A Pharmacologically Distinct Nicotinic ACh Receptor Is Found in a Subset of Frog Semicircular Canal Hair Cells

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Holt, Joseph C., Maria Lioudyno, and Paul S. Guth. A pharmacologically distinct nicotinic ACh receptor is found in a subset of frog semicircular canal hair cells. J Neurophysiol 90: 1526–1536, 2003; 10.1152/jn.00273.2002. Frog vestibular organs are endowed with a prominent cholinergic efferent innervation whose stimulation results in several different effects, thereby suggesting in the expression of postsynaptic acetylcholine (ACh) receptors. The application of ACh can mimic efferent stimulation in producing both an inhibition and a facilitation of afferent discharge which are thought to be mediated by at least two distinct ACh receptors present on vestibular hair cells, i.e., 9-containing nicotinic receptors (9nAChR) and muscarinic receptors (mAChR), respectively. Using patch-clamp and multiunit vestibular afferent recordings, we demonstrate the presence of an additional excitatory hair cell nicotinic ACh receptor pharmacologically distinct from both 9nAChR and mAChR. In order of increasing potency, this distinct receptor was activated by ACh, carbamylcholine, and particularly by the selective nicotinic agonist 1,1-dimethyl-4-phenyl-piperazinium (DMPP). This DMPP-sensitive nicotinic receptor (R9DMPP) was antagonized by the classic nicotinic antagonist d-tubocurarine, but refractory to strychnine, atropine, and propylbenzilylcholine mustard, at concentrations that completely block 9nAChR and/or mAChR. Activation of R9DMPP on application of ACh or DMPP to a subpopulation of isolated posterior semicircular canal (SCC) hair cells resulted in a large depolarization (18.0 ± 1.2 mV). The current underlying this depolarization was typically small (80.1 ± 21.6 pA) and showed an inward rectification starting around −45 mV. Given their respective EC50's (47 nM vs. 20 μM), R9DMPP was nearly 400 times more sensitive to ACh than 9nAChR and thus responded to concentrations of ACh considered too low to be effective at stimulating 9nAChR. Despite this remarkable sensitivity, exogenous ACh readily stimulated the mAChR in the intact posterior SCC hair cell transmitter release onto afferent terminals. These efferent responses are presumably mediated by a modulation of hair cell transmitter release onto afferent terminals. These contrasting effects may be attributed to the release of different efferent neurotransmitters and the activation of their corresponding postsynaptic receptors. Alternatively, it may be that these diverse effects are all produced by the predominant efferent transmitter ACh acting on different ACh receptors.

Consistent with the idea that these different efferent responses are mediated by different ACh receptors, cholinergic agonists, in the frog, mimic the various afferent responses seen during efferent stimulation. Among most frog vestibular endorgans, the prevailing response to exogenous ACh is a slowly developing facilitation of vestibular afferent discharge (Perin et al. 1998). This slow facilitation is also activated by muscarine and antagonized by atropine and propylbenzilylcholine mustard (PrBCM), suggesting that it is mediated by muscarinic acetylcholine receptors (mAChR) (Bernard et al. 1985; Norris et al. 1988; Perin et al. 1998). Similar observations have also been made in the lateral line (Bobbin et al. 1985). In the frog sacculle, however, ACh elicits a rapid inhibition of afferent firing that is antagonized by strychnine, d-tubocurarine (dTC), atropine, and apamin (Guth et al. 1994a; Holt et al. 2001; Sugai et al. 1992). This inhibition is thought to be mediated by 9-containing nicotinic acetylcholine receptors (9nAChR) functionally coupled to the activation of small-conductance, calcium-dependent potassium channels (SK), similar to what is observed in acoustic hair cells (Art et al. 1984; Fuchs and Murrow 1992; Nenov et al. 1996). We use the term “9-containing” as recent evidence has demonstrated that 9-nicotinic subunits in hair cells most likely form functional heteromultimers with α10-nicotinic subunits (Elgyothen et al. 2001; Rothlin et al. 2003). Although the 9/α10nAChR and the homomeric 9nAChR differ in their desensitization and permeability properties, their sensitivities to several antagonists, including strychnine and atropine, are very similar (Elgyothen et al. 2001).

A rapid excitation of frog posterior SCC afferents is also seen following the application of the cholinergic agonists 1,1-dimethyl-4-phenyl-piperazinium (DMPP) and carbamylcholine (Bernard et al. 1985). Like the presumed 9nAChR-mediated inhibition, this fast excitation was also blocked by dTC. It was suggested that perhaps a single nicotinic ACh receptor might be responsible for both the excitation and inhibition (Bernard et al. 1985). After all, activation of 9nAChR without the subsequent involvement of SK could result in a characteristic excitatory response commonly associated with the activation of most nicotinic acetylcholine receptors. In addition, DMPP has
been shown to activate α9nAChR in guinea pig outer hair cells (Erostegui et al. 1994). However, dTC is not selective for α9nAChR exclusively, and since several nicotinic receptor subunit mRNAs other than α9 and α10 have been demonstrated in vestibular endorgans (Anderson et al. 1997), it is uncertain whether the fast excitation and inhibition involve the same nicotinic ACh receptor or not. In this study, we compared the pharmacological and electrophysiological profile of this DMPP-sensitive, excitatory vestibular nicotinic ACh receptor to that of α9nAChR. Our results strongly suggest that the hair cell receptors involved in fast cholinergic excitation and inhibition are distinct.

Some of these results have appeared in abstract form (Holt et al. 2000).

**METHODS**

**Multunit afferent recordings**

Recordings of vestibular multunit afferent firing were performed as previously described (Guth et al. 1986, 1994a). Leopard frogs (*Rana pipiens*) were anesthetized (by chilling), doubly pithed, and decapitated. The labyrinth was exposed, and the nerve to either the posterior semicircular canal (SCC) or saccule was cut, thereby producing a decentralized stump estimated to contain 1,200 fibers (Honrubia et al. 1989). The isolated nerve was delicately pulled into a suction electrode filled with artificial perilymph (AP, see following text). Whole nerve discharge was amplified (1,000×) and filtered (low 

**Isolation of hair cells**

Dissociation of frog vestibular hair cells was optimized for the purpose of observing reliable ACh responses (Holt et al. 2001). The saccular macula or canal crista was dissected free from the whole labyrinth and transferred to a dish containing Hanks Balanced Salt Solution (Gibco, Life Technologies, Grand Island, NY) nominally devoid of calcium and magnesium but containing trypsin (0.05%) and EDTA (0.53 mM). Incubation times varied from 10 to 15 min for saccular maculae and 25–30 min for posterior SCC cristae. The sensory epithelium was transferred into dissociation solution (in mM: 105 NaCl, 2.5 KCl, 0.1 MgCl₂, 1.8 CaCl₂, 3.4 NaHCO₃, 0.5 NaH₂PO₄, 2.5 Na₂HPO₄, and 4 glucose) for the duration of the recording.

**Drug application**

Some aspects of drug application can be generalized for both multunit afferent recordings and isolated hair cells. Pharmacological agents were prepared in either AP or external solution and applied using a multibarrel pipette. In multunit recordings, the pipette was positioned 0.5 mm from the neuroepithelium where the delivery of the various solutions was controlled by a syringe pump (15–45 μl at 50 μl/min). In isolated cells, the pipette was placed approximately 300–600 μm from the selected hair cell where drug application was gravity-driven (3–4 μl/s). In either case, the pipette was capable of delivering any one of up to eight different solutions (i.e., AP or external with or without drugs). Each solution was maintained in a separate reservoir and delivery line just up to emptying from a single exit (26-gauge, 1.4 cm, volume = approximately 0.7 μl). In isolated hair cells, this small dead space introduced an additional approximately 200-ms delay between the application of different drugs, but the single port ensured that the geometry of any drug application for

The recording chamber containing isolated vestibular hair cells was clamped onto the movable stage of a Nikon Diaphot TMD inverted microscope (Nikon, Garden City, NY) equipped with DIC optics. The dish was perfused with external solution (1 ml/min) for the remainder of the experiment. Tight gigaohm seals (1–15 GΩ) were obtained using recording pipettes constructed from thick-walled 1.5-mm-OD borosilicate glass tubing (Longreach Scientific Resources, Orr’s Island, ME) pulled by an automated Flaming/Brown micropipette puller (Model P97, Sutter Instruments; Novato, CA) and heat-polished on a solutions, (MF-83, Narashige Scientific Instrument Laboratory, Tokyo, Japan) before their use. An Axopatch 1-B patch-clamp amplifier (Axon Instruments, Foster City, CA) was used in the voltage- and current-clamp configurations to record from vestibular hair cells. Records were low-pass filtered at 5 kHz with a four-pole Bessel filter. Command potentials and currents were controlled with a 12-bit D/A converter, and data were digitally sampled every 100 μs using a 12-bit A/D converter (Labmaster DMA, Scientific Solutions, Solon, Ohio) coupled to a PC (Gateway 233 MHz Pentium II, Gateway, North Sioux City, SD). Data were stored on hard media for off-line analysis (pCLAMP, ver. 6.04, Axon Instruments). The tip potential between the pipette’s internal solution and the bath was nullled 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). Command potentials were corrected accordingly for step protocols. Series resistance and cell capacitance measurements were constantly monitored and updated throughout all experiments. Series resistance was partially compensated (60–80%). As most of the protocols typically produced small currents (<2 nA), the voltage errors due to series resistance (AVG = approximately 15 mV) were generally <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).

The hair cell data shown here were recorded using the perforated-patch variant of patch clamping (Horn and Marty 1988). Three milligrams of amphotericin B were dissolved in 60 μl of DMSO and suspended in 5 ml of normal internal solution (in mM: 75 KCl, 2 MgCl₂, 30 K₂SO₄, 6 glucose, and 10 HEPES). This solution (kept shielded from the light with aluminum foil) was then used to back-fill the patch pipette. Successful seals were obtained without prior front-filling with normal internal solution. After perforation was complete and equilibrium conditions achieved, voltage- and current-clamp protocols were performed.
a given cell did not change. The application of each solution was manually controlled by an inline stopcock between the reservoir and delivery line. Depending on the proximity of the pipette to the hair cell, the exchange time between drug applications was on the order of 500–1,500 ms as determined by KCl-induced depolarization. AP or external solution was always maintained in one of the eight reservoirs and continually superfused before and after the application of cholinergic agonists as well as each agonist/antagonist series. This served both as a control for mechanical effects as well as to flush drugs from the tip and preparation. In this manner, the local solution could be rapidly exchanged allowing for repeated, predictable observations. Prior to co-application with any agonist, all cholinergic antagonists were also given alone. This allowed for observation of any effects that the antagonist itself might produce. Additionally, it permitted a standing concentration of the antagonist before administration of cholinergic agonists, and thus a better assessment of that antagonist’s potency. In multiunit recordings, antagonists were also applied by bath substitution unless otherwise noted. All reagents were obtained from Sigma-RBI except PrBCM (New England Nuclear, Boston, MA). All experimental protocols were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publications No. 80–23) revised 1978, and were approved by the Tulane University Advisory Committee for Animal Resources (ACAR).

Data analysis

Data were analyzed and plotted using pCLAMP6 Clampfit (Axon Instruments), Excel 7.0 (Microsoft, Redmond, WA), and Origin 6.0 (Microcal Software, Northampton, MA). Results are expressed as the mean ± SE unless otherwise noted. The data of both agonist and antagonist dose-response relationships were iteratively fitted with the Hill equation

\[ R_h = R_0 + \frac{(R_u - R_0)}{(1 + (X_{1/2}/X)^h)} \]

where \( X \) is the concentration of agonist or antagonist, \( X_{1/2} \) is the concentration that produces 50% of the maximal response (EC50 for agonists, IC50 for antagonists), \( R_h \) is the response to minimum concentrations (such that \( R_0 = 0 \) for agonists and \( R_0 = 100 \) for antagonists), \( R_u \) is response at maximal/supramaximal concentrations (such that \( R_u = 100 \) for agonists and \( R_u = 0 \) for antagonists), and \( h \) is the Hill or interaction coefficient. All responses (\( R_c \), \( R_a \), \( R_u \)) were first normalized by expressing as a percentage of the response elicited by near-maximal to maximal concentrations of the agonist of interest.

RESULTS

Effects of cholinergic agonists on multiunit afferent discharge

The application of various cholinergic agonists onto the frog saccule and SCC induced a total of three distinct effects on their multiunit afferent discharge. In the saccule (Fig. 1A1), 1 mM ACh routinely produced a robust inhibition (\(-264 ± 12 \) Hz; \( n = 20 \)) displaying a rapid activation (rise time = 23.2 ± 1.7 s), recovery (\( T_{1/2} = 32.8 ± 4.0 \) s), and duration (88.8 ± 8.2 s). This inhibition has been previously defined pharmacologically and most likely is triggered by activation of α9nAChR found on frog saccular hair cells (Guth et al. 1994a; Holt et al. 2001; Rothlin et al. 2003; Sugai et al. 1992). Activation of α9nAChR results in a calcium influx that subsequently activates colocalized small conductance, calcium-dependent, potassium channels (SK) (Art et al. 1984; Fuchs and Murrow 1992; Nenov et al. 1996). Activation of SK hyperpolarizes the hair cell thereby decreasing the release of the afferent transmitter and thus reducing the frequency of afferent discharge. As evidenced by the effect of ACh on saccular afferent discharge and hair cell currents, the frog saccule represents a good source for α9nAChR-expressing hair cells (Sugai et al. 1992; Guth et al. 1994a; Holt et al. 2001).

In the posterior SCC (Fig. 1A2), the application of 1 mM ACh resulted in a characteristic excitation of multiunit afferent firing (92.9 ± 3.7 Hz; \( n = 20 \)) with a notably slower activation (rise time = 49.5 ± 4.4 s), recovery (\( T_{1/2} = 190 ± 20 \) s), and duration (429 ± 43 s). This slow excitation has been pharmacologically defined as muscarinic, since it is activated by muscarine and antagonized by submicromolar concentrations of atropine and the irreversible, nonselective muscarinic antagonist PrBCM (Bernard et al. 1985; Norris et al. 1988; Perin et al. 1998).

Whereas the application of relatively high doses of ACh (0.3–3 mM) only induced a slow excitation in the posterior
SCC, another component was introduced following the application of the selective nicotinic agonist DMPP or the mixed nicotinic/muscarinic agonist carbachol (CCh) (Bernard et al. 1985). In our preparations, application of 10 μM DMPP (Fig. 1A) resulted in a rapid excitation of multiunit afferent firing (195 ± 7 Hz; n = 40). Its response kinetics (rise time = 12.2 ± 0.7 s; decay T_{1/2} = 23.1 ± 1.5 s; duration = 58.3 ± 3.2 s) were much faster than the mAChR-mediated excitation in the SCC (Fig. 1B) and slightly faster than the α9nAChR-mediated inhibitory response seen in the saccule (Fig. 1A). When 20–50 μM CCh was given (n = 6), the resulting response was composed of a rapidly occurring peak and a slower recovery to baseline suggesting that the DMPP-sensitive component and the mAChR-mediated slow excitation were both activated (Fig. 1A). In our preparations, a distinction between these two components was not obvious at CCh concentrations lower than 20 μM.

Effects of cholinergic antagonists on the DMPP response

The pharmacological and kinetic data presented so far would suggest that the rapid excitatory response in the posterior SCC elicited by DMPP and CCh is mediated by a nicotinic ACh receptor. Therefore we tested the effects of several cholinergic antagonists against this DMPP-sensitive component. The classic nicotinic antagonist, dTC, significantly reduced (1 μM) and abolished (10 μM) the response to 10 μM DMPP in four preparations (Fig. 1B). Since the mAChR-mediated response in the posterior SCC is not sensitive to these concentrations of dTC (Bernard et al. 1985), the blockade of the DMPP-sensitive component by dTC suggests that it is mediated by a nicotinic receptor. However, the DMPP-sensitive component in the posterior SCC cannot be distinguished from an α9nAChR-mediated response on these grounds alone as α9nAChR are also blocked by similar concentrations of dTC (Elgoyhen et al. 2001; Guth et al. 1994a; Verbitsky et al. 2000).

The strongest evidence that the DMPP-sensitive nicotinic component is different from α9nAChR is presented in Fig. 2, A–C. The application of 10 μM strychnine, a concentration shown to completely antagonize α9nAChR in many hair cell preparations (Elgoyhen et al. 2001; Guth et al. 1994a; Verbitsky et al. 2000), did not significantly affect the response to 10 μM DMPP in the posterior SCC (Fig. 2A). This distinction is best reflected in the 600-fold difference between the concentration of strychnine needed to block 50% (IC_{50}) of the corresponding peak response to ACh in the saccule and DMPP in posterior SCC (Fig. 2B). In the saccule, the IC_{50} for strychnine blockade of the inhibitory response to 300 μM ACh (filled circles) was 104 ± 7 nM (b = 3.67 ± 2.18) compared with an IC_{50} of 68.4 ± 24.8 μM (b = 1.00 ± 0.41) for the strychnine antagonism of 10 μM DMPP (filled squares) in the posterior SCC. As has been similarly reported for other nicotinic receptors (Garcia-Colunga and Miledi 1999), higher concentrations of strychnine eventually attenuated the DMPP response (Fig. 2B), but these concentrations were well above those needed to block the ACh-induced, α9nAChR-mediated response in the frog saccule or in Xenopus oocytes (Elgoyhen et al. 2001; Guth et al. 1994a; Verbitsky et al. 2000). Even at a strychnine concentration of 100 μM, a response to 10 μM DMPP was still observed. The relative ineffectiveness of strychnine suggests that the DMPP-sensitive component is different from α9nAChR.

In Fig. 2C, atropine, at a concentration previously shown to block both mAChR-mediated responses in the frog posterior SCC (Bernard et al. 1985; Norris et al. 1988) and α9nAChR-mediated responses in the frog saccule and in Xenopus oocytes (Elgoyhen et al. 2001; Guth et al. 1994a; Verbitsky et al. 2000), did not reduce the excitatory response to 10 μM DMPP alone. At strychnine concentrations above 10 μM, non-selective effects on baseline afferent firing were also noted. Error bars represent ± SE. C: similar to results with strychnine, the fast excitation selectively activated by 10 μM DMPP (15 s at 50 μl/min) was not antagonized following several hours exposure to a 10 μM atropine bath.

In addition to the distinct pharmacological profile generated using dTC, strychnine, and atropine, it might also be argued
that the DMPP-sensitive component could be distinguished from α9nAChR on the basis of their differential sensitivity to DMPP itself. Despite its ability to fully activate α9nAChR in guinea pig outer hair cells (Erostegui et al. 1994) and its profound excitatory effects on the posterior SCC multiunit afferent discharge, DMPP is a notably poor agonist in activating the inhibitory response in the frog saccule (Guth et al. 1994a; Sugai et al. 1992) as well as α9nAChR-mediated responses in Xenopus oocytes (Elgoyhen et al. 2001; Sgard et al. 2002). In the frog saccule, DMPP application does result in a small inhibitory response with similar kinetics as ACh, but always with a much lower potency often requiring concentrations of 0.1–1 mM to obtain demonstrable responses (Fig. 3A). Lower concentrations of DMPP (1–10 μM), however, were without effect on saccular multiunit afferent discharge, and at no time did the application of any concentration of DMPP result in a rapid excitatory response in the saccule like those seen in the posterior SCC. In three saccular preparations, this DMPP inhibitory response was completely blocked by strychnine and eliminated following protease application (data not shown). These response properties are consistent with the activation of α9nAChR that are similarly affected by these same treatments (Holt et al. 2001).

Although DMPP and CCh can easily activate the DMPP-sensitive component in the posterior SCC, the application of ACh only results in the activation of the mAChR-mediated slow excitation. The difference in the abilities of ACh, CCh, and DMPP to activate the fast excitatory response may be due, in part, to the different sensitivities of these agonists to degradation by acetylcholinesterase. DMPP and CCh are resistant, whereas ACh is not, and therefore may be appreciably degraded from its applied concentrations. To test this hypothesis, we used the acetylcholinesterase inhibitor physostigmine. Similar to the response seen with CCh (Fig. 1A4) (see Perin et al. 1998), the application of ACh following physostigmine treatment (10 μM) produced an excitatory response whose kinetics also suggested that multiple components were being activated (data not shown). When a posterior SCC preparation was co-treated with 1 μM PrBCM (Fig. 3B, arrow A) to irreversibly block the mAChR-mediated slow excitation (Fig. 3B2) and subsequently with physostigmine (10 μM) to block acetylcholinesterase (Fig. 3B, arrow B), the application of 1 mM ACh then elicited a rapid excitation that was indistinguishable from the response to 10 μM DMPP (Fig. 3B3; n = 7). Unlike ACh, the response to DMPP was unaffected by either treatment. These data suggest that 1) ACh does undergo a significant degradation by acetylcholinesterase as treatment with PrBCM alone did not unmask the rapid excitation (Fig. 3B2); and 2) the rapid excitatory response, whether elicited by either ACh or DMPP, is again not mediated by mAChR because it persists following treatment with PrBCM (n = 14). This comports well with the lack of blockade seen with atropine application (Fig. 2C). In the posterior SCC, ACh alone cannot activate the rapid excitatory response unless its concentrations were raised above 3 mM or physostigmine was used. Higher concentrations of physostigmine may further decrease the apparent difference in potencies between ACh and DMPP; however, higher concentrations of physostigmine were not tried as they also exert effects on background discharge (Norris et al. 1988).

It is likely that DMPP and ACh were stimulating the same receptor in the posterior SCC, because prolonged exposure (2–5 min) to DMPP resulted in a decreased responsiveness to subsequent applications of DMPP and ACh (after treatment with PrBCM and physostigmine). Similar observations have been made for DMPP and efferent responses in single unit recordings (Bernard et al. 1985). Furthermore, the kinetics of each response were nearly perfectly superimposable. The advantages to using low doses of DMPP (1–10 μM) over ACh was that 1) it appeared selective for the rapid excitatory response in the posterior SCC, thus avoiding the need for pre-treatments with physostigmine and PrBCM that were necessary to isolate the same response when using ACh, and 2) these same concentrations were not effective in activating the α9nAChR-mediated inhibitory response in the frog saccule (Guth et al. 1994a; Sugai et al. 1992), suggesting that similar receptors (namely α9nAChR) were not being activated in the
posterior SCC. Therefore in the remaining sections, the ACh receptor whose activation underlies the rapid excitatory response in the frog posterior SCC will now be tentatively referred to as the DMPP-sensitive nicotinic receptor ($R_{DMPP}$) to distinguish it from either $\alpha_9nAChR$- or mAChR-mediated responses.

Figure 3C illustrates the dose-response relationship between DMPP and the rapid excitatory response in multiunit afferent recordings from the posterior SCC. Six preparations were used to construct this curve where a minimum of three concentrations was applied at least twice in each preparation. The peak responses observed at each concentration were normalized to the peak response following the application of 10 $\mu$M DMPP. The sigmoid fit of the data were derived from the Hill Equation (see Methods). In our preparations, the excitatory effect of DMPP was first seen around 1 $\mu$M and reached saturation near 30 $\mu$M. The EC$_{50}$ was calculated to be $3.5 \pm 0.2$ $\mu$M ($h = 1.73 \pm 0.10$). Even when using relatively high doses of DMPP, the kinetics of the rapid excitatory response remained relatively unchanged (Fig. 3C, bottom), suggesting that this agonist, unlike ACh and CCh, was ineffective in activating the muscarinic receptors that underlie the slow excitation. Again, this is in agreement with the lack of blockade by either atropine or PrBCM (Figs. 2C and 3B).

**Evidence for two nicotinic ACh receptors in isolated vestibular hair cells**

Since frog vestibular efferents are thought to be predominately cholinergic and to only synapse with hair cells (Lopez and Meza 1988; Lysakowski 1996), ACh, whether released from efferent terminals or applied exogenously, is presumed to activate ACh receptors on these hair cells. Provided this is true, on ACh application one might expect to find response properties in isolated hair cells that complement those changes seen in multiunit afferent discharge. However, there are only a few reports of proposed hair cell ACh responses other than those driven by $\alpha_9nAChR$ in association with SK (Housley et al. 1990; Steinacker and Rojas 1988). In our recordings from saccular and SCC hair cells, two different responses were observed that could be initially distinguished from each other based solely on their kinetics.

In the majority of isolated saccular hair cells, the application of ACh generated a relatively large current that reversed near −80 mV and peaked between −30 and −10 mV (Fig. 4, A and B). This current-voltage ($I$-$V$) relationship was also reflected in the responses to single voltage step protocols. As expected, an increase in inward currents at −130 mV and outward currents at −60 mV were seen (Fig. 4C). Also consistent with the observed $I$-$V$ curve, ACh-induced, $\alpha_9nAChR$-mediated responses were most easily demonstrated using voltage steps to −10 mV where near-peak currents could be obtained (Holt et al. 2001). The generation of such outward currents would be predicted to hyperpolarize the hair cell. Indeed, a robust hyperpolarizing change in membrane potential (about 20 mV as shown in Fig. 4D) was induced by ACh under zero current-clamp conditions. This hyperpolarization is presumably due to the activation of $\alpha_9nAChR$ whose calcium influx subsequently activates SK (as suggested by the reversal potential close to $E_K$). These response properties were all antagonized by low concentrations of strychnine (Holt et al. 2001).

In contrast, the response to ACh or DMPP in some posterior SCC hair cells was notably different from the typical $\alpha_9nAChR$ response seen in frog saccular hair cells. In Fig. 5, A–F, the effects of ACh on the proposed $R_{DMPP}$ in posterior SCC hair cells are illustrated. In Fig. 5, A and B, 1 $\mu$M ACh, a concentration ineffective in generating $\alpha_9nAChR$-mediated responses in frog saccular hair cells (Holt et al. 2001), activated a small, inwardly rectifying current that reversed near −42 mV. Such inward rectification is a common feature of many neuronal nicotinic ACh receptors (Haghighi and Cooper 2000). In three SCC hair cells, the average reversal potential for the current activated by 1 $\mu$M ACh was $−45.9 \pm 2.9$ mV. Similar $I$-$V$ relationships were found when using 10–600 $\mu$M ACh ($E_{rev} = −45.0 \pm 1.3$ mV; $n = 12$) or after plotting the amplitudes of peak ACh-induced current during single voltage step protocols ($E_{rev} = −44.9 \pm 1.7$; $n = 3$). ACh application during depolarizing steps to −10 mV, which worked well for observing peak $\alpha_9nAChR$-mediated responses in saccular and outer hair cells (Holt et al. 2001; Nenov et al. 1996), failed to produce any detectable changes in currents from these posterior SCC hair cells (Fig. 5, A and B). Peak responses in these cells were best observed at more hyperpolarized potentials (−100 to −130 mV; Fig. 5, B and C). Again, in contrast to the $\alpha_9nAChR$-mediated response in saccular hair cells (Fig. 4, B and C), the ACh-activated current in these posterior SCC hair cells during hyperpolarizing steps was inward both at −60 and −130 mV. The peak currents produced by ACh (50–100 $\mu$M) or DMPP (0.3–1 $\mu$M) at −130 mV were quite small, averaging just 80.1 ± 21.6 pA when applied during a voltage step from −60 mV ($n = 15$). The voltage responses to these agonists, however, were on average quite large (18.0 ± 1.2 mV; $n = 34$), ranging from 5.9 to 31.2 mV (Figs. 5, D and E, and 7B). These large depolarizations might be expected given the pre-
predicted large input resistances shown by frog posterior SCC hair cells around their resting potential (Prigioni et al. 1996).

Another striking feature of this depolarizing ACh response in posterior SCC hair cells was its sensitivity to ACh. Voltage responses of a single posterior SCC hair cell to increasing concentrations of ACh are shown in Fig. 5E. Repeatable ACh-induced depolarizations of posterior SCC hair cells could be seen at as little as 10 nM ACh and peaked around 1 μM. The peak response to each concentration was expressed as a percentage of the peak response observed at 10 μM ACh and these normalized data (53 observations) were plotted for eight cells in Fig. 5F. At least three concentrations of ACh were used on each cell. The sigmoid fit was derived using the Hill equation described in the methods section. The predicted EC_{50} (47.9 ± 10.9 nM, h = 0.88 ± 0.16) is significantly lower than the EC_{50} calculated for ACh (approximately 10–20 μM) at 9nAChR in a variety of hair cell preparations (reviewed in Elgoyhen et al. 2001; Holt et al. 2001; Verbitsky et al. 2000).

Note that in Figs. 5, D and E, and 7B, there was an increase in the noise during application of ACh and DMPP. This may represent the opening and closing of membrane channels. As the ionic basis for this particular response has not been characterized, the underlying mechanism for these voltage fluctuations is currently unknown. They may represent the opening and closing of the underlying nicotinic receptor or the activation of some secondary conductance.

**DMPP response does not occur in all posterior SCC hair cells**

The excitatory response, as illustrated in Fig. 5, A–E, was only seen in a subpopulation of posterior SCC hair cells. In a sampling of 104 SCC hair cells, 41 (39.4%) responded to ACh or DMPP with the previously described depolarizing response (see Fig. 7B). Interestingly, cells in this subpopulation all displayed a rounded shape, similar to hair cells previously defined as pear-shaped localized to the intermediate zone of the crista ampullaris (Guth et al. 1994b; Masetto et al. 1994; Prigioni et al. 1996). In Fig. 6A, typical SCC “pear-shaped” hair cells are shown in panels 1–6. Such shapes were not an artifact of the isolation technique as they are routinely observed in cross-sections of the frog posterior canal (outlined and numbered in Fig. 6B; see also Guth et al. 1994b; Masetto et al. 1994). After harvesting from the intermediate region of the crista, isolated hair cells with this shape (average cell capacitance was 10 ± 0.5 pF; n = 34) were selected to isolate the excitatory ACh response. Interestingly, not all hair cells (33 of 74) classified as pear-shaped responded to ACh or DMPP. The
The excitation of multiunit afferent responses is driven by the same receptor that underlies the rapid Pharmacology of the hair cell DMPP-sensitive receptor. In seven of nine resting potential (H11002) shaped hair cells was (H9251) best described as small and round/pear-shaped. In fact, it was established that responsive hair cells could be identified before the application of ACh or DMPP based solely on their morphology and estimates of their resting membrane potential (right). The resting membrane potential of the responsive cells centered near ~70 mV. Scale bar (6) = 10 μm. Examples of this blockade are shown for one cell in Fig. 7A. In a cross-section from the frog crista, these pear- or round-shaped hair cells can be localized to the intermediate region (Masetto et al. 1994; Prigioni et al. 1996). These cells are outlined and numbered. Scale bar = 50 μm. B was kindly provided by Dr. Paola Perin (Department of Cell and Molecular Physiological and Pharmacological Sciences, University of Pavia).

average resting potential for those cells that did respond was -69.3 ± 3.2 mV (n = 41), whereas the average resting membrane potential of the remaining nonresponding pear-shaped hair cells was -46.1 ± 3.1 mV. These cells failed to respond even during injections of hyperpolarizing currents. Therefore SCC “pear-shaped” hair cells with a fairly negative resting potential (~65 to ~70 mV) could be predicted to respond to ACh or DMPP with a depolarizing response.

Pharmacology of the hair cell DMPP-sensitive receptor

Two pieces of evidence suggest that this depolarizing response is driven by the same receptor that underlies the rapid excitation of multiunit afferent firing. One is its blockade by dTC and the other is its sensitivity to DMPP. In seven of nine posterior SCC hair cells, application of 100 μM ACh resulted in the generation of an inwardly rectifying current that was completely antagonized by 1 or 3 μM dTC. The remaining two (of 9) posterior SCC hair cells were partially blocked. An example of this blockade is shown for one cell in Fig. 7A. In contrast to α9nAChR-mediated responses in saccular hair cells, these ACh-induced inward currents were not sensitive to 500 nM strychnine (n = 5; data not shown).

In contrast to responses in the intact epithelium, the depolarizing response in posterior SCC hair cells could be easily activated using low concentrations of both ACh and DMPP. In current clamp recordings (Fig. 7B), the application of 1 μM ACh and 0.1 μM DMPP produced nearly identical voltage responses in the same cell (n = 3). The discrepancy in response duration was simply a product of slightly different drug application times. As in the whole organ preparation, DMPP was the preferred agonist in hair cells because of its presumed selectivity for the fast depolarizing nicotinic ACh receptor over α9nAChR and mAChRs. Given that DMPP and ACh, at these concentrations, are not effective agonists at α9nAChR (Elgoyhen et al. 2001; Erostegui et al. 1994), the similarities between the depolarizing response to DMPP and ACh suggest that ACh is also only activating a single receptor in these cells, namely R_{DMPP}.

Because voltage responses to ACh or DMPP could be relatively large, the current-clamp configuration was also used for measures of pharmacological antagonism. Figure 7C shows voltage responses to 1 μM DMPP in one posterior SCC hair cell with and without antagonists known to block the various vestibular cholinergic responses. The three panels on the left demonstrate that application of 1 μM DMPP, like ACh (see also Fig. 7B), resulted in a robust depolarization. In the three panels on the right side of Fig. 7C, three different cholinergic antagonists (dTC, atropine, and strychnine, respectively) were tested against the voltage response to 1 μM DMPP. Only 3–10 μM dTC blocked the effect of 1 μM DMPP. Similar observations of dTC blockade were made for seven cells. In contrast, these three antagonists are known to block the α9nAChR-mediated response in a number of different preparations (Elgoyhen et al. 2001; Holt et al. 2001; Verbitsky et al. 2000). In fact, most α9nAChR-mediated responses in frog saccular hair cells were completely blocked by 500 nM strychnine (Holt et al. 2001), but this concentration had no effect on the depolarizing response in five SCC hair cells. In parallel with multiunit afferent recordings, the hair cell pharmacology distinguishes the DMPP response from either α9nAChR- or mAChR-mediated responses.

DISCUSSION

R_{DMPP} is pharmacologically distinct from α9nAChR and mAChR

The present evidence supports the notion that R_{DMPP} is different from other hair cell ACh receptors previously described. The points of distinction may be summarized as follows: 1) low concentrations (1–10 μM) of the selective nicotinic agonist DMPP activated an excitatory hair cell ACh receptor in the frog posterior SCC, resulting in a substantial increase in multiunit afferent discharge while having no effect on saccular multiunit afferent firing (despite its robust ACh-induced, α9nAChR-mediated inhibition); 2) R_{DMPP} was almost 400 times more sensitive to ACh (47.9 ± 10.9 nM) and displayed a different I-V relationship than α9nAChRs found in either saccular or auditory hair cells; 3) R_{DMPP} could be activated using concentrations of ACh and DMPP that are considered ineffective at activating α9nAChR; 4) the DMPP response was maintained following application of strychnine, atropine, or PrBCM at concentrations that block α9nAChR- and/or mAChR-mediated responses in the saccule and posterior SCC; and 5) the DMPP response in both multiunit afferent recordings and isolated SCC hair cells was antagonized by relatively
low concentrations (1–10 μM) of dTC, a classic nicotinic antagonist. The sensitivities to DMPP, ACh, and these cholinergic antagonists represent a unique pharmacological profile that discriminates between the present excitatory DMPP and the well-described α9nAChR or mAChR. This pharmacological profile is also consistent with DMPP being a nicotinic receptor.

It is recognized that the DMPP response shares many similarities with the activation of the mechanotransduction (MET) channels in hair cells including an overlap in relative current amplitudes, their inward nature, and susceptibility to blockade by dTC. However, DMPP differs from activation of MET channels in several ways. First, the I-V relationship of DMPP indicates an inward rectification, whereas the I-V curve for activation of MET channels is quite linear, reversing near 0 mV (Corey and Hudspeth 1979). Second, higher concentrations of dTC are needed to completely block MET currents (Glowatzki et al. 1997; Valli et al. 1974). Finally, the most critical distinction is that the activation of DMPP by ACh in isolated SCC hair cells was dose-dependent. It was only observed during the perfusion of ACh or DMPP but not external solution or the various antagonists, thus ruling out a role for mechanical activation in the generation of these responses. Furthermore, the pharmacology of the DMPP-induced excitation of multiunit afferent firing in the frog posterior SCC, which we attribute to DMPP, cannot be accounted for by the activation of MET channels, as the endolymphatic compartment is not accessible to dTC in this preparation. This supports the notion that DMPP and ACh are acting on a distinct ACh receptor.

A nicotinic ACh receptor like DMPP may also underlie the rapid excitatory afferent responses observed in turtle, toadfish, pigeon, chinchilla, and squirrel monkey following direct electrical efferent stimulation (reviewed in Goldberg et al. 2000). Considering that most vestibular efferent fibers are predominantly cholinergic (reviewed in Goldberg et al. 2000; Guth et al. 1998), multiple effects seen with efferent stimulation may indeed reflect a diversity in postsynaptic ACh receptors found on hair cells and afferent fibers. In the frog, DMPP, along with α9nAChR and mAChR, may represent one such example of that diversity. When considering that efferent responses can also be mimicked by application of cholinergic agonists, it is tempting to describe these efferent effects as cholinergic. In most cases, missing is the testing of this assertion by coupling the appropriate pharmacology with direct efferent stimulation. In frog, however, the overwhelming evidence suggests that both the efferent-mediated inhibition and excitation are nicotinic as they are both antagonized by dTC and abolished.
following prolonged exposure to cholinergic agonists including DMPP (Bernard et al. 1985). Taken together, the pharmacology presented in this paper suggests that the efferent-mediated inhibition and excitation, at least in the frog, are mediated by distinct nicotinic ACh receptors.

Localization of the DMPP-sensitive receptor

In afferents localized to the intermediate region of the turtle posterior crista, the predominant response to efferent stimulation is a rapid excitation (Brichta and Goldberg 2000). Although there are no studies in the frog posterior SCC crista that allow for a direct comparison, the DMPP-sensitive response is seen in distinctively shaped hair cells thought to be restricted to the intermediate region (Guth et al. 1994b; Masetto et al. 1994; Prigioni et al. 1996). These hair cells display both the largest BK-type calcium-dependent potassium currents and calcium currents among posterior SCC hair cells but express no inward rectifiers and little voltage-dependent outward currents (Perin et al. 2001; Prigioni et al. 1996). The rectification observed in the ACh-induced current around −45 mV suggest that the range of potentials in which this receptor could exert a physiological role is within the activation range of calcium currents, which activate around −60 mV, reach half-activation around −45 mV and peak around −20 mV (Perin et al. 2001). Given the large input resistance of these cells around −60 mV (Prigioni et al. 1996), the activation of $R_{DMPP}$ could generate a large voltage response (as evidenced here) that would depolarize the cell enough to significantly increase the resting discharge while not saturating afferent transmission. If efferent response properties are similarly arranged in frog as in turtle, the hair cell efferent receptor, which underlies fast excitation, and $R_{DMPP}$ may be the same. This is, once again, supported by the notion that prolonged (i.e., desensitizing) DMPP application reduces efferent excitation and that both are blocked by dTC (Bernard et al. 1985).

In the frog posterior SCC, distinct excitatory and inhibitory efferent effects have been observed, with some afferent fibers displaying biphasic responses (Myers et al. 1997; Sugai et al. 1991; Valli et al. 1986) similar to those observed in some turtle afferents (Brichta and Goldberg 2000). Similarly, when limiting focus to excitatory effects, the slow, muscarinic effect was sometimes observed together with the fast, nicotinic response in single afferents following carbachol application (Bernard et al. 1985). Given a large hair cell-to-afferent convergence, it is possible that multiphasic effects reflect the innervation by a single afferent of multiple hair cells showing contrasting responses to ACh as a product of $R_{DMPP}$, mAChR, and α9nAChR/SK activation. Biphasic responses could also be accomplished with multiple receptor subtypes on a single SCC hair cell, although we saw no evidence for such arrangement in our studies.

Differential synaptic placement

ACh application to the posterior SCC routinely activated mAChR without affecting $R_{DMPP}$ unless concentrations >3 mM were used or acetylcholinesterase was blocked with physostigmine. This is an interesting observation given that $R_{DMPP}$ in solitary hair cells responds to very low concentrations of ACh (EC$_{50}$ = 47.9 ± 10.9 nM). This suggests that $R_{DMPP}$ in the intact SCC may be heavily surrounded by acetylcholinesterase that prohibits exogenously applied ACh from stimulating it. The mAChR may be more sensitive to ACh and/or differentially localized to regions of the epithelium or the efferent synapse such that they are easier to stimulate with exogenous ACh. Differential placement is also suggested by efferent stimulation in the frog in that it appears to only activate the fast excitatory response, although, in these same preparations, a slower mAChR-mediated excitation was activated using cholinergic agonists (Bernard et al. 1985). Analogous to autonomic ganglia and glutamatergic synapses, cartography of the vestibular efferent synapse may be proposed where nicotinic receptors are typically clustered within the synapse and muscarinic receptors are located perisynaptically (Ramcharan and Matthews 1996; Wakade and Wakade 1983). Differences in localization of muscarinic versus nicotinic receptors may account for the difficulty in activating $R_{DMPP}$ with exogenously applied ACh and failure to activate slow excitatory responses using direct electrical stimulation of efferents. It will be of interest to determine under what conditions ACh released from efferent fibers activates these mAChRs.

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DISCLOSURES

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