TETRODOTOXIN-SENSITIVE, VOLTAGE-DEPENDENT SODIUM CURRENTS IN HAIR CELLS FROM THE ALLIGATOR COCHLEA

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ABSTRACT We have used whole-cell patch clamp techniques to record from tall hair cells isolated from the apical half of the alligator cochlea. Some of these cells gave action potentials in response to depolarizing current injections. When the same cells were voltage clamped, large transient inward currents followed by smaller outward currents were seen in response to depolarizing steps. We studied the transient inward current after the outward current had been blocked by external tetraethylammonium (20 mM) or by replacing internal potassium with cesium. It was found to be a sodium current because it was abolished by either replacing external sodium with choline or by external application of tetrodotoxin (100 nM). The sodium current showed voltage-dependent activation and inactivation. Most of the spiking hair cells came from the apex of the cochlea, where they would be subject to low-frequency mechanical stimulation in vivo.

INTRODUCTION

Time- and voltage-dependent ionic conductances of receptor cells have been shown to play an important role in sensory processing. The functional importance of a receptor cell's complement of ion channels has been clearly demonstrated in cochlear hair cells of turtles (1) and has been suggested by recent studies on hair cells of birds (Fuchs, P. A., T. Nagai, and M. G. Evans, manuscript submitted for publication) and frogs (3–5). In these animals a uniquely tuned electrical resonance in the membrane of each hair cell modulates the voltage response to movement of the ciliary bundle. This resonance behavior arises from the combined action of inward calcium current and outward calcium-activated potassium current (4, 6).

We have recently found that hair cells of the alligator cochlea are also electrically tuned, possessing calcium and calcium-activated potassium currents that underlie voltage oscillations similar to those in other electrically tuned cells (Fuchs, P. A., and M. G. Evans, unpublished data). In contrast to all other hair cells studied to date, however, a subpopulation of these cells (all of which were tall or inner hair cells [7]) also possess a sodium current that is voltage-dependent and can be blocked by tetrodotoxin (TTX). This sodium current contributes to the generation of action potentials in these cells and can occur in addition to the electrical tuning mechanism.

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METHODS

Whole-cell tight-seal recordings were made at room temperature (22°–24°C) on hair cells isolated from the apical half of the alligator cochlea. Six alligators, 2-mo-old and 40–50-cm long, were obtained from the Rockefeller Refuge of the Louisiana Department of Wildlife and Fisheries. Animals were decapitated and the skull split along the midline. The cochlea was removed from the otic capsule after cutting through the cartilage and bone surrounding the foramen of the VIIIth nerve. After the overlying tegmentum vasculosum was cut away, the isolated cochlea was exposed to 90 μg/ml subtilisin for 10 min followed by 0.5 mg/ml papain for 20 min. While in papain the tectorial membrane overlying the hair cells was removed and the sensory epithelium divided into sections of 200 μm top to bottom using a sharpened tungsten needle. Hair cells from a single section of the 5-mm-long cochlea were mechanically separated by pulling them off the basilar membrane using a suction pipette (30-μm tip diam). The cells were then gently dispersed into the glass-bottomed recording chamber and transferred to the stage of an inverted microscope (Nikon, Diaphot-TMD) where the experiments were done.

The recording chamber was continuously perfused with oxygenated saline containing (mM): NaCl, 142; KCl, 3.5; $MgCl_2$, 1.1; $CaCl_2$, 2.6; Hepes, 5; NaOH, ~2.5 (pH 7.6), and glucose 8. Monovalent ion substitutions were mole for mole. In Na-free saline NaCl was replaced by choline chloride and NaOH by Tris base. In tetraethylammonium (TEA) saline 20 mM NaCl was replaced by TEA Cl. In Ca-free saline Ca was replaced by 2.6 mM Mg and 1 mM EGTA was added. Ba saline was made by replacing Ca and 14.5 mM Na with 17.1 mM Ba. TTX and $CdCl_2$ were added without ion substitution. The pipette (internal) solution contained (mM): KCl, 112; $MgCl_2$, 2; $CaCl_2$, 0.1; EGTA, 11; Hepes, 10; KOH, ~30 (pH 7.4); K_2ATP, 2. Occasionally a Cs internal solution was used in which KCl was replaced by CsCl and KOH by NaOH. Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Standard patch clamp techniques for whole-cell recording were used (8, 9). Patch pipettes were pulled from borosilicate glass. The tapering region of the pipettes was coated with Sylgard (Dow Corning Corp.,

Midland, MI) to minimize capacitance, and the tips were lightly fire-polished. When filled they had resistances of 2–5 MΩ. The patch clamp amplifier was either an Axopatch 1A (Axon Instruments, Inc., Burlingame, CA) or a Dagan 8900 (DAGAN Corp., Minneapolis, MN). Before recording from a cell its dimensions were measured using an occular micrometer. Once in the whole-cell recording mode the cells were initially voltage clamped close to the resting potential. In most experiments the series resistance was not routinely compensated, but rather calculated from the cell capacitive current (9). Voltages were uncorrected for series resistance (5–30 MΩ) unless otherwise stated. Maximum clamp errors due to the voltage drop across the series resistance are given in the figure legends. Data were recorded on FM tape (3 dB low pass corner frequency 4.4 kHz) and later digitized for analysis with a DEC PDP 11/23+ computer.

Solutions were applied locally around the cell using a system of parallel glass capillaries (10) ~80 μm in diameter placed ~200 μm from the cell. In experiments using TEA (20 mM) to block the outward current, the fastest solution exchange time was found to be ~0.2 s, although some variability, depending on positioning, was found.

Membrane potentials have been corrected for the junction potential between the internal solutions and normal saline which was found to be −4 mV for the K internal solution and −3 mV for the Cs internal solution (measured with respect to a 3 M KCl bath electrode). Therefore an observed membrane potential of −70 mV was in reality −74 or −73 mV depending on which internal solution was used.

RESULTS

We recorded from 37 tall (inner) hair cells (7) under control conditions from the apical half of the alligator cochlea. In current clamp nine of these cells gave action potentials (spikes) in response to depolarizing current injection. These cells were found to have particularly large inactivating inward currents in voltage clamp, and had resting potentials of −64 ± 14 mV (mean ± SD). They ranged in length from 19 to 50 μm (mean 37 μm), excluding the ciliary bundle, and were usually found near the apical tip of the cochlea (seven of the cells were from the apical-most fifth). We report here experiments from these nine spiking cells and seven others in which internal K was replaced with Cs.

Fig. 1 *A* shows a voltage recording from a spiking cell. The cell had a resting potential of −75 mV. The spikes depolarized the cell 50 mV above the plateau voltage of the response to current injection and lasted ~15 ms. Taking data from all the spiking cells we found that the size of the action potentials was 49 ± 11 mV and time to peak was 4.6 ± 1.8 ms (mean ± SD, $n = 9$).

When the cell shown in Fig. 1 *A* was studied under voltage clamp, at a holding potential of −74 mV, depolarizing voltage jumps of >30 mV induced large, rapidly activating inward currents followed by smaller, maintained outward currents (Fig.1 *B*). Smaller jumps induced more slowly activating inward currents and no net outward current. The outward current could be completely blocked by external application of TEA (20 mM) as shown in Fig. 1 *C*. Under these conditions the inward current could be seen alone, and two components (transient and maintained) could be distinguished in response to all voltage jumps shown except the smallest (to −54 mV). For the larger voltage jumps (to −44, −34, and −24 mV), the initial activation of the transient current was very rapid with time to peak decreasing as the step increased (2.58, 1.74, and 1.32 ms for voltage jumps to −44, −34, and −24 mV, respectively). Although the decline of the inward current was complex, the time constant of decay derived from an exponential fit to the initial (most rapid) part of the decay also decreased as the step increased (7.46, 1.98, and 1.33 ms for −44, −34, and −24 mV, respectively).

The small maintained component of the inward current visible in Fig. 1 *C* was not studied in detail. It was most likely a Ca current since in other cells it was increased in the presence of external Ba and was insensitive to TTX (100 nM). The outward current seen in Fig. 1 *B* was probably a Ca-activated K current since in addition to being blocked by TEA it was also blocked by the Ca channel blocker Cd (1 mM), and in other cells it was shown to be dependent on external Ca. This finding also supports the identification of the maintained inward current as a Ca current.

The cell shown in Fig. 1 did not generate voltage oscillations during current injection, a result in common with most of the spiking cells. We also found cells that did show voltage oscillations like those seen in electrically tuned hair cells, but these cells did not usually produce large action potentials. These ringing, nonspiking cells were found in more basal regions, more than 1 mm from the apical tip.

The transient inward current was found to be a Na current since it could be eliminated either by complete removal of external Na or by external application of the Na channel blocker TTX. An experiment examining the effect of Na removal and TTX application is shown in Fig.

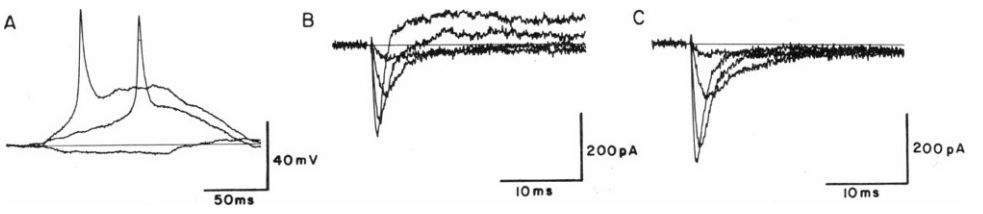

FIGURE 1 Responses of an apical hair cell in current and voltage clamp. (*A*) Current injections of +10, +4, and −4 pA were given for 100 ms while the cell was at its resting potential (−75 mV). Spikes can be seen riding on the plateaus of the depolarizing responses. (*B*) Currents from the same cell under voltage clamp are shown. 100-ms depolarizing voltage jumps (to −54, −44, −34, and −24 mV) were given at 1 Hz from a holding potential of −74 mV. The maintained outward currents were blocked by 20 mM TEA in *C*, same clamp protocol. The largest currents in this figure were subject to clamp errors of ~3 mV. The capacitive transients were removed from the current records after digitization.

2 . In this experiment the outward current was blocked by using the Cs internal solution. Fig. 2 *A* shows the current–voltage (I-V) relation for the peak inward current in this cell. The transient current was first visible at −53 mV and reached a maximum near −20 mV before gradually declining with increased depolarization. Although we did not reverse the current the extrapolated reversal potential was +33 mV, not far from the Na equilibrium potential of +40 mV. (Calculated from the Nernst equation and assuming that the Na concentration inside the cell was equal to that of the Cs internal solution, which was 30 mM.) Also shown is the I-V relation in the presence of 100 nM TTX or Na-free saline. Both these procedures completely eliminated the transient inward current (see Fig. 2, *B* and *C*).

Sodium substitution was complete within a maximum time of 5 s and was quickly reversible. In contrast, the block produced by 100 nM TTX developed more slowly (20 s) and was only partly reversed even after 10 min of wash. The residual inward current seen in the presence of TTX activated quickly and was maintained throughout the step. This current was probably a Ca current as briefly described above. The slower onset of this current in Na-free saline (Fig. 2 *C*) may have resulted from transient outward movement of Na (internal Na was 30 mM), which would obscure the earliest inward current flow.

The voltage dependence of both the time to peak and initial rate of decay of the transient inward (Na) current described above suggested voltage-dependent activation and inactivation kinetics as first described by Hodgkin and Huxley (11) for the Na conductance in the squid giant axon. To examine the effect of membrane potential on the Na current recorded at a fixed potential we used a two-pulse voltage-clamp protocol. In two cells we examined the effect of changing the voltage of the first (conditioning) pulse on the Na current recorded during the second (test) pulse to −24 mV. One of these experiments is shown in Fig. 3 in which we used 20-ms depolarizing prepulses in the presence of 20 mM external TEA. As the conditioning voltage was made more depolarized the Na current measured at −24 mV became smaller, consistent

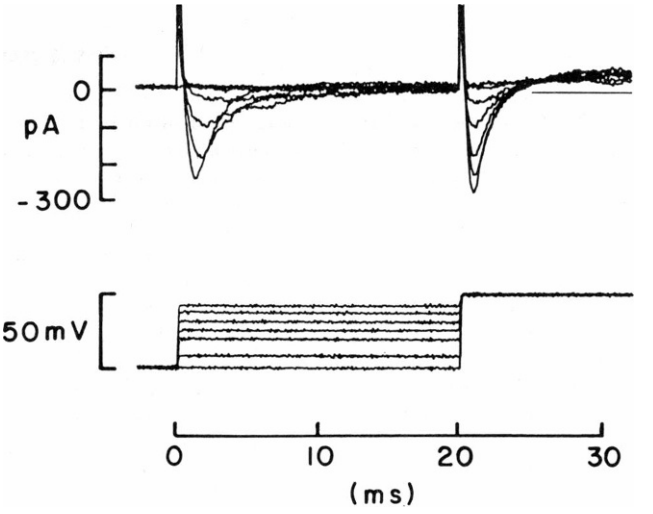

FIGURE 3 Inactivation of the Na current. Current recorded (*top*) in response to a two-pulse voltage-clamp protocol (*bottom*). The cell was held at −74 mV, and 20 ms depolarizing prepulses were given before clamping to −24 mV. As the prepulse depolarizations were increased the resulting inward (Na) current recorded at −24 mV was reduced. Inactivating inward current was also seen at the beginning of the prepulses where it increased with depolarization. The largest currents in this figure were subject to ~2 mV clamp error. The outward current was greatly reduced by 20 mM TEA in the external solution. Capacitive currents are shown truncated. The horizontal line at the right of the current record denotes the zero current level.

with increased steady-state inactivation at depolarized potentials. In the second experiment (not shown), both depolarizing and hyperpolarizing prepulses were given in a cell internally perfused with the Cs solution. As the conditioning voltage was made more negative the Na current at −24 mV markedly increased, consistent with a removal of inactivation. In this experiment the maximum Na current was 2.2 nA (conditioning voltage −210 mV). At voltages of −118, −103, and −71 mV the steady-state inactivation was 22, 50, and 96%, respectively (voltages corrected for the uncompensated series resistance of 10 MΩ). By varying the duration of a conditioning prepulse to −118 mV the time to remove half of the steady-state inactivation was found to be 11.6 ms. These experiments

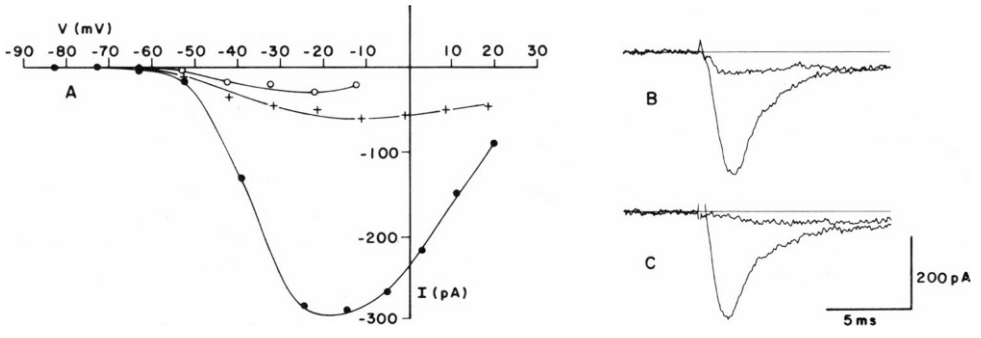

FIGURE 2 (*A*) Current–voltage relation of a cell internally perfused with the Cs solution. Peak inward currents were plotted against voltage-clamp potential, holding potential −73 mV. Currents in the presence of 100 nM TTX (*crosses*) and Na-free saline (*open circles*) are shown as well as the control (*solid circles*). Leakage subtraction and series resistance compensation were performed after digitization. Examples of currents recorded in response to depolarizing voltage steps to −33 mV in the presence of TTX (*B*) and Na-free saline (*C*) are shown superimposed on the corresponding controls. The largest currents in *B* and *C* were subject to ~8-mV clamp error.

indicated that the Na conductance was largely inactivated over the physiological voltage range. Nonetheless it was clear that even the small Na conductance remaining at the resting potential could contribute to excitability in these cells. An understanding of the significance of the very negative inactivation curve must await further investigation.

DISCUSSION

We have demonstrated that a population of tall (inner) hair cells isolated from the apical half of the alligator cochlea have a Na conductance, which has voltage-dependent activation and inactivation and is blocked by 100 nM TTX. The shape of the I-V relation of the Na current is similar to that reported using a variety of voltage clamp techniques in other cells (12–14). The sensitivity of the Na current to low concentrations of TTX indicates a close similarity to Na current in nerve (15) and muscle (16).

In addition to the Na currents described here, tall alligator hair cells possess Ca and Ca-dependent K currents which are similar to those reported for frog (4), turtle (6) and chick (17). Presumably then the repolarization of the action potential in alligator hair cells depends on an interaction between Ca-activated K current and Ca current, and this may account for the relatively long duration of the spikes.

We did not directly examine the effects of TTX or Na-free saline on the action potentials, nevertheless it seems probable that the Na conductance contributes to the upshoot of the action potential found in this population of alligator hair cells. Slow action potentials have been reported previously in hair cells of turtles (6) and frogs (18) and in an analogous population of hair cells from the apex of the morphologically similar chick cochlea (19). The underlying conductance changes were not investigated in detail, but presumably involve the inward Ca currents known to occur in these cells. The finding that action potentials in frog hair cells were insensitive to TTX (18) supports this contention.

Voltage-dependent Na currents have not been found in other hair cells. It is perhaps surprising to find a fundamentally nonlinear excitation mechanism in sensory receptor cells. The functional significance of these currents may be hinted at by our observation that Na currents were found in tall cells from more apical and therefore lower frequency locations, whereas cells that appeared to be electrically tuned (data not shown) were isolated from more basal, higher frequency locations (20). Hair cell action potentials would ensure time-locked afferent activity to low-frequency sounds, and could enhance time-dependent operations such as sound localization. That spiking hair cells have been reported in the vestibular and auditory systems of other species, including the apex of the chick cochlea, could mean that regenerative action potentials may be generally used in low-frequency mechanotransduction.

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