Transmission Between Type II Hair Cells and Bouton Afferents in the Turtle Posterior Crista

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Holt, Joseph C., Jin-Tang Xue, Alan M. Brichta, and Jay M. Goldberg. Transmission between type II hair cells and bouton afferents in the turtle posterior cristal. J Neurophysiol 95: 428–452, 2006. First published September 21, 2005; doi:10.1152/jn.00447.2005. Synaptic activity was recorded with sharp microelectrodes during rest and during 0.3-Hz sinusoidal stimulation from bouton afferents identified by their efferent-mediated inhibitory responses. A glutamate antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) decreased quantal size (qsize) while lowering external Ca²⁺ decreased quantal rate (qrate). Miniature excitatory postsynaptic potentials (mEPSPs) had effective durations (qdur) of 3.5–5 ms. Their timing was consistent with Poisson statistics. Mean qsizes ranged in different units from 0.25 to 0.73 mV and mean rates from 200 to 1,500/s; there was an inverse relation across the afferent population between qrate and qsize. qsize distributions were consistent with the independent release of variable-sized quanta. Channel noise, measured during AMPA-induced depolarizations, was small compared with quantal noise. Excitatory responses were larger than inhibitory responses. Peak rates, which could approach 3,000/s, led peak excitatory mechanical stimulation by 40%. Quantal parameters varied with stimulation phase with qdur and qsize being maximal during inhibitory stimulation. Voltage modulation (vmod) was in phase with qsize and had a peak depolarization of 1.5–3 mV. On average, 80% of vmod was accounted for by quantal activity; the remaining 20% was a nonquantal component that persisted in the absence of quantal activity. The extracellular accumulation of glutamate and K⁺ are potential sources of nonquantal transmission and may provide a basis for the inverse relation between qrate and qsize. Comparison of the phases of synaptic and spike activity suggests that both presynaptic and postsynaptic mechanisms contribute to variations across afferents in the timing of spikes during sinusoidal stimulation.

INTRODUCTION

As was first shown at the neuromuscular junction, neurotransmitter is released from the presynaptic terminals in multimolecular packets or quanta (del Castillo and Katz 1954b). Before release, the quanta are packaged in synaptic vesicles (Heuser and Reese 1973; Katz 1969; Stevens 2003). Quantal transmission is present at conventional synapses, where release is episodically triggered by action potentials (Stevens 2003), and also in sensory cells, including hair cells (Furukawa et al. 1978; Rossi et al. 1994), photoreceptors (Hirasawa et al. 2001; Maple et al. 1994), and retinal bipolar cells (Freed 2000; Tian et al. 1998), where release is continual and modulated by ongoing receptor and/or synaptic potentials. Many continually releasing synapses have a distinctive morphology, with the presynaptic terminal being organized around a synaptic ribbon or body, an electron dense structure to which synaptic vesicles are tethered (Lenzi and von Gersdorff 2001). Despite the functional and morphological differences between synapses involved in episodic and continual release, they resemble one another in many respects, including their molecular machinery (Safieddine and Wenthold 1999; von Kriegstein et al. 1999) and their response to continual depolarization (del Castillo and Katz 1954a; Liley 1956; Neher and Sakaba 2001a,b). A basic tenet of conventional quantal theory is that individual quanta are released independently. Recently, the principle has been questioned, and the proposal has been made that there is a coordinated release of multiple quanta (Edmonds et al. 2004; Glowatzki and Fuchs 2002; Parsons and Sterling 2003; Singer et al. 2004).

Quantal transmission between hair cells and their afferents has been studied in the goldfish sacculus (Furukawa et al. 1978; Starr and Sewell 1991), lateral-line organs (Flock and Russell 1976; Sewell 1990), the mammalian cochlea (Glowatzki and Fuchs 2002), and vestibular organs of frogs (Annoni et al. 1984; Rossi et al. 1994), lizards (Schessel et al. 1991), and fish (Locke et al. 1999). The purpose of this study was to extend the analysis of an in vitro preparation of the turtle posterior cristal. We were attracted to this preparation because of our interest in determining the distinctive roles of type I and type II hair cells in vestibular transduction. Type II hair cells, found in the vestibular organs of all vertebrates, are cylinderically shaped and are innervated by bouton endings derived from several afferent and efferent fibers (Lysakowski 1996; Wersäll 1956; Wersäll and Bagger-Sjöbäck 1974). In many respects, type II hair cells resemble hair cells in nonvestibular organs. Type I hair cells are distinctive. Each is amphoraphaped and innervated by a single afferent terminal in the shape of a calyx ending. Efferent innervation is exclusively postsynaptic onto the calyx ending. These hair cells are peculiar to the vestibular organs of reptiles, birds, and mammals. In reptiles and birds, type I hair cells are confined to a central or striolar region of each end organ, whereas in mammals, they are found throughout the neuroepithelium (for review, see Lysakowski and Goldberg 2004).

An advantage of the turtle posterior cristal, besides the presence of the two kinds of hair cells, is that we know a great deal about its afferents, including their branching patterns (Brichta and Peterson 1994), their responses to rotational stimulation (Brichta and Goldberg 2000a), and their responses to electrical activation of efferent fibers (Brichta and Goldberg 2000b). Intra-axonal labeling studies have identified the distinctive discharge properties of afferents differing in their...
branching patterns, kinds of endings, and zones of the crista they innervate (Brichta and Goldberg 2000a). In addition, the electrophysiology of its hair cells has been studied (Brichta et al. 2002; Goldberg and Brichta 2002). Perhaps the greatest advantage of the preparation is that it provides an opportunity to relate the details at individual stages of the transduction process with the overall results of transduction as judged by the discharge properties of its afferents (Brichta et al. 2002; Goldberg and Brichta 2002). Moreover, afferent discharge is similar in the in vitro preparation and in intact, anesthetized animals (Brichta and Goldberg 2000a).

This study characterizes synaptic transmission between type II hair cells and bouton afferents. Besides being of interest in its own right, the topic provides a context for studies involving type I hair cells and calyx afferents. In this paper, we first develop techniques to identify afferents as calyx or bouton. Next, we show that discharge is not perturbed by the surgical procedures required to record from afferents near their terminal in the neuroepithelium. Third, we study quantal activity recorded from bouton afferents during rest and during sinusoidal mechanical stimulation that simulates angular head rotations (Dickman and Correia 1989; Rabbitt et al. 1995). To characterize quantal activity, we have adapted quantitative methods previously developed in studies of the neuromuscular junction (Fesce et al. 1986; Segal et al. 1985), the frog posterior crista (Rossi et al. 1989, 1994), and central synapses (Neher and Sakaba 2001a, b, 2003).

We concentrated on four issues related to synaptic transmission between type II hair cells and bouton afferents. First, we determined if quantal activity is consistent with a so-called “standard” model, based on shot-noise theory (Rice 1944), in which neurotransmission involves the independent, random release of quanta possibly differing in size. To accomplish this, we compare experimental records with computer simulations incorporating the assumptions of the model. Second, we described variations in quantal size and shape as quantal rate is varied by mechanical stimulation. Third, we investigated whether transmission between hair cells and their afferents includes nonquantal components, as had been previously suggested for transmission involving type I hair cells and calyx endings (Goldberg 1996; Yamashita and Ohmori 1990). Fourth, by comparing the phases of spike and quantal activity, we estimated the presynaptic (hair-cell) and postsynaptic (afferent) contribution to variations in the timing of discharge across the afferent population.

METHODS

Tissue preparation

Red-eared turtles (Trachemys scripta elegans, 100–300 g, 7–14-cm carapace length) of either sex were decapitated, and the head was split parasagitally. The left half-skull and brain were placed in an environment (21–23°C). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Chicago.

In the in vitro preparation and in intact, anesthetized animals (Brichta and Goldberg 2000a).

To expose the posterior ampullary nerve for recording, the left brain stem was blocked transversely between the levels of the trigeminal and glossopharyngeal nerves. The bony channels containing the glossopharyngeal and vagal nerves were opened and the two nerves were removed, exposing the bone directly over the posterior ampulla. A fenestra was made, revealing the ampullary nerve, including the separate branches to the two hemi cristae. Epineurium was removed with a tungsten hook. Glass microelectrodes, filled with 3 M KCl and having typical impedances of 40–80 MΩ, were connected to the head-stage of a negative capacitance preamplifier (Biomedical Engineering, Thornwood, NY) and were advanced into the nerve in 5-μm steps with an inchworm drive (Burleigh, Victor, NY) mounted on a three-axis micromanipulator.

An indenter was used to stimulate the posterior canal. To accommodate the tip of the indenter, a hole was made in the temporal bone, exposing the membranous duct of the posterior canal. The indenter consisted of the fire-polished, flattened end of a glass tube (contact area, 0.1–0.2 mm²) mounted on an aluminum rod, in turn tethered to the center of a trilaminate piezoelectric plate (Model PI-140.10, Polytec PI, Auburn, MA), clamped at its ends so that it bent as a function of the voltage difference between its outer layers. An amplifier (model E-650.00, Polytec PI) controlled the voltage. A linear variable differential transformer (LVDT: Model DC-750-050, Macrosensors, Pennsauken, NJ), mounted inline along the length of the rod, measured the resulting displacement. The entire assembly was advanced with a three-axis micromanipulator. After establishing contact with the posterior canal duct, the indenter was slowly advanced by 150 μm to prevent loss of contact during controlled indenter withdrawal. Maximum displacement was ±100 μm.

Electrical stimulation of efferent fibers was used to classify afferents. All efferent fibers destined for the posterior crista travel in a so-called cross-bridge, the nerve bundle connecting the anterior and posterior branches of the VIIIth nerve (Fayyazuddin et al. 1991). The cross-bridge was exposed by removing a small section of the roof of the mouth, immediately rostral to the bony protuberance housing the lagena. An insulated silver wire with a 0.5-mm chlorided tip was placed on the cross-bridge. A similar chlorided silver wire was placed in the roof of the mouth. Trains of 100-μs constant-current shocks were delivered from a World Precision Instrument 1850A stimulus isolator (Sarasota, FL) to the two electrodes. The cross-bridge electrode was the cathode. Severe the facial nerve prevented muscle contractions during electrical stimulation.

The half-head was mounted in a recording chamber and viewed with a Zeiss Stemi 2000 dissecting microscope (Carl Zeiss Microimaging, Thornwood, NY). The exposed nerve was continually superfused with the oxygenated control solution provided from a gravity-fed, multi-barrel pipette capable of delivering solutions at 3 μl/s from any one of four 10-ml reservoirs.

On-line computer processing

Experiments were controlled by custom Spike2 scripts run on a Pentium IV computer with a Micro1401 interface (Cambridge Electronic Design, Cambridge, UK). The microelectrode signal was low-pass filtered at 1 kHz (4-pole Bessel filter, model 432, Watertek, San Diego, CA) and sampled at 7–10 kHz by a 12-bit A/D converter. Other A/D converters sampled the indenter LVDT monitor and currents delivered to the microelectrode. Indenter displacement was controlled by the output from a 12-bit D/A converter, which was passed through a three-digit attenuator. Timing of electrical shocks to efferent fibers was controlled from a digital-output port.

Solutions

The control solution consisted of (in mM) 105 NaCl, 4 KCl, 0.8 MgCl₂, 2 CaCl₂, 25 NaHCO₃, 2 sodium pyruvate, and 10 glucose. The final pH was 7.2–7.3 after bubbling with 95% O₂-5% CO₂. A low-calcium solution was prepared by reducing Ca²⁺ in the normal control solution from 2 to 0.1 mM, elevating Mg²⁺ from 0.8 to 5 mM, and adding 5 mM EGTA. These modifications reduced free Ca²⁺ to low nanomolar concentrations as calculated by the Webmaxc Standard Program (http://www.stanford.edu/~cpatton/webmaxc/S), while keeping divalent charge screening approximately constant (Hille
A high-potassium solution was prepared from the control solution by elevating KCl from 4 to 10 mM and adjusting the NaCl accordingly. All chemicals and drugs used in this study were obtained from Sigma-Aldrich (St. Louis, MO) except 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), d-2-amino-5-phosphonovalerate (AP5), cyclohexiazide (CTZ), and dl-threo-benzyloxyaspartate (dl-TBOA) (all from Tocris Cookson, Ellisville, MO) and 4,4′-dithiothreitol (DIDS) (Molecular Probes, Eugene, OR). CTZ was stored as a 100 mM stock solution in dimethyl sulfoxide (DMSO). Unless otherwise stated, drugs were added to the control solution.

Physiological testing

Both extracellular and intracellular recordings were made from afferent nerve fibers. In either case, we first studied the response to electrical stimulation of efferent fibers. Trains of 20 shocks, spaced 5 ms apart, were delivered to the cross-bridge. Several trains were presented, and shock amplitude was adjusted to result in a clear response in the absence of antidromic activation. We collected a 5-s sample of background activity followed by the response to 6–12 cycles of 0.3-Hz sinusoidal indentation of the canal duct. To study synaptic activity in isolation, spikes were blocked with TTX (200–500 nM) in the control solution or with the charged membrane-impermeant local anesthetic, QX-314 (40 mM), in the micropipette solution. To reduce hair cell neurotransmitter release, the control solution was replaced with the low-calcium solution. To block quantal activity postsynaptically, the non-N-methyl-d-aspartate (NMDA) blocker, CNQX (3–300 μM), was used by itself or with the NMDA antagonist AP5 (100–300 μM). Glutamate transporters were blocked with TBOA (100–300 μM). The role of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor desensitization was assessed with CTZ (100–300 μM). DIDS (30–300 μM) and the high-potassium solution were used to examine the effects of potassium homeostasis on transmission.

Off-line computer processing

Computer files were transferred to Macintosh computers and processed with custom programs written in Igor Pro (WaveMetrics, Lake Oswego, OR). Statistical tests were done in Microsoft Excel and regressions were calculated in Igor Pro (Wavemetrics, Lake Oswego, OR). Statistical tests were done in Microsoft Excel and regressions were calculated in Igor Pro (Wavemetrics, Lake Oswego, OR). Statistical tests were done in Microsoft Excel and regressions were calculated in Igor Pro (Wavemetrics, Lake Oswego, OR).

Quantal analysis

The following sections describe quantitative methods used to analyze resting activity, which is presumed to be stationary. We then describe how the procedures were modified to handle the nonstationary activity occurring during sinusoidal stimulation.

Quantal shape. A spectral analysis of unstimulated (resting) activity was used to deduce quantal shape. Fast fourier transform (FFT) power spectra were obtained from 2,048-point segments taken from 5-s records. The several power spectra were averaged. A control spectrum, calculated from segments when quantal activity was minimized by inhibitory indentations, was subtracted from the resting spectrum. After logarithmic transformation, the resulting difference spectrum was fit by the logarithmic formula whose linear counterpart is

\[ G(f) = \frac{G(0) \alpha^2}{(2\pi f + \alpha)^2} \]  

The resting and control spectra were well separated at low frequencies but converged above 500 Hz. For this reason, the fit was confined to the bandwidth below 500 Hz. Equation 1 is the power spectrum corresponding to the so-called alpha function

\[ g(t) = (at)^{-1} \exp(-at) \]  

Unfortunately, the parameters of the fit were not particularly useful because they covaried so that a large variation in the rate constant, \( \alpha \), could be compensated for by a large, similarly directed variation in the exponent, \( k \). To avoid this problem, we obtained the best fitting \( \alpha \) after fixing \( k = 2 \).

Quantal shape, determined by spectral methods, was checked by automatically identifying individual quanta and averaging their waveforms. Deconvolution based on optimal (Wiener) filtering was used to detect individual quanta. The method was useful not only in specifying quantal shape but also in describing the timing of quanta and the distribution of their sizes.

Deconvolution. The algorithm was modified from one previously described (Fesce 1990; Rossi et al. 1994). The original record is convolved with the acausal filter (Press et al. 1992), whose transfer function is

\[ I(f) = \frac{1}{G(f)} \frac{S(f)}{S(f) + N(f)} \]  

In calculating the transfer function, the sum, \( S(f) + N(f) \), was set to the power spectrum of the original record, whereas \( S(f) \) was obtained by subtracting the control spectrum from the original spectrum. Although convolution is used, the result is deconvolution because each miniature excitatory postsynaptic potential (mEPSP) is replaced by a symmetric pulse, \( h \times d(t) \), occurring at the start of the mEPSP and having an amplitude equal to the quantal size, \( h \). The width of \( d(t) \) increases as the signal-to-noise ratio declines but is always narrower than the mEPSP shape, \( g(t) \). Because \( d(t) \) is narrower than \( g(t) \), the replacement improves the resolution in determining quantal times and allows the detection and specification of closely spaced mEPSPs. Typically, deconvolution improved the time resolution fivefold.

To detect candidate mEPSPs in the deconvolved record, we averaged the uninterrupted declines to either side of each maximum. A mEPSP was considered present when the average decline exceeded a threshold. For most purposes, thresholds were set so that the procedure, when applied to extracellular control waves, resulted in a detection frequency 4–6% of that for the corresponding intracellular record. A higher threshold was used in determining mEPSP shape; here, the threshold was set to detect 50–100 mEPSPs from the 5-s rest period.

Quantal size and rate. These parameters were estimated from shot-noise theory, specifically from the extension by Rice (1944) of Campbell’s theorem, which describes the relation between the \( nth \)
Semi-invariant (cumulant) of the record ($\lambda_n$), quantal rate ($\xi$), and quantal size ($h$) as

$$\lambda_n = \xi \langle h^n \rangle \int_0^\infty g'(t) dt$$  (4)

g(t) is the time-course of an event of unity amplitude and $\langle h^n \rangle$ is the mean value of $h^n$. The first three cumulants are the corresponding central moments ($\mu_1, \mu_2, \mu_3$): the mean, variance, and skew. Higher cumulants are given by Cramér (1961) and include the fourth cumulant, $\lambda_4 = \mu_4 - 3 \mu_2^2$.

The formulas for adjacent cumulants can be used to estimate $\xi$ and $h$. Ignoring variability in $h$, so that $\langle h^n \rangle \approx \langle h \rangle^n = h^n$, we have the following approximations

$$\dot{h} = \frac{\lambda_{n+1} I_n}{\lambda_{n+1}}$$  (5)

and

$$\dot{\xi} = \frac{\lambda_n}{h^3 I_n} = \frac{\lambda_{n+1} I_{n+1}}{\lambda_{n+1} I_n}$$  (6)

where the integral

$$I_n = \int_0^\infty g'(t) dt$$  (7)

Here, the superscripts are exponents and the subscripts are indexes. In evaluating the integrals, $I_n$, the original function, $g(t)$, is normalized to unity amplitude before being raised to the $n$th power. When using high-pass filtering, the normalization is done before the filtering and it is the filtered version of $g(t)$ that is substituted into Eq. 7.

$h$ and $\xi$ have been estimated from the mean and variance ($n = 1$ in Eqs. 5 and 6) (Ashmore and Copenhagen 1983; Freed 2000), but there are advantages in using the variance and skew ($n = 2$) (Fesce et al. 1986; Neher and Sakaba 2001b; Segal et al. 1985). The most obvious of these is that high-pass filtered records can be used, which eliminates the effects of fluctuations in resting membrane potential. In our case, the values of $\mu_2 = \lambda_2$ and $\mu_3 = \lambda_3$ were measured in records that were first convolved off-line with a first-order high-pass digital filter having a corner frequency of 159 Hz (1,000 rad/s).

In obtaining Eqs. 5 and 6, we have used the approximation, $\langle h^n \rangle = \langle h \rangle^n$, which is strictly true only if there is no variation in $h$. To continue using $\langle h^n \rangle$, we need correction factors, $D_n = \langle h^n \rangle / \langle h \rangle^n$, so that $\langle h^n \rangle = D_n \langle h \rangle^n$. $D_n$ can be evaluated by expanding the expected value of the expansion, $(h - \langle h \rangle)^n$. Actual values are related to the approximations of Eqs. 5 and 6 ($n = 2$) by

$$\langle h \rangle = \frac{D_2 \dot{h}}{D_1}$$  (8)

and

$$\dot{\xi} = \frac{D_1^2 \ddot{\xi}}{D_1}$$  (9)

Any two adjacent cumulants can be used in the same way. A difficulty in the use of higher cumulants is the unreliability of their statistical estimates. There are, nevertheless, advantages in using $\lambda_3$ and $\lambda_4$ (Fesce et al. 1986; Neher and Sakaba 2001b, 2003). One advantage is in separating quantal and channel noise with the latter arising from the opening and closing of individual channels. Channel noise results in Gaussian-distributed records (Neher and Sakaba 2001b), in which case it will contribute to $\lambda_2$ but not to $\lambda_1$ and $\lambda_3$. The latter two cumulants can be used to estimate the quantal contribution to the variance

$$\lambda_{2v} = \frac{\lambda_2}{\lambda_2 - \lambda_{2,nc}}$$  (10)

so the difference, $\lambda_{2,nc} = \lambda_2 - \lambda_{2,v}$, is an estimate of the channel-noise contribution.

Higher cumulants can also be used to estimate the variability in quantal size (Fesce et al. 1986), provided that $h$ has a gamma probability density

$$p(h) = \frac{h^{k-1} \exp(-h/\lambda_2)}{\langle h \rangle^k}$$  (11)

with a mean, $\langle h \rangle$, and a coefficient of variation $cv = \langle h \rangle^k$. Correction factors for this distribution are $D_2 = (k + 1)/(k + 2), D_3 = (k + 1)/(k + 2)/(k + 3)$, and $D_4 = (k + 1)(k + 2)/(k + 3)^2$. The dimensionless ratio

$$r = \frac{(\lambda_4 I_4)^2}{(\lambda_2 I_2)(\lambda_3 I_3)}$$

$$= \frac{D_4^2}{D_2 D_3}$$

$$= \frac{k + 2}{k + 3}$$  (12)

from which the $cv$ can be calculated from $k = (3r - 2)/(1 - r)$. Notice that $r$ is restricted to values between 0.75 ($k = cv = 1$) and 1 ($k \to \infty, cv = 0$).

NONQUANTAL NOISE. As measured from records, $\lambda_2$ contains three components: one attributable to quantal activity, another to channel noise, and the third of instrumental origin. It is necessary to eliminate the last two contributions. To do this, we exploited Eq. 5, in which there is a linear relation between $\lambda_1$ and $\lambda_3$, provided that quantal size ($\dot{h}$) and the shape integrals ($I_2$ and $I_3$) remain constant. Furthermore, of the various sources of variance, only quantal activity should produce a positive skew. We identified as the residual variance ($\sigma_{res}^2$) the value of $\lambda_2$ remaining after the relation between $\lambda_1$ and $\lambda_3$ was linearly extrapolated to zero skew. The skew–variance relation was obtained from high-pass records during sinusoidal indenter stimulation. Because of the possibility of nonlinearities, we only used points in the relation that approached zero skew, in particular the point of minimal variance and three points to either side of it.

Instrumental noise is a major contributor to $\sigma_{res}^2$. Channel noise could also contribute because of the possible persistence of neurotransmitter in the absence of quantal activity. However, unlike instrumental noise, which should remain constant, channel noise ($\sigma_C^2$) will increase in parallel with mean synaptic voltage ($V$). A simple binomial model of receptor activation gives

$$\sigma_C^2 = \Delta V \cdot \bar{V} - \bar{V}^2 ln$$  (13)

where $\Delta V$ is the voltage resulting from the opening of individual channels and $n$ is the number of channels (Sigworth 1980). Provided that $\Delta V$ remains constant, the formula should hold even when much of the neurotransmitter is contributed by quantal activity (Neher and Sakaba 2001b).

We used two methods to estimate $\sigma_C^2$. First, we computed the variance based on the third and fourth cumulants (Eq. 10). Second, we applied Eq. 13 to records in which bath application of the

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glutamate agonist, AMPA, introduced its own depolarization in the absence of quantal activity. Only the first term in Eq. 13 was needed to estimate $\sigma^2_c$.

**QUANTAL CONTRIBUTION TO MEMBRANE DEPOLARIZATION.** There is another advantage in using high-pass filtered records besides the elimination of low-frequency voltage fluctuations. In particular, the depolarization contributed by quantal activity can be estimated. Once $\xi$ and $<h>$ have been obtained from the filtered record and quantal shape ($I_q$) from a spectral analysis of the unfiltered record, the expected depolarization can be calculated from the product, $\xi(h)I_q$ (Eq. 4, $n = 1$). The predicted value can be compared with the actual depolarization measured from the unfiltered (low-pass) record. This provides a way of evaluating the possible contribution of nonquantal transmission. Such evaluations were best done on stimulated activity where we could compare predicted and actual periodic voltage modulations.

**ANALYSIS OF STIMULATED ACTIVITY.** To analyze the nonstationary activity occurring during 0.3-Hz sinusoidal stimulation, we divided each sine-wave cycle into 24 segments, each occupying 15°. To eliminate transient effects, the first cycle was excluded. A power spectrum was obtained for each segment and the spectra from corresponding segments in successive cycles were averaged. In a similar way, averaged central moments, $\mu_2$ and $\mu_n$, were obtained from the high-pass record. Spectra and central moments were plotted versus stimulus phase. The phase relations were smoothed by averaging each point and the three points to either side of it. Difference spectra, obtained by subtracting the smoothed spectrum having minimal power from the spectra for other individual segments, were fit by Eq. 1. Fits were only obtained for segments whose spectra before subtraction had cumulative power more than twice that of the minimal-power spectrum. From the spectral fits, we calculated the corresponding alpha functions (Eq. 2) and the integrals, $I_1$, $I_2$, and $I_3$ (Eq. 7). Integrals were fit with single-cycle sine-wave functions, and the fits were used to estimate the values for segments not meeting the minimal-power criterion. $\lambda_2$ (after subtraction of the residual variance) and $\lambda_3$ were used to calculate quantal size (Eq. 5) and quantal rate (Eq. 6).

**SIMULATIONS.** Monte Carlo simulations were used to study how various factors influence the estimation of quantal parameters. The pseudorandom number generator, $\sigma_{noise}$, provided by Igor Pro, produced a set of numbers uniformly distributed between 0 and 1, which were used in conjunction with the cumulative Poisson distribution function to determine the number of events in each of several bins, $\Delta t = 0.144$ ms. For each event, another iteration of the same number generator was used with a cumulative size distribution to determine quantal size. The simulation was convolved with an mEPSP alpha function of unity amplitude. Residual noise was simulated by placing in each bin an impulse whose height was determined by Igor Pro’s Gaussian pseudorandom number, $\sigma_{noise}$. The Gaussian distributed impulses were convolved with an alpha function whose parameters were chosen to simulate the desired power spectrum.

**RESULTS**

**Classification of units**

It was important to distinguish bouton (B) from calyx-bearing (CD) units. We explored two ways of identifying the two unit classes. First, as was established previously (Brichta and Goldberg 2000b) and confirmed here, B units near the torus (T) and those in midportions (M) of the hemicrista, collectively referred to as BT/BM units, differed qualitatively in their efferent responses from CD units. Efferent stimulation resulted in a reduction in discharge rate of presumed BT/BM units and an increase in discharge rate of presumed CD units. Second, the two classes of units differed in their responses to 0.3-Hz sinusoidal rotations (Brichta and Goldberg 2000a) and in their responses to canal-duct indentations.

We first consider efferent responses. These were evoked by trains of 20 shocks presented at 200/s. During intracellular recordings, we observed underlying synaptic events, as well as spike responses. Synaptic events, which had not been previously described, were particularly important in recognizing the many CD and the fewer BT/BM units that were silent at rest. In BT/BM units, spikes are inhibited by efferent stimulation and this is associated with a cessation of background afferent mEPSPs (Fig. 1A). In contrast, CD units show an efferent-evoked excitation, including a postsynaptic efferent EPSP and no cessation of afferent mEPSPs (Fig. 1B). One other class of units was encountered. These units had a regular pattern of background spikes ($cv_s < 0.4$), usually occurring at a resting rate of 10–30 spikes/s. Based on our previous labeling studies (Brichta and Goldberg 2000a), these are bouton (B) units located near the planum of the hemicrista. Presumably because of their thin axons (Brichta and Goldberg 2000a), BP units were difficult to impale. For this reason, they were included in our surveys of extracellular response properties but not in intracellular studies of synaptic transmission.

We now consider afferent responses to indenter stimulation. Units were first sorted into B and CD categories based on their efferent responses. The sample consisted of 83 extracellularly recorded units (50 B and 33 CD) obtained in three prepara-
tions. As had previously been found with head rotations (Brichta and Goldberg 2000a), the gains of B units are related by a power-law to the normalized coefficient of variation, $cv^*$, of spike discharge, while the phases are related by a semi-logarithmic relation to $cv^*$. Again confirming results with rotations, CD units behaved differently: they were irregularly discharging and their gains and phase leads were distinctly lower than those of B units with the same $cv^*$. Not only were the rotational and indenter data qualitatively similar, they were in quantitative agreement. The power-law for B units, $gain = a \times cv^*^b$, has an exponent that is statistically indistinguishable from the previously published value. A comparison between leading coefficients is not possible because of the different modes of stimulus delivery, indentation in this study, and rotation previously. No such limitation exists for the semi-logarithmic relation, phase = $a + b \log(cv^*)$. Once again, present and past results are not significantly different. The relative gains and absolute phases of B and CD units matched in $cv^*$ are also statistically indistinguishable from past data.

Because of the relation between afferent and efferent responses, either could be used to classify units. Afferent responses had the disadvantage that, because of the variability in indenter effectiveness between animals, many units had to be collected from each preparation before gains could be normalized and used to classify units. In contrast, a classification based on efferent responses could be done on a unit-by-unit basis and made this the preferred method.

An isolated labyrinth preparation was used in this and in past studies. Previously, no openings were made in the bony labyrinth, whereas here the posterior ampullary nerve and the base

![FIG. 2. Spike and synaptic activity. Intracellular recordings from an axon during a rest period followed by stimulation with 0.3-Hz sinusoidal canal-duct indentations. A: indenter displacement as measured by an in-line monitor. Inward deflections inhibit and outward deflections excite. B: spike discharge during rest and for 2 cycles of indentation. C: TTX blocks spikes revealing high-frequency synaptic activity riding on a sinusoidal modulation of the membrane potential. D: to isolate the high-frequency synaptic activity, the record in C is high-pass filtered off-line. E: expanded records drawn from C show synaptic activity during excitation, rest and inhibition. Individual mEPSPs are clearly seen during inhibition. As quantal rate increases during rest and excitation, mEPSPs overlap, but occasionally distinct mEPSPs can be discerned (arrows).](http://jn.physiology.org/doi/10.1152/jn.00110.2005)
of the posterior crista were exposed and superfused. Previous recordings were in the inferior division of the VIIIth nerve near Scarpa’s ganglion, whereas these recordings were made in the ampullary nerve at the base of the crista, =0.25 mm from the neuroepithelium. The similarity in extracellular results indicates that the more invasive exposure and the different recording locus did not greatly alter transduction machinery. Brichta and Goldberg (2000a) showed that the discharge properties of the in vitro preparation were similar to those obtained in a conventional in vivo preparation. This similarity can be extended to the current preparation.

Quantal nature of synaptic activity

Figure 2 includes a set of typical records. There was an unstimulated (rest) period followed by sinusoidal indentation at a frequency of 0.3 Hz. Inward displacements, by compressing the canal duct, move the cupula toward the utriculus and thereby inhibit; outward displacements have the opposite effect (Fig. 2A). Immediately after impalement, the resting membrane potential was −49 mV, and spikes, rather than overshooting, only reached −9 mV. Spike rate ranged from 28/s (rest) to zero (inhibition) to 64/s (excitation) (Fig. 2B). As the impalement continued, the resting potential grew more negative, eventually reaching −55 mV. As will be considered later, part of the hyperpolarization was associated with the application of TTX, which was used to block spikes. This allowed us to observe underlying events, including periodic modulations in the rate of high-frequency events as in the underlying voltage (Fig. 2C). Both modulations were synchronized to the indenter stimulus. High-pass filtering of the record (Fig. 2D) eliminated the voltage modulation, leaving the high-frequency events.

That the high-frequency events represent quantal activity is best seen when quantal rate is reduced during inhibitory indentations (Fig. 2E, bottom). Under those conditions, individual mEPSPs can be discerned. At higher quantal rates, such as occur during rest (Fig. 2E, middle) or during excitation (Fig. 2E, top), quantal events overlap. Occasionally, however, they are sufficiently distinct (Fig. 2E, top and middle, arrows) that their resemblance to isolated quanta can be recognized.

Consistent with the quantal nature of this activity, it is decreased by lowering external Ca²⁺. Figure 3A shows the effects on high-pass records of replacing our control solution (Fig. 3A1) with a low-Ca²⁺ solution containing EGTA (Fig. 3A2). The variances during rest and during peak excitation were substantially reduced. Both effects were reversible (Fig. 3A3). In other units (data not shown), the EGTA solution buffered to our control Ca²⁺ and Mg²⁺ concentrations had no discernible effect. Quantal activity was also reduced by the non-NMDA, ionotropic glutamate receptor antagonist, CNQX (Fig. 3B); effects were obtained at concentrations as low as 3 μM and were reversible. The NMDA blocker, AP5, at concentrations of up to 300 μM, was ineffective. In a later section, we will show that lowering Ca²⁺ reduces quantal rate, whereas CNQX reduces quantal size.

Quantal analysis of resting activity

A complete description of steady-state quantal activity requires the specification of quantal shape, the timing of individual quanta, quantal size, and quantal rate. These are described here, as is an unanticipated inverse relation between the last two variables. Because of its impact on the shot-noise estimates of quantal size and rate, we also consider the variability of quantal size. Unless otherwise stated, results are based on a detailed analysis of 16 BT/BM units. Results for these 16 units are summarized in Supplemental Table S11, available at the Journal of Neurophysiology web site.

QUANTAL SHAPE. Spectral methods were used to estimate mEPSP shape. An FFT power spectrum was calculated from the low-pass intracellular record (Fig. 4A). The corresponding power spectrum during maximal inhibition served as a control for residual noise. A difference spectrum was calculated and fit by Eq. 1, both with the exponent free and with it fixed to k = 2 (Fig. 4B). As was typical, the fit obtained with k fixed was almost as good as that when k was allowed to vary. For the power spectrum of Fig. 4B, the best fitting rate constant with k = 2 was 901 ± 26/s.

1 The Supplementary Material for this article (four tables) is available online at http://jn.physiology.org/cgi/content/full/00447.2005/DC1.
Deconvolution was used as a second method to estimate mEPSP shape. Several events in the unfiltered record were detected because their size in the Wiener-filtered record exceeded a threshold (Fig. 4C). There was good agreement between the spectral and deconvolution estimates (Fig. 4D).

In the population of 16 units, there was some variation in mEPSP shape. This is shown in Fig. 5A, which includes alpha functions \((k = 2)\) based on spectral analyses for three units characterized by fast, intermediate, and slow mEPSPs. The rate constants were 1,018 ± 7.3 (fast), 770 ± 3.3 (intermediate),
and 591 ± 6.8/s (slow). Over the population of 16 units, there was a close agreement between the spectral and deconvolution estimates of the rate constants (Fig. 5B). Mean values were 745 ± 128/s (SD; spectral) and 756 ± 181/s (deconvolution).

TIMING OF QUANTAL RELEASE. An assumption of quantal theory is that individual quanta are released independently. For continual transmission, this implies that release obeys Poisson statistics. Such statistics are to be expected for the following reason. Our afferents have many bouton endings that can be assumed to be involved in quantal transmission (Brichta and Goldberg 2000a). As a result, the quantal traffic may be likened to the superposition of several independent renewal processes.

In such a circumstance, the summed release statistics will approximate a Poisson process regardless of the release statistics at each active site (Cox 1962).

In short, we expected that quantal release would obey Poisson statistics but felt that this should be verified. A demonstration of Poisson statistics requires that two conclusions be established: 1) waiting time (interval) statistics should be exponentially distributed and 2) successive intervals should be statistically independent. Individual quanta were detected in deconvoluted records. An interval histogram obtained during rest is seen in Fig. 6A. Except for the absence of short intervals, the distribution is close to exponential. Furthermore, adjacent intervals are statistically independent; the serial correlation coefficient had a statistically insignificant value of 0.010 ± 0.028.

The lack of short intervals is a detection problem. In the unit shown in Fig. 6, the half-width ($t_{1/2}$) of the mEPSP was 3.6 ms compared with a $t_{1/2} = 0.50$ ms for its deconvolution counterpart. Two waves closer than $t_{1/2}$ will yield a summed response with a single peak and will be recognized by our detection program as a single event. We expect a shortage of intervals <1 ms, and this is what we observed. To confirm the interpretation, we deconvolved a simulation matched to the experimental record. When the detection algorithm was applied to the simulated record, the interval histogram was nearly exponential except for the first 1-ms bin (Fig. 6B, Uncorrected). In contrast, when the intervals were obtained directly from the simulation program, the discrepancy disappeared (Fig. 6B, Corrected).

Although not an essential feature of quantal theory, it would be a simplification if quantal size were independent of the interval from the preceding mEPSP. This seems to be the case for intervals >1 ms. For shorter intervals, there is an ≈25% reduction in size compared with the values for long intervals. As was confirmed by simulations, the size decrease is a technicality because of the interaction between overlapping standard pulses.

The timing of quantal events was studied in all 16 units. Interval histograms had exponential tails extending down to the first or first and second 1-ms bins. The units in which the deviation was confined to the first bin had narrower delta functions (mean $t_{1/2} = 0.54 ± 0.020$ ms, $n = 5$) than did units where there were fewer than expected entries also in the second bin ($t_{1/2} = 0.94 ± 0.040$ ms, $n = 11$). On average, the Wiener-delta half-width of 0.81 ± 0.056 ms was 4.7 times smaller than the mEPSP half-width of 3.78 ± 0.20 ms. Serial correlations were small and positive, averaging 0.050 ± 0.010, and were statistically significant ($P < 0.05$) in only 5/16 units. Such positive correlations reflect variability in quantal rate over the 5-s sampling interval as was confirmed by calculating serial correlograms (Fig. 6C). Typically, correlation coefficients remained positive for separations of 100 intervals or more. Finally, quantal size was unrelated to interval for all intervals (3 units), for intervals down to 1 ms (7 units), and for intervals down to 2 ms (6 units).

RELATION BETWEEN QUANTAL RATE AND QUANTAL SIZE. Quantal size (qsize, h) and quantal rate (qrate, $\xi$) during rest were calculated from Eqs. 5 and 6. qsize ranged from 0.25 to 0.73 mV; corresponding values for qrate were 180–1,540/s. There was an inverse relation between the two variables, which was well fit by a power law with an exponent, $-2.18 ± 0.31$ ($P < 0.001$; Fig. 7A).

Estimates of the two variables are not statistically independent. Rather, from Eq. 6 ($n = 2$), $\xi$ is inversely proportional to
Because of the lack of independence, the regression between the two variables cannot be taken at face value. To evaluate the influence of random variations in the two estimates, we repeated 20 simulations with identical input parameters. We also studied the covariation between quantal size and quantal rate during repeated trials from six individual units in each of which five or more repeats were available. An example is seen in Fig. 7B (x). In both the simulated and empirical data, the relations were less steep than seen in Fig. 7A. More importantly and in both cases, the random variations in qrate and in qsize estimates were considerably smaller than the variations across the unit sample.

Another potential source of the relation has to do with quantal-size variability. This will be considered in the next section.

QUANTAL-SIZE DISTRIBUTIONS. It has been suggested that neurotransmitter is released from ribbon synapses as the coordinated exocytosis of multiple quanta (Glowatzki and Fuchs 2002; Parsons and Sterling 2003). We studied the proposition by comparing empirical qsize distributions, obtained from deconvolved samples of resting activity, with simulations where quantal release was assumed to be independent and governed by Poisson statistics. Two other considerations motivated the analysis. The shot-noise equations used to estimate quantal parameters require corrections for qsize and qrate that depend on qsize variability (Eqs. 8 and 9). Second, were the qsize cv to vary from unit to unit, this could contribute to the inverse relation between qsize and qrate described in the preceding section (Fig. 7A).

We compared empirical and simulated size distributions rather than directly using empirical distributions, because the latter can be misleading because of the presence of residual noise and of closely spaced, yet undetected, events. Figure 8, A–C, compares an empirical qsize distribution with those from simulations. In the simulations, gamma distributions were assumed for qsize variability. To match empirical and shot-noise calculations, qsizes and qrates were modified in the simulations according to Eqs. 8 and 9 as cv was varied. The best fit of the empirical size histogram, obtained with cv = 0.5, was quite good (Fig. 8B). For smaller assumed cv’s (Fig. 8A), the simulated size histograms were too compact and not sufficiently skewed. For cv’s above the optimum (Fig. 8C), the theoretical distributions were too skewed with an overabundance of small sizes just above the detection threshold.

In all 16 units, simulations were run with cv varying between 0 and 1 in steps of 0.1. Goodness of fits was measured by the mean squared error, expressed as a percentage of mean squared values of the empirical distribution (Fig. 8D). Best fits were obtained in different units at cv values ranging from 0.1 to 0.8 with a mean of 0.38 ± 0.05 (SE). Despite the spread of optimal fits, good fits were obtained at cv = 0.4 in 15 of the 16 units. Optimal fits were quite good, with the residual mean-square error averaging 6.4 ± 1.4%. The excellence of the fits implies that release can be described by Poisson statistics accompanied by variability in qsize. There is no need to invoke cooperative release of multiple quanta.

Some of the variations in qsize could reflect the relative locations of various release sites on intraepithelial branches relative to the recording locus. Smaller mEPSPs might be expected to arise at more remote sites, in which case their rising and falling phases could be slowed by cable properties. To examine this possibility, we sorted mEPSPs detected by variability in quantal size. There is no need to invoke cooperative release of multiple quanta.

Adopting a cv = 0.4 as typical requires substantial corrections of shot-noise calculations. qsize estimates should be multiplied by 0.76 and qrate estimates by 1.50. The oppositely directed corrections in qsize and qrate tend to cancel in the calculation for quantal voltage, whose correction is the product of the other two corrections. We corrected qrates and qsizes for each unit using the correction factor appropriate for the cv giving an optimal fit. Mean values of the corrected values followed by the uncorrected values are 0.35 ± 0.029 versus

FIG. 7. An inverse relation between quantal size and quantal rate. A: quantal rate (qrate) vs. quantal size (qsize) for 16 units. There is a power-law relation between the 2 variables. B: inverse relation cannot be explained by random variability in the estimates of the 2 variables. This is shown for 20 simulations and for 9 repetitions from an axon. C: for each of the 16 units in A, variability of qsize was estimated by comparing empirical and simulated size distributions (Fig. 8). From estimated qsize cv’s, shot-noise estimates of qsize and qrate were corrected. This weakened, but did not eliminate, the inverse relation between qrate and qsize.
0.45 ± 0.032 mV for qsize and 810 ± 150 versus 540 ± 100/s for qrate.

A change in qsize cv is associated with oppositely directed changes in the shot-noise estimates of qsize and qrate. Holding the true mean values constant, an increase in qsize cv will lead to a decrease in the shot-noise estimate of qsize and an increase in the shot-noise estimate of qrate, changes which could contribute surreptitiously to the negative relation between qrate and qsize. To evaluate this possibility, we replotted the power-law relation of Fig. 7 after correcting qsize and qrate unit-by-unit (Fig. 7C). The exponent was reduced from 2.18 to 1.34. From the reduction, which was significant (2-tailed t-test, P ≤ 0.05), it would appear that a variation in qsize cv did contribute to the relation. At the same time, this could not be the only contributor as the relation remained highly significant (same test, P ≤ 0.001).

**Higher Cumulants and the Estimation of Quantal Size Variations and Channel Noise.** We attempted to use Eq. 12, based on shot-noise theory, to provide a second estimate of qsize variability. The attempt was unsuccessful because the calculated values of r, the dimensionless ratio in the equation, fell outside the theoretically expected bounds of 0.75–1. In many (11/16) units, r was <0.75 and, in the entire sample of 16 units, averaged 0.66 ± 0.066 compared with a value of 0.90 ± 0.016 predicted from the qsize variability described in the preceding section. It is unlikely that the choice of gamma distributions is to blame for the discrepancy because they provide such good fits to empirical qsize distributions. Simulations pointed to nonstationary qrates as a more likely culprit. This is shown in Fig. 9A, which shows two simulated voltage distributions: one from a concatenation of simulations with different qrates and the other from a single simulation at the same average qrate. The concatenated simulation has a more peaked voltage distribution and a larger value of kurtosis (λ_4); values of the variance (λ_2) and skew (λ_3) are virtually identical for the two simulations.

One way of reducing the influence of qrate variations is to segment the records. When this was done, λ_4 estimates were reduced but their statistical variability became too large for them to be useful. As a result, we adopted an alternative, three-step approach. 1) We first determined how much the empirical estimate of λ_4 had to be reduced so that the qsize cv deduced from Eq. 12 matched the deconvolution estimate. 2) Simulations were used to determine the qrate variability that would justify the desired λ_4 reduction. 3) Finally, we attempted to measure actual qrate variability. To obtain the desired reduction of λ_4 for each unit, we substituted experimental values of λ_2 and λ_3 into Eq. 12 and used the value of the dimensionless ratio, r, based on the deconvolution estimate of qsize variability. Averaged over the 16 units, the desired λ_4...
was consistent with a qrate, the variance associated with each cumulant. Of the various expectation that such a variation should result in an increase in filtered records can be used. Unlike the variance of the skew ($\mu_3$), larger in the experimental records, presumably because of nonstationary qrates. Straight lines in B and C are best-fitting linear regressions passing through the origin; slopes are $1.01 \pm 0.018$ (B) and $1.44 \pm 0.100$ (C); dashed line in C, unity slope.

To estimate actual qrate variability, we made use of the expectation that such a variation should result in an increase in the variance associated with each cumulant. Of the various cumulants, $\lambda_3$ has obvious advantages. Unlike $\lambda_1$, high-pass filtered records can be used. Unlike $\lambda_2$, it should not be affected by channel noise (Neher and Sakaba 2001b, 2003). And unlike $\lambda_4$, its mean value is insensitive to qrate variations (Fig. 9A). We matched experimental and simulated records based on experimental shot-noise estimates of qsize and qrate corrected for qsize variations (Eqs. 8 and 9). In the simulation for each unit, qrate was held constant. For each of the 16 units, the simulated and experimental high-pass records were each divided into 200-ms segments and the mean values and SD of $\lambda_3$, calculated. qrate variations should lead to increases in the experimental, compared with the simulated, SD. The expected increase was observed. As seen in Fig. 9B, there was a good match between the experimental and simulated mean values of $\lambda_3$. In contrast, a regression between the two SD values gave a slope of $1.44 \pm 0.10$ (Fig. 9C), which simulations indicated was consistent with a qrate $cv$ of $0.47 \pm 0.053$, statistically indistinguishable from the value needed to explain the overestimate of $\lambda_3$.

We conclude that shot-noise estimates of qsize variability are consistent with those obtained from deconvolution once correction is made for qrate variability. The results also have a bearing on estimates of channel noise. The overestimation of $\lambda_4$ caused by qrate variation will deflate $\lambda_{2,c}$ (Eq. 10) and inflate $\lambda_{2,c}$. Using the uncorrected values of $\lambda_4$ provided an estimate of $27 \pm 6.7\%$ as the average channel-noise contribution to the high-pass variance. In a later section, an independent method provided an estimate of $2.2\%$ for the channel-noise contribution. The two estimates of channel noise can be brought into agreement were the empirical value of $\lambda_4$ reduced by a factor of 0.75, almost identical to the factor, $0.73 \pm 0.067$, needed to reconcile shot-noise and deconvolution estimates of qsize variability.

Variations in quantal parameters during afferent excitation and inhibition

Previous sections described quantal activity during resting conditions. Here, we consider how such activity varies during the excitation and inhibition produced by 0.3-Hz sinusoidal canal-duct indentations. A simple situation would occur where such stimulation to modulate qrate without affecting qsize or quantal shape. As we now show, this was not the case. Rather, there were systematic changes in the other quantal parameters linked to variations in qrate. As was the case in treating resting activity, the order in which the parameters are considered is dictated by the shot-noise equations used to estimate them. Thus quantal shape is needed in the estimation of qsize and qrate. To obtain estimates at selected phases, the sine-wave cycle was divided into 24 equally spaced bins. Data in the corresponding bins of successive sine-wave cycles were averaged and smoothed (for details, see METHODS). Data points were fit by sine waves according to the formula

$$y = \Delta y \sin \left[2\pi(\theta - \theta_{\text{MAX}} + 90)/360\right] + y_0$$

where $\theta$ is the midpoint of each bin in degrees; $\Delta y$ is the modulation amplitude around an average value, $y_0$; and $\theta_{\text{MAX}}$ is the phase of the maximum value, which can be compared with maximum indenter inhibition ($90^\circ$) and excitation ($270^\circ$). When there were obvious asymmetries between excitation and inhibition, only the excitatory half-cycle was fit. No attempt was made to correct for random variations in qsize. Results are shown for a single fiber in Fig. 10. Mean values based on all 16 fibers are summarized in Supplemental Table S2.

Quantal shape was estimated by spectral methods and the deduced alpha function provided estimates of two shape variables, effective quantal duration ($I_1, \text{qdur}$) and peak mEPSP time (spread). As the control spectrum, we chose the inhibitory bin with minimal power. Because the reliability of spectral estimates depends on the signal-to-noise power ratios, we only
For the entire population, maximal qrates were phase advanced from peak excitatory indentation by an average of 32°. Supplemental Table S2 gives mean values for $y_0$ and $\Delta y$. Because the distributions for these variables were positively skewed, the following median values (with interquartile ranges in parentheses) provide useful summaries of central tendencies: $y_0$, 319/s (113–764/s); $\Delta y$, 770/s (586–1,844/s); peak quantal rate, 1,430/s (990–2,650/s).

INPUT–OUTPUT RELATIONS. If quantal rate were a linear function of indenter input, it should be proportional throughout the indenter cycle to the full sine-wave based on the excitatory half-cycle sine-wave fit. This was not the case because inhibitory responses were always smaller than excitatory responses. To characterize the nonlinearity, we plotted the actual response, defined as the displacement from a baseline qrate, against the expected linear response. In calculating the baseline, we averaged the values for the two points 180° apart with a minimal difference in qrates. The resulting input–output curve for the individual unit is concave upward (Fig. 11A) and is fit by a polynomial, the sum of a linear and a quadratic term.

Based on the best fitting polynomial, the peak excitatory response (767/s) is three times larger in magnitude than the peak inhibitory response (–258/s), giving an asymmetry ratio of 767/258 = 3.0. An obvious source of nonlinearity is the silencing of quantal release, but other mechanisms must be involved. This is indicated by fact that limiting the analysis to absolute qrates >50/s does not significantly alter the polynomial fit.
2,002/s); peak inhibitory response, −289/s (−102 to −625/s); asymmetry ratio, 3.3 (2.0−8.6). In 12 units, we were able to determine that restricting absolute rates to >50/s did not alter the coefficients of the polynomial regression.

For the small indentations used here, the input−output curves showed no signs of excitatory saturation and, in some cases, only a partial inhibitory saturation. Larger stimulus amplitudes were tested in four units. Indentations of sufficient cases, only a partial inhibitory saturation. Larger stimulus asymmetry ratio, 3.3 (2.0–8.6). In 12 units, we were able to (32.0

The voltage modulation raises the possibility that it is responsible for some of the periodic variation in qsize and other quantal parameters. Specifically, the depolarization, by reducing the driving force, could diminish qsize. Calculations indicate, however, that such an effect would be small. AMPA receptors (Jonas et al. 1993; Ozawa et al. 1998), including those in hair-cell afferent terminals (Glowatzki and Fuchs 2002), have reversal potentials near 0 mV. The ratio between the mean excitatory modulation, and the average magnitude of the resting potential gives a driving-force reduction of <4%, much smaller than the average qsize modulation of 18%. A second, more plausible effect of vm is that it could modulate voltage-sensitive conductances whose activation could reduce qsize. To evaluate the influence of such an effect, we plotted the relation between qsize and vm for individual units. Typically, two-thirds of the total variation in qsize occurred at the two points 180° apart with a negligible difference in vm, implying that vm can account for only a fraction, possibly one-third of the variation in qsize.

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VOLTAGE MODULATION. During mechanical stimulation, there is a periodic modulation in membrane potential (vm) correlated with grate modulation. Because the excitatory modulation was larger than its inhibitory counterpart, we fit sine waves only to excitatory half cycles. For the individual unit (Fig. 10D), the half-cycle modulation led excitatory indentation by 42.2 ± 1.1°, similar to the grate phase lead of 41.9 ± 2.2° (Fig. 10C). As summarized in Supplemental Table S3, the 16 units had a mean (±SE) excitatory modulation of 2.2 ± 0.2 mV and a mean phase re peak excitatory indentation of 37.6 ± 2.4°, slightly larger than the phase of excitatory grate modulation (32.0 ± 3.8°, Supplemental Table S2).

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Nonquantal transmission

We compared the experimentally observed total voltage modulation (T) with the quantal modulation (Q) the latter calculated from the formula, Q = ξ(h/I), for each of the 24 bins making up the 360° cycle (Fig. 10D). As might be expected, both T and Q are proportional to ξ (Fig. 11B). T was almost always larger than Q, suggesting the existence of a nonquantal (NQ) contribution to the modulation. A possible external source of NQ is a microphonic, either one arising from hair cells or one due to the mechanical displacement of the prepa-ration. To exclude these possibilities, we always inspected the control record obtained after the microelectrode came out of the axon (Fig. 10D, Control). In all cases, the control vm was negligible.

This last observation eliminates the possibility of an external source of NQ. At the same time, it was appreciated that our estimates of NQ are based on a complicated set of calculations. For that reason, we sought to separate the quantal and non-quantal components pharmacologically. This was done with CNQX and lowered external Ca2+ , both of which decrease quantal activity (Fig. 3). The quantal analysis also allowed us to ascertain if, as is commonly assumed, the effects of CNQX and of lowered external Ca2+ were consistent with a reduction in qsize and grate, respectively.

TOTAL VERSUS QUANTAL VOLTAGE MODULATION. Because both T and Q showed rectification, we fit sine waves only to excitatory half cycles. For the individual unit (Fig. 10D), the half-cycle modulations had amplitudes of 1.71 ± 0.036 (T) and 1.16 ± 0.031 mV (Q) and almost identical phase leads with respect to peak excitatory indentation of 42.2 ± 1.1° (T) and 41.1 ± 1.4° (Q). Subtracting Q from T gave a NQ component of 0.51 ± 0.045 mV and a phase lead of 45.6 ± 4.7°.

Fifteen of 16 units gave a calculated quantal component smaller than the observed total component. Supplemental Table S3 summarizes the magnitudes and phases of the T, Q, and NQ. Q and NQ have been corrected unit-by-unit for qsize variation. The three components lead indenter excitation on average by 37.6 ± 2.4° (T), 35.6 ± 3.4° (Q) and 43.6 ± 6.3 (NQ). On average, NQ makes up 20.5 ± 5.8% of the total modulation.

EFFECTS OF CNQX. In many units, a nonquantal component persisted after quantal transmission was blocked by CNQX. In the example seen in Fig. 12, the high-pass variance (Fig. 12A,
A proportional reduction in expected relation is a power law with linear relation between the two statistics. In the latter case, the reduction in quantal activity is due to a reduction in qsize, a residual variance. As a first step in the analysis, we plotted the skew against the variance to decide whether the reduction in quantal activity is due to a reduction in qsize or qrate. Were the former the case, there should be a linear relation between the two statistics. In the latter case, the expected relation is a power law with a ratio proportional to \( \mu_3 \) with an exponent \( k = 1.5 \). A log-log regression gave \( k = 1.53 \pm 0.13 \), consistent with a qsize reduction (Fig. 12B). Were \( T \) entirely due to quantal activity, it should decrease during drug action in proportion to the square root of the peak-excitatory variance, \( \mu_2^{1/2} \), because the latter is proportional to qsize. A proportional reduction in \( T \) is not observed, indicating the presence of an \( NQ \) component. To estimate \( NQ \), we plotted \( T \) versus \( \mu_2^{1/2} \) (Fig. 12C). The y-intercept of a linear regression between the two variables provides a value of 1.04 \( \pm 0.14 \) mV for \( NQ \), similar to that of 1.20 \( \pm 0.02 \) mV obtained from a shot-noise analysis of a record taken before drug application.

A similar analysis was done in 10 axons, including the one just shown. In all cases, CNQX greatly reduced quantal activity. Results are summarized in Supplemental Table S4. Power-law regressions between peak values of the skew (\( \mu_3 \)) and variance (\( \mu_2^{1/2} \)) gave exponents with an average 1.48 \( \pm 0.03 \). In seven cases, a highly significant y-intercept or \( NQ \) component \( (P < 0.001) \) was present after CNQX block. In only three cases was the \( NQ \) component similar to the shot-noise estimate from pre-CNQX control data. For another six units, the CNQX estimate was considerably smaller than the control estimate. On average, the \( NQ \) component estimated from the CNQX exposure was 30–35% of the shot-noise control value. The results support the presence of a nonquantal component but indicate that there is a discrepancy between the CNQX and pre-CNQX control estimates of \( NQ \).

**EFFECTS OF LOWERED EXTERNAL \( \text{Ca}^{2+} \).** The discrepancy may indicate that some of the nonquantal modulation is due to the accumulation of glutamate. Were this the case, the \( NQ \) component should also decline when quantal transmission is reduced by lowering external \( \text{Ca}^{2+} \). We first note that the quantal reduction with low \( \text{Ca}^{2+} \) is caused by a decrease in qrate. This can be seen in Fig. 13A, which plots qsize and qrate before, during, and after the control solution was replaced by our low-Ca solution. Included in the figure are shot-noise estimates of qrate during rest and of both qrate and qsize during the maximal excitation produced by 0.3-Hz indentations. There was a reversible decline in maximal excitatory qrates from 1,770 to 440/s and in resting qrates from 580 to 10/s. During this time, qsize increased only slightly, whereas quantal shape hardly changed (Fig. 13B). That the main effect of lowered \( \text{Ca}^{2+} \) is a reduction in qrate is confirmed when skew is plotted against variance wave-by-wave (Fig. 13C). A log-log regression gave an exponent, \( k = 1.05 \pm 0.05 \), close to the expected.
value of $k = 1$ for a qrate variation. To estimate the $NQ$ component remaining were qrate reduced to zero, we plotted the excitatory half-wave amplitude of total (T) vmod versus the corresponding half-wave amplitude of the variance, the latter proportional to the excitatory modulation of qrate (Fig. 13D). A linear fit gave a $y$-intercept of $0.14 \pm 0.045$; dashed line, unity slope. $D$: excitatory half-wave amplitude of the low-pass voltage ($\mu_1$) is plotted vs. the half-wave amplitude of the high-pass variance ($\mu_2$), the latter a measure of the excitatory modulation of qrate. Extrapolation to the $y$-intercept gives an estimate of the nonquantal component (bottom horizontal arrow) considerably smaller than the nonquantal component estimated by shot-noise calculations from a control trial (top horizontal arrow).

E: for each of 7 units, the ratio of quantal sizes (qsizes) from low-Ca$^{2+}$ and from control records is plotted against the corresponding ratio for qrate (qrates). There is an increase in qsizes confined to units whose qrates were reduced to $<15\%$ of control. A linear regression between the 2 variables is statistically significant ($P < 0.05$).

FIG. 13. Nonquantal component can be reduced in low Ca$^{2+}$. $A$: shot-noise calculations. Lowering external Ca$^{2+}$ reduces both the resting (unstimulated) and the maximal qrate during 0.3-Hz sinusoidal stimulation. Quantal size is slightly increased. Each trial consists of a 5-s resting period and 12 sine-wave cycles. There is a control trial and 6 trials in low Ca$^{2+}$ followed by a return to the control solution. $B$: mEPSP shape (Eq. 2) based on spectral analysis of resting activity from control and low Ca$^{2+}$ (2nd post-Ca$^{2+}$ trial) records. $C$: quantal skew ($\mu_1$) vs. variance ($\mu_2$) plotted in double-logarithmic coordinates. Each point is based on sine-wave fits to a single cycle of a 0.3-Hz sinusoidal stimulus; points are the peak values. Solid line, log-log regression line has a slope, $1.04 \pm 0.045$; dashed line, unity slope. $D$: excitatory half-wave amplitude of the low-pass voltage ($\mu_1$) is plotted vs. the half-wave amplitude of the high-pass variance ($\mu_2$), the latter a measure of the excitatory modulation of qrate. Extrapolation to the $y$-intercept gives an estimate of the nonquantal component (bottom horizontal arrow) considerably smaller than the nonquantal component estimated by shot-noise calculations from a control trial (top horizontal arrow).

E: for each of 7 units, the ratio of quantal sizes (qsizes) from low-Ca$^{2+}$ and from control records is plotted against the corresponding ratio for qrate (qrates). There is an increase in qsizes confined to units whose qrates were reduced to $<15\%$ of control. A linear regression between the 2 variables is statistically significant ($P < 0.05$).
the five units, DL-TBOA decreased $Q$, increased $NQ$, and resulted in a phase lag in $NQ$ but not in $Q$. Quantal parameters were estimated by shot-noise analyses. In all cases, there was a reduction in $qsize$. There was no consistent influence on $grate$. It might be expected that transmitter persistence would increase quantal duration ($qdur$), but this was seen in only two of the five units. On average, DL-TBOA led to the following statistically significant results ($P < 0.01$). $qsize$ was reduced 47 ± 6%, $Q$ was reduced 61 ± 10%, and $NQ$ was increased 88 ± 37%. $NQ$ was phase lagged, on average, by $33.9 ± 5.3°$.

Quantal size was reduced in the prestimulus (rest) period, suggesting that glutamate accumulation during transporter block persists beyond the stimulation period. That accumulation is exacerbated by stimulation is also indicated by a depolarizing shift that develops during stimulation and lasts throughout the stimulus and poststimulus periods (Fig. 14C). This effect was seen in all five units once their resting potentials had stabilized at near-maximal negative values.

### GLUTAMATE AGONIST (AMPA)

The effects of DL-TBOA, including the decrease in $qsize$, are likely to be the result of glutamate accumulation, which presumably has its effect by way of receptor occupancy. To verify this interpretation, we bath applied AMPA in concentrations of 30–100 μM. In all cases, AMPA resulted in a depolarization coupled with an abolition of quantal activity. An example is shown in Fig. 15. In this case, AMPA (30 μM) was applied immediately before the start of the record. There was a 6-mV depolarization (Fig. 15A), associated with the almost complete elimination of the stimulus-evoked modulation of the high-pass variance (Fig. 15B) and skew (Fig. 15C). The elimination of quantal activity is indicated by the skew reaching zero. In the absence of quantal activity, a nonquantal modulation of the membrane potential persists (Fig. 15A, inset) and has a peak-to-peak amplitude of 0.6 mV, approximately one-third the size of the modulation seen before AMPA began to work. Because AMPA blocks neurotransmitter action, the remaining nonquantal modulation is likely caused by some nonglutamatergic mechanism.

The application of AMPA also allowed us to estimate channel noise. To do so, we calculated the high-pass variance in the absence of quantal activity. Even before AMPA had done so, quantal activity was eliminated during the inhibitory portion of the response to large indentations. As the AMPA-induced depolarization developed, the variance during peak inhibition showed a slight increase before it declined (Fig. 15B). The relation between the variance and the mean potential (Fig. 15D) was fit by Eq. 13 and gave an estimate of $\Delta V = 0.81 ± 0.10 \mu V$ for the initial slope of the relation. Similar measurements, done in a total of five units, gave a mean $\Delta V$ of $1.24 ± 0.36$ (SE) μV.

This value of $\Delta V$ can be used to estimate the contribution of channel noise to the high-pass variance, a subject we encountered in estimating $qsize$ variability. We confine our remarks to resting activity. Based on average values of $grate$, $qsize$, and quantal shape, we can calculate from Eq. 4 ($n = 2$) that the quantal contribution to the variance is 0.045 mV$^2$. From Supplemental Table S1, we can subtract the minimum from the resting membrane potential to obtain $V = 0.90$ mV as the average depolarization during resting activity. From the mean value of $\Delta V$, we obtain a channel-noise variance, $\Delta V \times V =$
The complete block of glutamate neurotransmission by CNQX, by low external Ca$^{2+}$, or by AMPA application. An obvious candidate is K$^+$ accumulation since receptor currents leaving hair cells are carried by K$^+$ ions. Were these to accumulate extracellularly, they could depolarize hair cells and afferent terminals. To examine this possibility, we studied the effects of increasing extracellular K$^+$ from 4 to 10 mM and of bath application of DIDS (30–100 μM), a blocker of K-Cl (KCC) cotransporters (Russell 2000).

Both procedures had similar effects. These can be illustrated by an example of DIDS application, which resulted in a reduction of quantal transmission with a smaller reduction in vmod (Fig. 16, A and B). The increase in extracellular K$^+$, either produced by its direct application or by blocking its uptake, might work by depolarizing hair cells, which would increase qrate, leading to the extracellular accumulation of glutamate and a reduction of qsize. Alternatively, a postsynaptic depolarization might reduce qsize by activating voltage-dependent currents and lowering the impedance of the afferent terminal. Shot-noise analysis is consistent with a qsize reduction: the skew-variance relation has a power-law exponent of 1.47 ± 0.06 (Fig. 16C). There is no indication of an accompanying qrate increase, which when coupled with a qsize reduction should lead to an exponent >1.5. The voltage modulation (T) is separated into quantal (Q) and nonquantal (NQ) components in Fig. 16D. While Q decreases almost 10-fold, there is only a 36% reduction in T. NQ first increases and then decreases to slightly less than control values.

Similar results were obtained in a total of six units: three with high K$^+$ and three with DIDS. In no case did the power-law exponent of the skew-variance relation exceed 1.5. There was a marked reduction in Q in all units, a much smaller decrease in T, and no consistent fall in NQ. At least a transient increase in NQ was seen in five of six units. The increase in NQ, which averaged 20%, might be partially masked because of antagonistic influences. NQ might be expected to increase because of a presumed block of K$^+$ uptake and to decrease as a result of a lowering of the afferent terminal’s input impedance.

Relative phase leads of spike and synaptic activity

There is a large diversity in the response dynamics of vestibular afferents (Goldberg 2000; Lysakowski and Goldberg 2004), which in the case of the fibers innervating the turtle posterior crista can be measured by the phase lead of the response to 0.3-Hz sinusoidal stimulation (Brichta and Goldberg 2000a). By comparing phase leads of spike and synaptic activity, we were able to dissect the spike phase into presynaptic (hair cell) and postsynaptic (afferent) contributions. Specifically, the synaptic phase lead should measure the hair cell contribution, whereas any discrepancy between the phase leads of synaptic and spike activity should represent the afferent contribution. The dissection is justified by noting that the kinetics of postsynaptic receptor mechanisms, as judged by mEPSP shapes, makes a negligible contribution to response dynamics: a phase lag of 0.3° at 0.3 Hz. A synaptic delay of 1 ms adds a phase lag of 0.1°.

Figure 17A includes two phase-histograms for an axon, one based on spike activity (SP) and the other on the total (T) voltage modulation (vmod), the latter obtained after spikes were blocked with TTX. SP and T both lead indenter excitation
with phase leads of $\Phi_{SP} = 66 \pm 3$ and $\Phi_T = 41 \pm 1^\circ$, respectively. Comparisons between $\Phi_{SP}$ and $\Phi_T$ ($r = 0.85, P \ll 0.001$). Spike activity leads synaptic activity by 5–35° [19.1 ± 1.6° (SE)]. The phase difference, $\Delta \Phi = \Phi_{SP} - \Phi_T$, must arise postsynaptically and is likely to be caused by spike adaptation (Goldberg et al. 1982; Rabbitt et al. 2004b). There is a tendency for $\Phi_{SP}$ to be larger, the larger is the value of $\Phi_T$. As a result, the presumed adaptation contributes to the phase diversity across the afferent population. A linear regression between $\Phi_T$ and $\Phi_{SP}$ was used to estimate pre- and postsynaptic contributions to response dynamics. Of the total variation in spike phase of 62° seen in Fig. 17B, the linear regression indicated that 17.7 ± 1.6° (66%) attributable to postsynaptic mechanisms, leaving 40.6 ± 1.5° (66%) attributable to presynaptic mechanisms. A similar analysis, done with quantal (Q) vmod replacing $T^{vmod}$, led to statistically indistinguishable results.

The analysis assumes that TTX does not affect the timing of synaptic activity. To examine the assumption, we used a computer program to delete spikes from the records obtained before TTX application. This was done both to the original and to the high-pass records. An example of spike deletion from a high-pass record is shown in Fig. 17C. The spike-deleted pre-TTX record and the TTX record were compared in terms of the phases of their $T^{vmod}$ and high-pass variances. In Fig. 17D, high-pass variances are compared for the individual axon of Fig. 17C and are seen to be nearly in phase. As in the specific example, phase differences in a sample of 11 units were small, with mean phase differences of $-2.5 \pm 2.6^\circ$ (SE) for $T^{vmod}$ and $+3.8 \pm 1.5^\circ$ for the high-pass variance. The signs indicate that the pre-TTX record leads the TTX record for $T^{vmod}$, whereas the reverse is true for the variance.

TTX typically resulted in a small hyperpolarization of 1–4 mV and a reduction of 25–50% in the variance ($h^2$) of the no-stimulus (resting) synaptic activity. The hyperpolarization implies the presence of a persistent sodium (NaP) current, whereas the variance reduction suggests that NaP might amplify $h^2$. The presumed effect on $qsize$ is small. Assuming a proportionality between $\lambda_2$ and $h^2$, the variance reduction seen with TTX would be consistent with a $qsize$ reduction of only 10–30%.

FIG. 16. DIDS reduces quantal transmission while enhancing nonquantal transmission. A and B: compares the low-pass mean voltage ($\mu_1$) and the high-pass variance ($\mu_2$) for control conditions and after bath application of DIDS (30 mM), which reduces the sinusoidal modulation of $\mu_2$ 7-fold, but that of $\mu_1$ by only one-third. C: high-pass skew ($\mu_2$) and variance ($\mu_2$) plotted against each other. Points depict max-
DISCUSSION

We have used quantitative methods, including spectral techniques, deconvolution, and shot-noise theory, to characterize synaptic transmission in the turtle posterior crista during rest and during mechanical stimulation. Recordings were made in afferent nerve fibers near their termination in the neuroepithelium. A simple physiological test, based on the distinctive responses of different afferent groups to efferent activation, allowed us to determine the type of endings possessed by an afferent, as well as the place in the neuroepithelium it innervates (Brichta and Goldberg 2000b). Of the two kinds of afferents found in the turtle posterior crista, this study dealt with bouton, rather than with calyx-bearing, afferents, and of the various kinds of bouton afferents, we only considered those inhibited by efferent activation. Such so-called BT/BM units are found toward the nonsensory torus rather than toward the planar extremes of each hemicrista. They are characterized by relative high gains and large phase leads in their responses to rotations (Brichta and Goldberg 2000a) or indentations (the present study).

An obvious advantage of the in vitro preparation, as judged by the discharge properties of its afferents, is that it approximates the behavior of a conventional in vivo preparation (Brichta and Goldberg 2000a). A drawback is that we could only record from myelinated axons, ~250 μm from the neuroepithelium, because attempts to impale fibers nearer their peripheral terminations were unsuccessful. In recording from myelinated axons, we used sharp, rather than patch, electrodes. Activity was recorded in current clamp, which lacks the analytic rigor of voltage-clamp recordings. At the same time, we were able to deduce some of the synaptic mechanisms responsible for the modulation of afferent discharge.

The distance from the recording site to the end of myelination, ~250 μm, should introduce only a small attenuation of synaptic voltages. Given the measured electrical properties of myelinated axons (BeMent and Ranck 1969; Rushton 1951; Tasaki 1955) and the size of our axons (Brichta and Goldberg 2000a), the distance should be just over a quarter of a length constant. Two observations suggested that the cable properties of unmyelinated, intraepithelial branches did not severely attenuate or slow the mEPSPs recorded in the parent axon. First, the rising phases and overall shapes of mEPSPs were uncorrelated with qsize. Second, similar qsize distributions have been seen in individual CD and BT/BM units (Xue et al. 2002), even though the former units have much more compact terminal trees (Brichta and Goldberg 2000a; Brichta and Peterson 1994). It is unclear the extent to which the dissociation of qsize and quantal shape in BT/BM afferents reflects passive cable properties and/or the shaping of mEPSPs by active conductances.

A second drawback concerns the interpretation of drug actions, which were used in an attempt to elucidate the mechanisms of nonquantal transmission. This is a tissue preparation and any particular drug could conceivably target hair cells, afferent nerve fibers, and/or supporting cells. In some cases, quantal analysis was consistent with a specific interpretation. For example, the power-law relations between the skew and variance in high-pass records were consistent with a presynaptic action of low external Ca2+ and a postsynaptic action of CNQX. That DL-TBOA was targeted to supporting cells is indicated that SP has a larger phase lead than T. B: relation between the phase leads, ΦT and ΦSP, for total (T) depolarization and spike discharge, respectively, is plotted for 25 units. ΦSP varies over a 62° range across the population. ΦT is smaller than ΦSP. Best-fitting linear regression (——) has a slope less than unity (— —), implying that the difference in phase leads grows as the ΦSP increases. C: original high-pass record (top trace and right ordinate) and the same trace after spikes have been removed by a computer program (bottom trace and left ordinate). D: variance of the spike-removed record in C is compared with the record obtained after spikes are blocked by TTX. Only 6 of the 12 sine-wave cycles are shown.
sitized states (Partin et al. 1993; Wong and Mayer 1993), should increase qsize and qdur. We did not observe the expected effects when CTZ was added to the bath at 100 μM. CTZ at this concentration had the expected result in the mammalian cochlea (Glowatzki and Fuchs 2002), suggesting that our negative results might reflect limited drug access.

Quantal size and rate

We observed an inverse relation across the afferent population between the estimates of qsize (h) and qrate (ξ) for resting (unstimulated) synaptic activity. However, because the same statistical variables enter into the estimates of both parameters, they are not statistically independent. Confirming this, we found a covariation between the two estimates during repeated simulated or actual records. At the same time, the variance of either variable during repeated samples was smaller than that observed across the unit population. Another source of spurious correlation involves estimates of h and ξ that do not correct for qsize variability. This factor contributed to, but did not explain, the entire correlation between the two estimates.

While these remarks suggest caution, other results suggest that the observed inverse relation is not spurious. There is an inverse relation between the two variables during sinusoidal stimulation with qrate peaking during excitation, while qsize reaches a maximum during inhibition. Several mechanisms may contribute to the latter relation. One of these is a modulation (vmod) in the afferent’s membrane potential during stimulation. However, by plotting h versus vmod, we found that approximately two-thirds of the qsize modulation is seen at a single voltage. Hence, other mechanisms must be involved. Excitation is associated with an elevated release of glutamate from hair cells. In addition, there should be a rise in basolateral K⁺ currents. Increasing glutamate or K⁺ concentrations was found to decrease qsize. Glutamate could work by increasing receptor occupancy and/or by reducing the input impedance of the afferent terminal. An impedance change should also accompany K⁺ efflux. All of these effects should reduce qsize.

The same mechanisms involved in the excitatory modulation of qsize and qrate may also be responsible for the inverse relation seen across the afferent population. We can suppose that, based on uncontrolled factors, our preparations vary in excitability with the result that there are variations in qrate, which are associated with changes in extracellular glutamate and K⁺ concentrations. These, in turn, will result in oppositely directed variations in qsize. An attractive feature of the proposal is that it can be extended to other preparations. In particular, there have been several studies of quantal transmission in the frog posterior crista (Annoni et al. 1984; Cochran 1995; Rossi et al. 1994). Despite the similarity in methods with those of this study, results were quite different. Rates in the frog were much smaller, whereas qsizes were much larger. In addition, spike recordings done in the same preparation (Rossi and Martini 1986) indicate that the resting discharge and sensitivity of afferents are also much lower than is seen in intact preparations (Honrubia et al. 1989). These results suggest that overall transduction is depressed in the in vitro frog preparation and that the presumed reduction in rates may be responsible for the large qsizes.

The highest stimulated rates seen in our preparation were near 3,000/s, about 5–10 times higher than seen in the frog (Rossi et al. 1994). On average, the BT/BM afferents impaled in our studies have 50 bouton endings (Brichta and Goldberg 2000a), most of which make synaptic contact with hair cells (Lysakowski 1996). Expressed per ending, our rates of 60/s are considerably smaller than the maximal release rates estimated for single synapses in capacitance studies of hair cells (Edmonds et al. 2004; Parsons et al. 1994).

Shot-noise model and multivesicular release

Our results, as well as those in the frog posterior crista (Rossi et al. 1994) are consistent with a shot-noise model of quantal transmission in which there is independent release of individual quanta of varying size. A different interpretation was offered to explain qsize variability in afferents innervating rat inner hair cells (Glowatzki and Fuchs 2002). In the latter study, in which quantal activity was recorded in voltage clamp, qsize distributions were particularly broad and positively skewed. Results were interpreted as indicating that quantal activity involves the coordinated release of multiple quanta. It was suggested that the underlying qsizes were normally distributed with a low cv and that such multivesicular release (MVR) led to a skewed distribution with a large cv. Glowatzki and Fuchs (2002) provided several observations consistent with MVR, but they also found that a model based on their interpretation gave a poor fit to their own data.

Our results, including the broad, positively skewed qsize distributions, can be explained without invoking the coordinated release of multiple vesicles. A similar conclusion has been reached in studies of many single-site synapses (for review, see Franks et al. 2003). There are, nevertheless, several lines of evidence suggesting that, under some circumstances, multivesicular release can occur at ribbon synapses (Edmonds et al. 2004; Parsons and Sterling 2003; Singer et al. 2004). It is worth emphasizing that broad qsize distributions are consistent with either the independent release of individual quanta or multivesicular release.

Nonquantal transmission

Such transmission was hypothesized as an adjunct source of depolarization of the type I hair cell and its calyx ending (Goldberg 1996). Because of the long, uninterrupted cleft between these two structures, K⁺ ions and glutamate released by the hair cell at its base would persist in the cleft and depolarize both elements. As shown in this study, nonquantal transmission also occurs between type II hair cells and bouton endings. First shown by shot-noise calculations, nonquantal transmission was confirmed by the presence of a periodic depolarization during sinusoidal mechanical stimulation even after glutamate neurotransmission was blocked by CNQX or by lowering external Ca²⁺. The periodic depolarization remaining after the block usually fell short of that predicted by shot-noise calculations, which was taken as evidence that part of the nonquantal effect was caused by spillover of glutamate from nearby synapses. Such spillover involving the activation of AMPA receptors has also been seen among central synapses (Carter and Regehr 2000; DiGregorio et al. 2002). In hair cell neuroepithelia, glutamate is removed from the extracellular space by the glutamate transporter GLAST, located in supporting cells (Ottersen et al. 1998). Blocking GLAST with DL-
TBOA enhanced nonquantal transmission, consistent with the role envisioned for glutamate in nonquantal transmission.

The nonquantal transmission remaining after CNQX could possibly be explained by glutamate acting on NMDA or metabotropic glutamate receptors. Presumably, low Ca$^{2+}$ reduces glutamate release from hair cells so glutamate accumulation provides an unlikely origin for the persistence of nonquantal release under those conditions. K$^+$ accumulation is a likely alternative. Much of the ionic current passing through transduction channels is carried by K$^+$ (Corey and Hudspeth 1979) and the same is true of the ionic current leaving the base of the hair cell (Eatock et al. 2002; Brichta et al. 2002). During sinusoidal mechanical stimulation, there should be a periodic modulation of K$^+$ ions in the extracellular space, and this should result in a similar modulation of the afferent’s membrane potential.

We used two agents to explore the possible involvement of K$^+$ in nonquantal transmission. Raising extracellular K$^+$, which was done in the hopes of partially saturating K$^+$ uptake processes, enhanced nonquantal transmission. Second, there was an enhancement with DIDS, a blocker of KCC cotransporters. Neither result is entirely convincing because the mechanisms of K$^+$ uptake in vestibular organs are far from clear. In particular, while KCC3 and KCC4 have been immunolocalized to supporting cells in the mammalian cochlea and have been implicated in K$^+$ homeostasis there (Boettger et al. 2002, 2003), the role of these and other transport mechanisms in the vestibular neuroepithelium remains to be examined.

Given their continual release from hair cells during transduction, it is not surprising that K$^+$ and glutamate could accumulate in the extracellular space and give rise to nonquantal transmission. Our observation that elevation of K$^+$ or the bath application of AMPA greatly reduces quantal transmission emphasizes the importance of the homeostatic mechanisms controlling extracellular concentrations of K$^+$ and glutamate. Although afferent discharge seems normal in the in vitro turtle ear, homeostatic mechanisms may not be entirely normal. This suggests caution in concluding that nonquantal transmission occurs normally. If it does, it can be of functional significance. Nonquantal ($NQ$) depolarization of afferents at peak excitation is on the order of 20% of total ($T$) depolarization and might be expected to make a proportionate contribution to spike discharge.

Channel noise and quantal activity

Many of our results were based on shot-noise calculations involving the high-pass variance. In estimating the variance caused by quantal activity, it is necessary to eliminate the contributions of both instrumental and channel noise. Instrumental noise is easily handled because, if one is careful to exclude mechanical artifacts, it should remain constant during the stimulus cycle. Channel noise is harder to deal with because it should be proportional to the postsynaptic depolarization caused by the neurotransmitter, whether this arises from quantal or nonquantal transmission (Neher and Sakaba 2001a,b). Estimates of channel noise became particularly important in considering qsize variability. The deconvolution methods used to estimate this variability were far from ideal because they could not be used to estimate small quanta buried in the recording noise and also because of their inability to distinguish closely spaced mEPSPs. Shot-noise calculations, based on Eq. 12 (Fesce et al. 1986), do not suffer from these drawbacks but require that grates remain constant over the sampling interval. While we did not achieve this condition in our experimental records, we did develop quantitative techniques to estimate and correct for grate variability. With these corrections, we could reconcile the deconvolution and shot-noise estimates of qsize variability.

An implication of the agreement between the two approaches was that channel noise was small relative to quantal noise. To measure channel noise, we used the relation between the variance and mean depolarization produced by bath-applied AMPA in the absence of quantal activity. Note that the estimate is based on the internal relation between the mean and variance and, as such, should be insensitive to the location of the recording electrode. Furthermore, the estimate is not invalidated by the possible activation of voltage-sensitive channels because the latter would contribute to the channel noise we record in current clamp. Channel noise was found to make a small (<3%) contribution to the typical variance occurring during rest.

This has implications for the etiology of discharge regularity, an important property of vestibular afferents (Goldberg 2000). The variability of interspike intervals requires a source of synaptic noise. It has been assumed that quantal, rather than channel, noise is a major source of the needed voltage fluctuations (Goldberg 2000; Smith and Goldberg 1986). These observations provide the first experimental support for the assumption.

Response dynamics

Vestibular nerve fibers differ in the ways their gains and phases change with the frequency of head rotations. In mammals (Baird et al. 1988; Goldberg and Fernández 1971), turtles (Brichta and Goldberg 2000a), and other animals (reviewed by Highstein et al. 2005; Lysakowski and Goldberg 2004), the response dynamics of regularly discharging fibers resemble those of the torsion-pendulum model, which in turn reflects the macromechanics of the cupula and endolymph. Irregular units show both low- and high-frequency deviations from predicted macromechanics. An important, unresolved problem in vestibular physiology is the identification of the transduction stages at which the differences in response dynamics arise.

In this study, we assigned differences to presynaptic (hair cell) or postsynaptic (afferent) mechanisms based on the phase leads during sinusoidal mechanical stimulation of synaptic input and spike discharge. It made little difference whether we used the total ($T$) or quantal ($Q$) modulation as a measure of synaptic input. To isolate these modulations, we first blocked spikes with TTX. Evidence was presented that TTX did not appreciably alter the timing of synaptic activity. Approximately two-thirds of variation in response phase was assigned to presynaptic mechanisms, leaving one-third of the variation to be explained by postsynaptic mechanisms. The estimate was done at a single frequency, 0.3 Hz, which was chosen because it leads to the largest differences across afferents in their response dynamics (Brichta and Goldberg 2000a). Based on the mechanisms to be considered next, we can suppose that postsynaptic mechanisms would become more important at lower frequencies and less important at higher frequencies.
Can presynaptic and postsynaptic mechanisms be identified more precisely? Concerning a presynaptic contribution, it is doubtful that macromechanics are involved (Rabbit et al. 2004b) or have appropriate differences been seen in transduction (Vollrath and Eatock 2003) or basolateral currents (Brichta et al. 2002). This leaves two possibilities: the micromechanical linkage between hair bundles and the cupula or otoconial membrane (Kachar et al. 1990; Lim 1984) and adaptive rundown of synaptic transmission (Furukawa and Matsuura 1978; Furukawa et al. 1982). A comparison of the receptor potentials produced by mechanical stimulation and by intracellular currents in the frog utricular macula pointed to micromechanics as an important factor (Baird 1994). In contrast, a recent study in the toadfish horizontal crista emphasized the potential importance of synaptic rundown (Rabbit et al. 2004a).

As for the postsynaptic contribution, afferent discharge can be expected to show adaptation to prolonged depolarization (Goldberg and Fernández 1971; Taglietti et al. 1977). Our results imply that the adaptation would become more prominent in irregularly discharging units with more phasic response dynamics. Such a difference was not seen with sinusoidal galvanic currents that directly activate the postsynaptic spike encoder (Ezure et al. 1983; Goldberg et al. 1982). Rather, the currents resulted in a phase lead of 10–15° in all units. In the toadfish, the phase lead observed at lower frequencies in the spike discharge of phasic afferents could be reduced by GABA_A antagonists (Holstein et al. 2004b). This was related to the observation that some hair cells are GABAergic (Holstein et al. 2004a). GABA_A actions include a reduction in transmitter release by the suppression of presynaptic Ca²⁺ currents and a slow inhibitory postsynaptic potential (IPSP) usually caused by the activation of postsynaptic K⁺ currents (Bowery et al. 2002). Both effects could result in adaptation. However, it is unclear how either mechanism could explain our observations because both of them might be expected to reduce, if not eliminate, the phase discrepancy between the total voltage modulation and spike activity. In addition, an IPSP should, contrary to our findings, also introduce a phase discrepancy between the total and nonquantal voltage modulations. While these remarks do not eliminate the possible involvement of GABA_A neurotransmission, they point out the need to identify postsynaptic mechanisms in more detail.

Concluding remarks

We became attracted to the turtle preparation in the hopes of unraveling the mechanisms of synaptic transmission between type I hair cells and calyx endings. This study, by clarifying transmission at bouton synapses, provides a context for understanding the former, more complicated situation. At the same time, we have been able to study cellular mechanisms responsible for afferent discharge in an in vitro preparation in which spike discharge resembles in vivo conditions. Perhaps the most unanticipated result of this study was the presence at bouton synapses of nonquantal transmission, which we had thought might be peculiar to the type I–calyx synapse (Goldberg 1996). Nonquantal transmission, in turn, emphasizes the need for homeostatic mechanisms to preserve quantal transmission in the face of the busy synaptic traffic seen in a continually transducing sensory organ.

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