_9 \)nAChR-mediated response appears to be well preserved from both a pharmacological and functional standpoint. In fact, the \( \alpha_9 \)nAChR in vestibular hair cells joins several different nicotinic receptors, including those found in the neuromuscular junction, the electric organ of the \textit{Torpedo californica}, and \textit{Aplysia} neurons, which remain functional following trypsin application (Albuquerque et al., 1968; Betz and Sakmann, 1971, 1973; Lindstrom et al., 1980; Conti-Tronconi et al., 1982; Oyama et al., 1990). Unlike those treated with papain, trypsinized saccular hair cells display outward currents sensitive to 4-aminopyridine (4-AP) and TEA (unpublished observations, J.C. Holt). Furthermore, 4-AP perfusion reveals a rapidly inactivating outward current that is blocked by subsequent TEA perfusion remarkably similar to the recently described BK identified in frog saccular cells not exposed to proteolytic enzymes (Armstrong and Roberts, 1998, 1999). In general, it appears that trypsin dissociation exerts a much milder effect on some membrane proteins than either papain or the bacterial proteases.

The effects of \( \alpha_9 \)nAChR stimulation in both auditory and vestibular hair cells among vertebrates are well conserved. As far as we know, there has been no exception. In both types of cells, this activation results in the subsequent activation of SK. In fact, the most striking disparity among hair cell \( \alpha_9 \)nAChRs thus far reported has been the difference in 1,1-dimethyl-4-phenylpiperazinium sensitivity (Chen et al., 1996) which might be attributed to incorporation of the recently described \( \alpha \)10nAChR subunit (Vetter et al., 2000; Lustig et al., 2000). Because of the remarkable similarities and conservation of function among \( \alpha_9 \)nAChRs from different auditory and vestibular preparations, it is likely that papain, protease VIII and protease XXIV would exert similar effects on the \( \alpha_9 \)nAChR in hair cells of other organs as those observed in the frog saccule. In this regard, the susceptibility of the \( \alpha_9 \)nAChR/ SK channel complex will depend on the concentration of enzymes used, the length of time exposed to that concentration, and \( \alpha_9 \)nAChR/SK density.

Tall hair cells from the chick cochlea, obtained non-enzymatically, have also been shown to respond to ACh (Shigemoto and Ohmori, 1991); however, ACh responses were not observed in chick cochlear tall hair cells following enzymatic dissociation using a bacterial protease (Murrow and Fuchs, 1990; Fuchs and Murrow, 1992a). Because proteolytic enzymes (i.e. protease XXIV) were used to dissociate the cells from the chick cochlea in these studies, these observations may be a product of proteolytic degradation of the components underlying the \( \alpha_9 \)-nicotinic response. Frog saccular hair cells isolated using either papain, protease VIII, or protease XXIV rarely (<10%) responded to ACh. In those few instances where \( \alpha_9 \)-nicotinic responses were observed, they were always small in amplitude (<200 pA) and difficult to maintain over short periods of time, consistent with continued degradation by the proteolytic enzymes. Papain was also likely to be responsible for the small ACh-induced currents identified in SCC hair cells by Housley et al. (1990). The amplitude of \( \alpha_9 \)nAChR-induced currents in chick cochlear hair cells may have been underestimated in those earlier studies using protease XXIV. In fact, the \( \alpha_9 \)nAChR-mediated response may be underestimated in any hair cells isolated using protease VIII, protease XXIV, or papain although this idea has not been tested.

4.1. Mechanisms underlying the loss of the ACh response

The loss of the inhibitory (hyperpolarizing) response to ACh in vestibular cells following treatment with papain or the bacterial proteases (VIII, XXIV) most likely results from proteolysis of the \( \alpha_9 \)-containing nicotinic receptor and/or its downstream effector, the SK. It has been shown that papain apparently eliminates the functional contributions of SK which would be expected to ultimately alter the dynamics of the \( \alpha_9 \)-nicotinic ACh response in saccular hair cells (Armstrong and Roberts, 1998). Although protease VIII and XXIV (based on comparable substrate preference – Ottesen and Svendsen, 1970) are likely to work similarly to papain in eliminating the ACh response, there is currently no-in-
formation regarding the mechanisms (or sites) of their effects.

Although we present no direct evidence that the α9-nicotinic receptor is functionally spared following exposure to either papain or the proteases, it has been shown that the ACh sensitivity of the endplate nicotinic receptor found in skeletal muscle is not affected following several hours of incubation with pronase, trypsin, chymotrypsin, papain, or protease VII (similar to VIII and XXIV) (Albuquerque et al., 1968; Betz and Sakmann, 1971, 1973). Nicotinic ACh receptors purified from T. californica and reconstituted in model lipid vesicles retain 'extreme functional stability' following extensive proteolytic cleavage with either trypsin or papain. Despite a pronounced degradation of nicotinic receptor subunits, these membrane preparations were still capable of mediating a carbachol-induced cation flux (Lindstrom et al., 1980; Conti-Tronconi et al., 1982). Finally, the activation of a second nicotinic receptor identified in vestibular organs, which is pharmacologically distinct from the α9nAChR, is also not affected by protease VIII or papain treatment (unpublished observations, J.C. Holt and P.S. Guth). If trypsin, papain, and the proteases all exhibit similar effects on the α9nAChR subunits, then it may be suggested that the loss of the α9nAChR-mediated response in saccular hair cells isolated with papain or protease VIII, XXIV is a result of the proteolysis of SK. In a recent study in acutely dissociated rat hippocampal pyramidal neurons (Hirschberg et al., 1999), an 'abrupt, complete, and irreversible' run-down of SK channel activity was observed in excised inside-out patches. In all the patches examined, SK channel activity was abolished within the first 10 min of recording. In a fraction of the patches (30%), SK activity was eliminated during the first minute of recording. The authors suggest that this run-down may be attributed to the loss of some cytoplasmic factor. It must also be noted that protease XXIII was used to dissociate the cells. Perhaps, the loss of SK channel activity is the result of enzyme treatment. As in our cells, enzyme activity appears to continue even after removal of the enzyme.

Of the three known members of the SK channel family (Ishii et al., 1997; Bond et al., 1999), SK2 is proposed to underlie the α9nAChR-activated potassium conductance in auditory and vestibular hair cells. Its candidacy is supported both by its apamin sensitivity, cDNA library expression, and in situ hybridization studies (Elgoyhen et al., 1994; Yoshida et al., 1994; Nenov et al., 1996b; Shao et al., 1998; Dulon et al., 1998). Analysis of the amino acid sequence for the rat SK2 subunit reveals five possible sites along its four extracellular loops that might be possible targets for hydrolysis by trypsin (Bond et al., 1999). An arginine residue in the third loop is neighbored by a glutamate residue which might slow or impair proteolytic degradation. Although most of the peptide bonds found in the extracellular domain of SK would be 'fair game' for papain and the bacterial proteases, trypsin's enzymatic interaction with SK2 would be limited only to a few sites which may or may not be accessible due to conformational or steric considerations. Our studies would indicate that any interaction that trypsin might have does not compromise the functional integrity of this potassium channel. The same cannot be said for papain or protease VIII, XXIV.

5. Conclusions

A three-pronged approach using pharmacology, molecular biology, and immunocytochemistry supports the hypothesis that the inhibitory ACh response in isolated vestibular hair cells is most likely produced by an α9-containing nAChR. Furthermore, brief exposures to protease VIII, protease XXIV, and papain abolish the α9nAChR-mediated response in both the intact organ and in acutely isolated, tryspinized frog vestibular hair cells.

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