The temporal characteristics of Ca$^{2+}$ entry through L-type and T-type Ca$^{2+}$ channels shape exocytosis efficiency in chick auditory hair cells during development

Snezana Levic and Didier Dulon
Equipe Neurophysiologie de la Synapse Auditive, Unité Mixte de Recherche, Institut National de la Santé et de la Recherche Médicale US87 et Université Bordeaux Segalen, Institut des Neurosciences de Bordeaux, Centre Hospitalier Universitaire Pellegrin, Bordeaux, France

Submitted 25 June 2012; accepted in final form 6 September 2012


During development, synaptic exocytosis by cochlear hair cells is first initiated by patterned spontaneous Ca$^{2+}$ spikes and, at the onset of hearing, by sound-driven graded depolarizing potentials. The molecular reorganization occurring in the hair cell synaptic machinery during this developmental transition still remains elusive. We characterized the changes in biophysical properties of voltage-gated Ca$^{2+}$ currents and exocytosis in developing auditory hair cells of a precocial animal, the domestic chick. We found that immature chick hair cells (embryonic days 10–12) use two types of Ca$^{2+}$ currents to control exocytosis: low-voltage-activating, rapidly inactivating (mibefradil sensitive) T-type Ca$^{2+}$ currents and high-voltage-activating, noninactivating (nifedipine sensitive) L-type currents. Exocytosis evoked by T-type Ca$^{2+}$ current displayed a fast release component (RRP) but lacked the slow sustained release component (SRP), suggesting an inefficient recruitment of distant synaptic vesicles by this transient Ca$^{2+}$ current. With maturation, the participation of L-type Ca$^{2+}$ currents to exocytosis largely increased, inducing a highly Ca$^{2+}$ efficient recruitment of an RRP and an SRP component. Notably, L-type-driven exocytosis in immature hair cells displayed higher Ca$^{2+}$ efficiency when triggered by prerecorded native action potentials than by voltage steps, whereas similar efficiency for both protocols was found in mature hair cells. This difference likely reflects a tighter coupling between release sites and Ca$^{2+}$ channels in mature hair cells. Overall, our results suggest that the temporal characteristics of Ca$^{2+}$ entry through T-type and L-type Ca$^{2+}$ channels greatly influence synaptic release by hair cells during cochlear development.

Exocytosis: low-voltage-activating, rapidly inactivating (mibefradil sensitive) T-type Ca$^{2+}$ currents and high-voltage-activating, noninactivating (nifedipine sensitive) L-type currents. Exocytosis evoked by T-type Ca$^{2+}$ current displayed a fast release component (RRP) but lacked the slow sustained release component (SRP), suggesting an inefficient recruitment of distant synaptic vesicles by this transient Ca$^{2+}$ current. With maturation, the participation of L-type Ca$^{2+}$ currents to exocytosis largely increased, inducing a highly Ca$^{2+}$ efficient recruitment of an RRP and an SRP component. Notably, L-type-driven exocytosis in immature hair cells displayed higher Ca$^{2+}$ efficiency when triggered by prerecorded native action potentials than by voltage steps, whereas similar efficiency for both protocols was found in mature hair cells. This difference likely reflects a tighter coupling between release sites and Ca$^{2+}$ channels in mature hair cells. Overall, our results suggest that the temporal characteristics of Ca$^{2+}$ entry through T-type and L-type Ca$^{2+}$ channels greatly influence synaptic release by hair cells during cochlear development.

coclea; hair cells; Ca$^{2+}$ channels; exocytosis

NEUROTRANSMITTER RELEASE at the auditory hair cell ribbon synapses is mainly triggered by Ca$^{2+}$ influx flowing through dihydropyridine (DHP)-sensitive L-type channels, these Ca$^{2+}$ channels being primarily constituted by Ca$_v$1.3 ($\alpha_{1D}$) subunits (see for review Fuchs et al. 2003). Indeed, Ca$_v$1.3-null mice (Ca$_v$1.3$^{-/-}$) are profoundly deaf because they bear cochlear hair cells with a 90% reduction in voltage-gated Ca$^{2+}$ current and, as a consequence, their hair cells have a largely reduced voltage-evoked Ca$^{2+}$-dependent synaptic exocytosis (Brandt et al. 2003; Dou et al. 2004; Platzer et al. 2000). Notably, most of the residual Ca$^{2+}$ current in Ca$_v$1.3$^{-/-}$ hair cells is insensitive to DHP antagonists, suggesting that this current is carried by non-L-type Ca$^{2+}$ channels. This residual Ca$^{2+}$ current is also insensitive to specific peptide toxins directed against N-, P/Q-, and R-type Ca$^{2+}$ channels (Brandt et al. 2003). However, it is completely inhibited by low concentrations of Ni$^{2+}$, suggesting that it could be carried by T-type channels and possibly by Ca$_v$3.1 T-type subunits, which have been shown to be expressed in the mouse and chick inner ear (Levic et al. 2007; Nie et al. 2008). Notably, T-type Ca$^{2+}$ currents are mainly, and transiently, expressed in early-developing auditory hair cells of the chick basilar papilla (Levic et al. 2007), whereas in mature (posthearing) chick auditory hair cells, most of the voltage-gated Ca$^{2+}$ current is carried by L-type DHP-sensitive Ca$^{2+}$ channels (Fuchs et al. 1990; Spassova et al. 2001). Furthermore, L-type Ca$^{2+}$ channels drive hair cell exocytosis with a Ca$^{2+}$ efficiency increasing with auditory maturation (Levic et al. 2011).

Low-voltage-activated transient T-type Ca$^{2+}$ current has been shown to initiate spontaneous action potential firing in early immature developing chick auditory hair cells (Levic et al. 2007). However, it is not known whether T-type Ca$^{2+}$ currents can directly trigger Ca$^{2+}$-dependent exocytosis in developing chick hair cells. If so, the question arises as to how efficiently T-type Ca$^{2+}$ channels are coupled to synaptic release during development. The question is important since spontaneous firing by immature hair cells is believed to instruct, presumably through Ca$^{2+}$-evoked exocytosis of trophic factors, the refinement of synaptic connections in the peripheral and central auditory system (Gabrielle et al. 2000; Hashisaki et al. 1989; Tritsch et al. 2010). The present study investigates for the first time the relative contribution of T-type and L-type Ca$^{2+}$ currents to Ca$^{2+}$-dependent exocytosis in auditory chick hair cells during development.

MATERIALS AND METHODS
Preparation of semi-intact chicken basilar papilla. The present investigation was performed in accordance with the guidelines of the Animal Care and Use Committee of the European Communities Council Directive (86/609/EEC), and the University of Bordeaux (ethics committee: Direction Régionale de l’Alimentation, de l’Agriculture et de la Forêt d’Aquitaine, permit number B 33075) approved this study. It included chickens at different stages of embryonic development ranging from embryonic days 10–12 (E10–E12) as well as posthatched 2-day-old chickens (P2). Fertilized eggs were incubated at 37°C in a Marsh automatic incubator (Lyon Electric,
Chula Vista, CA). Chicken embryos were euthanized and staged according to the following criteria: from E8 to E12, based on visceral arches, feather eggs, and eyelids; after E12, based on the length of the beak (Hamburger and Hamilton 1992). Basilar papillae were isolated as described previously (Levic et al. 2007, 2011). The preparations were dissected in oxygenated chicken saline containing (in mM) 155 NaCl, 6 KCl, 4 CaCl2, 2 MgCl2, 5 HEPES, and 3 glucose, pH 7.4. The tectum vasculosum and the tectorial membrane were removed without any prior enzymatic treatment, using a fine minuten pin. Chicken basilar papillae were stored in a 37°C incubator in minimum essential medium (Invitrogen) before recordings were made from hair cells in situ. All experiments were performed at room temperature (21–23°C) within 5–45 min of isolation. All reagents were obtained from Sigma Chemicals, unless otherwise specified. Recordings were made in neutral (taller) hair cells from the low-frequency coding region corresponding to the top one-third of the part wide of the basilar papilla. These tall hair cells, which are mainly innervated by afferent fibers, correspond to the inner hair cells in mammals.

Electrophysiology. Calcium currents were recorded in whole cell voltage-clamp configuration using 3–5 MΩ resistance pipettes. Currents were recorded with an EPC 10 amplifier (Heka Electronik, Lambrecht/Pfalz, Germany) and filtered at a frequency of 2–5 kHz through a low-pass Bessel filter. Only recordings with holding current <20 pA were retained for analysis.

Real-time changes in membrane capacitance (ΔCm) were recorded using the EPC 10 amplifier. A 2-kHz sine wave of 10 mV was applied to the cells from a holding potential of ~90 mV. ΔCm signals were low-pass filtered at 80 Hz. ΔCm were measured 0.05–0.5 s after the end of the depolarizing pulse, and values were averaged over a period of 0.3–20 s. Membrane (Rm) and series resistance (Rs) were monitored during the course of the experiment. Only recordings with stable Rm and Rs values were considered for further analysis. Rs values ranged within 5–20 MΩ. The seal resistance was typically 5–20 GΩ. Liquid junction potentials were measured and corrected online. Extracellular solution for measuring Ca2+ currents contained (in mM) 125 NaCl/choline-Cl, 6 KCl, 5 CaCl2, 25 TEA, 4 4-aminopyridine, 10 t-glucose, 1 MgCl2, and 10 HEPES; pH 7.3, 310 mosmol/l. Intracellular solution contained (in mM) 75 NMDG, 70 CsCl, 5 Na2ATP, 2 MgCl2, 10 HEPES, 0.5–10 EGTA, and 10 glucose; pH 7.3, 300 mosmol/l. The stock solutions of all toxins/drugs used were composed of either ddH2O or DMSO and stored at –20°C. Stock solutions were reconstituted and perfused in the recording chamber. The final DMSO concentration in the recording bath solution was ~0.001%.

For some experiments, hair cells were also stimulated with native E12 prelabeled action potentials (AP) having the following characteristics (Levic et al. 2007): total spike duration, 184.4 ms; half-width, 4.6 ms; maximum right slope dV/dt, 5.4 V/s; maximum left slope dV/dt, 5.6 V/s; maximum spike amplitude, 55 mV; and holding potential, –80 mV.

Data analysis. The number of cells (n) is given with each data set. Data were analyzed using Clampfit (Axon Instruments) and Origin 7.0 (Microcal Software). Pooled data are presented as means ± SD. Significant difference between groups of cells or between different embryonic stages of development were evaluated using a two-tailed Student’s t-test; with P < 0.05 or 0.001 indicating statistical significance. Time constants (τ) were obtained from fits using Origin software. Time constants were obtained by fitting multiple exponential equations to the activation decay of the current. The equation was of the form I = Io + A1 exp(−t/τ1) + A2 exp(−t/τ2) + A3 exp(−t/τ3) + . . . , where Io is the initial current magnitude, τ1, τ2, . . . , τn are the time constants, and A1, A2, . . . , An are the proportionality constants. Synaptic transfer functions relating Ca2+ current (ICa) and ΔCm or QCa2+ and ΔCa were calculated using an integral of total ICa, including the tail currents. The data were fitted using first-order power functions: ΔCm = s[ICa]n or ΔCm = s[QCa2+]n, where s is slope factor (IF/PA or IF/PC) and N is the power index.

Immunohistochemistry. Tissues were fixed with 4% paraformaldehyde in PBS for ~3 h, rinsed, and immunostained with polyclonal goat antibody directed against mouse CtBP2 (1:200; Sigma) and polyclonal rabbit antibody against rat CaV3.1 [1:250; epitope peptide MDEEDGAGAESGQPRSTQ(L), corresponding to amino acid residues 1–22 of rat CaV3.1; Alomone Labs]. Immunostaining was visualized with anti-goat secondary antibody conjugated to Alexa 488 (green, CtBP2) and anti-rabbit secondary antibody conjugated to Alexa 546 (red, CaV3.1). Omission of the primary antibodies or preincubation with the epinephrine peptides eliminated staining in all preparations examined. Hair cell actin was counterstained with phalloidin conjugated to Alexa Fluor 568 (1:200; Molecular Probes). Fluorescent images were collected and analyzed with a confocal laser scanning upright microscope (Leica DMR DCS SP2 AOBS, Biorad Imaging Center). Images of ribbons were taken in the basal synaptic area (step size 0.4 μm). Counting of T-type-labeled ribbons was performed from three different experiments and from three different sections. From each section, each counted field included about 20 hair cells.

RESULTS

T-type and L-type Ca2+ current-driven exocytosis. To determine the relative contribution of T- and L-type currents in hair cell exocytosis, we used 10 μM mibebradil as a T-type channel blocker (Levic et al. 2007; McDonough et al. 1998) and 50 μM nifedipine as an L-type channel blocker (Fuchs et al. 1990, Spassova et al. 2001). The effects of mibebradil and nifedipine on ΔCm responses were examined at four developmental periods of cochlear synaptogenesis: embryonic stages E10, E12, and E16 (in ovo) and 2 days posthatching (P2). The first embryonic period, E10, corresponds to an early stage of synaptogenesis when the first presynaptic specializations (synaptic bodies or ribbons) can be detected in hair cells (Rebillard and Pujol 1983) and when the afferent fibers first come into contact with their base (Whitehead and Moster 1985). At stage E12, low-frequency hearing starts in the chick embryo (Rebillard and Rubel 1981; Saunders et al. 1973). Stage E16 (5–3 days before hatching) corresponds to the final step of synaptogenesis and hair cell maturation. Finally, P2 corresponds to nearly adult hearing values.

The contribution of T-type Ca2+ currents in Ca2+-evoked exocytosis peaked at E12, whereas that of L-type Ca2+ channels continuously increased from E10 to P2 (Fig. 1). As expected for low-voltage-activated Ca2+ channels, the Boltzmann fitting characteristics of the ICa voltage dependence in the presence of nifedipine (T-type) showed a 10 mV more negative half-maximal voltage activation (V1/2) compared with ICa in the presence of mibebradil (L-type current). At E12, a mean V1/2 of –21.5 ± 1.0 mV (n = 9) was measured for L-type currents compared with a V1/2 of –33.5 ± 1.0 mV (n = 9) for T-type currents. Whereas the voltage dependence of the T-type current did significantly change with age, that of L-type current showed a negative shift of 10 mV between E12 and P2. Furthermore, hair cell T-type currents showed much faster inactivation compared with L-type currents (Fig. 1A), as previously described in detail by Levic et al. (2007). Notably, the extracellular application of 10 μM Ni2+, a specific blocker of T-type current (see Carbone et al. 2006), produced a similar block on voltage-activated Ca2+ currents and exocytosis to 50 μM mibebradil (data not shown).
Fig. 1. Comparative exocytosis evoked by L- and T-type Ca\(^{2+}\) currents in embryonic day 10, 12, and 16 (E10, E12, E16) and posthatching day 2 (P2) chick hair cells. A: examples of Ca\(^{2+}\) current (I\(_{\text{Ca}}\)) and changes in membrane capacitance (\(\Delta C_m\)) recorded in 2 apical E12 hair cells in response to a 100-ms step potential from \(-90\) to \(0\) mV, before and after the addition of 10 \(\mu\)M mibebradil (left) or 10 \(\mu\)M nifedipine (right). The subtracted currents (control minus drug application) corresponding to the T- and L-type currents are also shown for each condition. B: current-voltage relationships of \(\Delta C_m\) and I\(_{\text{Ca}}\) responses of control, mibebradil-sensitive (T-type), or nifedipine-sensitive (L-type) currents of E10 (n = 6), E12 (n = 9), E16 (n = 9), and P2 (n = 4) hair cells. Bottom row of panels shows the Boltzmann fit of the T-type (gray stars) and L-type (black triangles) Ca\(^{2+}\) current voltage-activation curve at different developmental stages: E10 (T-type \(V_{1/2} = -35 \pm 1.2\) mV and \(s = 3.5 \pm 1\); L-type \(V_{1/2} = 19.1 \pm 2.7\) mV and \(s = 3.5 \pm 1.4\)), E12 (T-type \(V_{1/2} = -33.5 \pm 1.0\) mV and \(s = 2.5 \pm 0.9\); T-type \(V_{1/2} = -21.5 \pm 1.0\) mV and \(s = 2.5 \pm 0.9\)), E16 (T-type fit: \(V_{1/2} = -30 \pm 1.5\) mV and \(s = 3 \pm 0.8\); L-type fit: \(V_{1/2} = -19 \pm 0.6\) mV and \(s = 3.5 \pm 0.9\)), and P2 (T-type not fitted; L-type fit: \(V_{1/2} = -30 \pm 1.4\) mV and \(s = 4 \pm 1.3\)), where \(V_{1/2}\) is half-maximal voltage activation and \(s\) is slope factor.

\(\text{Ca}^{2+}\) efficiency. The Ca\(^{2+}\) dependence of exocytosis driven by each type of Ca\(^{2+}\) current was then investigated at different stages of development by measuring \(\Delta C_m\) responses (fF) as a function of \(I_{\text{Ca}}\) charge integral (Q; pC) generated by a constant 100-ms voltage step at different membrane potentials from a holding potential of \(-90\) mV (Fig. 2, A and B). Ca\(^{2+}\) efficiency of exocytosis (\(\Delta C_m/I_{\text{Ca}}\)) increased for both different \(I_{\text{Ca}}\) subtypes during maturation [e.g., Ca\(^{2+}\) efficiency for T-type current: E12, 0.3 \pm 0.3 fF/pC (n = 9); P2, 3.6 \pm 0.2 fF/pC (n = 4), \(P < 0.001\); for L-type current: E12, 0.4 \pm 0.3 fF/pC (n = 9); P2, 8.9 \pm 1.5 fF/pC (n = 4), \(P < 0.001\)]. However, the increase in Ca\(^{2+}\) efficiency during development was much more pronounced for L-type currents than for T-type currents (Fig. 2C). The cooperative index, \(N\) power fit of Ca\(^{2+}\) dependency, decreased similarly throughout development for both channels (Fig. 2D).

To determine more precisely the proportion of T-type and L-type current at each development stage, we compared exocytosis and \(I_{\text{Ca}}\) current amplitude evoked for voltage steps from \(-90\) to \(-30\) mV (a voltage mainly activating low-voltage transient T-type Ca\(^{2+}\) currents) or to \(-10\) mV (voltage activating mainly high-voltage activating, sustained L-type Ca\(^{2+}\) currents; see Fig. 1B). At E12, with stimulation by a 100-ms step from \(-90\) to \(-30\) mV in the presence of nifedipine, T-type Ca\(^{2+}\) current represented 81 \(\pm\) 8\% of total \(I_{\text{Ca}}\) peak and contributed to 60 \(\pm\) 2\% of \(\Delta C_m\). At the mature stage P2, T-type currents represented only 10.0 \(\pm\) 1.0\% of total \(I_{\text{Ca}}\) and evoked 15.3 \(\pm\) 1.5\% of \(\Delta C_m\). On the contrary, at \(-10\) mV in the presence of mibebradil, L-type current accounted for 27.8 \(\pm\) 1.8\% of total \(I_{\text{Ca}}\) at E12 and 90.8 \(\pm\) 5.6\% at P2, which contributed to 15.6 \(\pm\) 0.5\% and 97.8 \(\pm\) 6.5\% of \(\Delta C_m\), respectively.
**RRP and SRP exocytosis.** The kinetics of exocytosis were then studied for a voltage step at $-30$ mV (mainly activating T-type) and $-10$ mV (activating both L-type and T-type) at increasing durations from 10 to 1,000 ms (Fig. 3, A and B). At E12, a stage at which both types of current are present, kinetics of exocytosis showed a similar fast component up to 200 ms at both voltages (Fig. 3C), likely corresponding to the fusion of a readily releasable pool of vesicles (RRP). However, at $-30$ mV, exocytosis rapidly saturated at steps longer than 200 ms and did not display the mobilization of the secondary slowly releasable pool of vesicles (SRP) normally recorded at long steps. It is worth recalling that the SRP is believed to involve the mobilization of vesicles that are located far from the ribbon release sites (Levic et al. 2011; Spassova et al. 2001). These results suggest that SRP vesicles cannot be recruited by T-type Ca$_{2+}$ channels, presumably because of the transient nature (fast inactivation) of the Ca$_{2+}$ current that limits spatial Ca$_{2+}$ diffusion from its entry point during sustained stimulation. Notably, the Ca$_{2+}$-dependence of the RRP ($\Delta$C$_{m}$ against $I_{Ca}$ integral) established at a voltage of $-30$ and $-10$ mV for brief stimulus durations between 10 and 200 ms (Fig. 3D) showed a similar relationship at E12. These results suggest that T-type and L-type Ca$_{2+}$ channels are similarly organized at the sites of vesicle release of the hair cell ribbon synapses.

**Action potential-driven exocytosis.** Finally, we determined the contribution of L- and T-type Ca$_{2+}$ currents in $\Delta$C$_{m}$ responses to a prerecorded native action potential (AP), a physiologically relevant stimulus to developing hair cells (Fig. 4, A and B; Levic et al. 2007). T-type current contribution to AP-induced exocytosis was found to increase from E10 to E16 and then to decrease considerably at P2 (Fig. 5A). Ca$_{2+}$ efficiency of exocytosis for T-type current slightly increased throughout development [e.g., E10, 1.4 ± 0.7 pF/pC (n = 9); E16, 3.3 ± 1.5 pF/pC (n = 10); P2, 5.1 ± 0.5 pF/pC (n = 6); Fig. 5C, P < 0.05]. At all stages of development, the contribution of L-type current to AP-induced exocytosis was much larger and largely increased from E10 to E12 and then plateaued from E12 to P2 (Fig. 5B). Notably, AP-driven exocytosis was more efficiently driven by L-type Ca$_{2+}$ channels than by T-type Ca$_{2+}$ channels (Fig. 5C). The Ca$_{2+}$ efficiency of exocytosis triggered by L-type current increased considerably with maturation up to E16 and then decreased at P2 [e.g., E10, 6.7 ± 1.5 pF/pC (n = 9); E16, 17.6 ± 4. (n = 10); P2 9.1 ± 3.2, (n = 6); Fig. 5C, P < 0.05]. When the Ca$_{2+}$ efficiency of exocytosis generated by L-type currents during AP and step stimulus (100-ms duration from $-90$ to $-10$ mV) is compared, developing hair cells displayed a much greater Ca$_{2+}$ efficiency with AP stimuli at all stages up to E16 [e.g., measured at E12: AP, 12.3 ± 2.3 pF/pC (n = 12); step, 2.5 ± 0.5 pF/pC (n = 9); Fig. 5D, P < 0.05]. Surprisingly, at the mature stage P2, the exocytotic Ca$_{2+}$ efficiency evoked with APs declined to reach values similar to that evoked by voltage steps. In contrast, exocytosis driven by T-type currents evoked during AP and voltage steps showed similar Ca$_{2+}$ efficiency at all developmental stages.

$\Delta$C$_{m}$ and Ca integral. L-type Ca$_{2+}$ channels are believed to be organized in clusters at the synaptic ribbon where they constitute Ca$_{2+}$ microdomains that trigger exocytosis. Our results showing a T-type-triggered RRP with kinetics similar to that triggered by L-type at E12 suggest that T-type channels are also closely associated with the synaptic ribbons. Although developing chick hair cells have been shown to express T-type Ca$_{3.1}$ mRNA (Levic et al. 2007), it is still unknown where the Ca$_{3.1}$ protein is localized in hair cells. For this reason, we used high-resolution confocal fluorescent microscopy to image the immunolabeling of the Ca$_{3.1}$ protein (alpha Ca$_{3.1}$) in developing chick hair cells. In good agreement with our electrophysiological and pharmacological results, Ca$_{3.1}$ (T-type subunit) immunolabeling could be detected in chick hair cells as early as E10. Immunolabeling with an antibody directed against CtBP2 (a marker of the synaptic ribbon)
Our data suggest that exocytosis in developing hair cells is triggered by the summation of Ca\(^{2+}\) entering through L- and T-type voltage-gated Ca\(^{2+}\) channels, similar to retinal bipolar cells, olfactory bulb neurons, chromaffin cells, and mechanoreceptors of the dorsal root ganglia (Egger et al. 2003; Pan et al. 2001; Shin et al. 2003; and for review see Carbone et al. 2006). With hair cell development, the participation of T-type Ca\(^{2+}\) current progressively decreases, whereas that of L-type increases. Loss of low-voltage-activated T-type Ca\(^{2+}\) current has also been documented in developing neurons (Chambard et al. 1999) and chromaffin cells (Levitsky and López-Barneo 2009).

Interestingly, in immature hair cells, Ca\(^{2+}\) influx from both types of Ca\(^{2+}\) channels seems to address the same synaptic machinery up to E12, since the respective synaptic transfer function showed a similar relationship in terms of Ca\(^{2+}\) efficiency and Ca\(^{2+}\) cooperative index when activated by depolarizing voltage steps. However, with maturation from E12 to P2, exocytosis evoked by L-type Ca\(^{2+}\) channels showed a much steeper increase in Ca\(^{2+}\) efficiency compared with T-type, suggesting that L-type Ca\(^{2+}\) channels become more closely associated with the synaptic machinery.

We found that exocytosis evoked by T-type Ca\(^{2+}\) current lacked the slow release or sustained component (SRP), a component involving the mobilization of vesicles that are likely located quite far (>200 nm) from the ribbon release sites (Levic et al. 2011; Spassova et al. 2001). This result is in good agreement with the transient (fast inactivating) nature of the T-type Ca\(^{2+}\) current, which probably limits the size of the T-type Ca\(^{2+}\) microdomains upon voltage activation. Furthermore, the lack of SRP is also in good agreement with a restricted localization of T-type Ca\(_V^{3.1}\) channels at the ribbon, as indicated by immunoreactivity.

The use of two distinct classes of Ca\(^{2+}\) currents in chick hair cells contrasts with what occurs in developing hair cells of the altricial mammals mouse and gerbil, where the spatial distribution and number of L-type Ca\(^{2+}\) channels change, but not so much the channel properties (Johnson and Marcotti 2008; Zampini et al. 2010). Moreover, immature chick hair cells apparently lack the fast-inactivating Na\(^{+}\) currents (Levic et al. 2007), which are transiently present in early developing mouse hair cells (Marcotti et al. 2003). Note that Na\(^{+}\) currents are reported to contribute to the initial phase of AP in developing mouse (Marcotti et al. 2003), whereas T-type currents are reported to play this role in chick hair cells (Levic et al. 2007).

The use of AP stimulation as physiologically relevant stimuli for developing hair cells provided interesting insights into the respective contribution of L- and T-type channels to hair cell exocytosis. In immature hair cells up to E16, exocytosis associated with L-type channels, but not T-type channels, showed a much higher Ca\(^{2+}\) efficiency during AP stimulation compared with voltage steps of similar duration. This indicates that the temporal statistics of the Ca\(^{2+}\) stimuli play a key role in stimulus-secretion coupling in immature hair cells. At central synapses such as the calyx of Held, presynaptic APs have very fast kinetics (half-width between 0.27 and 0.4 ms) and each AP is estimated to open only a small fraction (35–50%) of the total Ca\(^{2+}\) channels that will in turn activate only a small fraction of the release sites (see Wang et al. 2009). This fast presynaptic AP in central synapses provides an impulse-like signal to trigger vesicle release, allowing then an efficient preservation of timing information. In immature hair cells, in

**DISCUSSION**

The present report characterizes the functional changes occurring during progressive maturation of the hair cell synaptic machinery after the onset of hearing in a precocial vertebrate, the chick, where sound-evoked cochlear nuclei activity can be measured as early as E12 in ovo, with high threshold and broadband frequency coding, and reaches full maturation 1–2 days posthatching (Saunders et al. 1973). Remarkably, exocytosis of chick hair cells progressively displays faster kinetics and higher Ca\(^{2+}\) efficiency with maturation of the auditory system (Levic et al. 2011). Similar changes have been shown in hair cells of altricial prehearing animals such as gerbil and mouse (Johnson et al. 2005, 2009). For the first time, we provide strong evidence for the involvement of both T-type and L-type Ca\(^{2+}\) channels in triggering synaptic exocytosis in developing chick auditory hair cells. Finally, we found that AP-driven hair cell exocytosis is more efficiently triggered by L-type Ca\(^{2+}\) channels than by T-type Ca\(^{2+}\) channels in immature hair cells.

**Fig. 3.** Kinetics of exocytosis evoked by L- and T-type Ca\(^{2+}\) currents. A: example of \(I_{\text{Ca}}\) and \(\Delta C_m\) recorded in E12 tall apical hair cells at 100-ms, 500-ms, and 1-s steps from −90 to −10 mV. B: example of \(I_{\text{Ca}}\) and \(\Delta C_m\) recorded in E12 tall apical hair cells at 100-ms, 500-ms, and 1-s depolarizing steps from −90 to −30 mV (activating mainly T-type channels). C: kinetics of exocytosis from 20 ms to 1,000 ms for constant step potentials to −30 mV (\(n = 5\)) and −10 mV (\(n = 5\)) in E12 hair cells. D: Ca\(^{2+}\) efficiency; synaptic transfer functions relating \(Q_{\text{Ca}}\) and \(\Delta C_m\) measured at different stimulus durations elicited from −30- and −10-mV step potentials in E12 hair cells. Synaptic transfer functions were fitted with power functions where \(N = 0.7\) at −30 mV and \(N = 0.9\) at −10 mV.
contrast, native spontaneous APs have slow kinetics (half-width ~5 ms; Levic et al. 2007). These slow APs should therefore maximally activate all Ca\(^{2+}\) channels in hair cells, similarly to sustained depolarizing steps to -10 mV (Patillo et al. 1999). However, the major difference between stimulating with a step potential and a slow AP is that Ca\(^{2+}\) channels are instantaneously activated during a voltage step, whereas they are progressively recruited within a 5-ms period during an AP.

What mechanisms underlie a better Ca\(^{2+}\) efficiency for exocytosis during AP stimulation compared with voltage step in immature hair cells? With a diffusion coefficient for Ca\(^{2+}\) in cytoplasm ranging between 200 and 400 \(\mu m^2/s\) in our EGTA recording conditions (Roberts 1994; Wang et al. 2009; Wu et al. 1996) and an approximate total duration of 10 ms for the hair cell AP, the Ca\(^{2+}\) shell from individual Ca\(^{2+}\) channels upon single AP stimulation can be estimated as having a rather large diameter of about 70–140 nm. Considering that the hair cell ribbon active zone is about 250 nm in diameter and contains about 80 channels (Martinez-Dunst et al. 1997; Roberts 1994), our results suggest that exocytosis during a single AP displays a maximum Ca\(^{2+}\) efficiency when individual Ca\(^{2+}\) domains overlap at the ribbon active zone. This overlap of Ca\(^{2+}\) channel activity is likely essential for maximizing the exocytosis efficiency during an AP in immature hair cells. Furthermore, it is worth mentioning that the release kinetics of synaptic vesicles in hair cells are likely limited by the rate of site clearance and endocytosis of the fused vesicles at the active zone (Beutner and Moser 2001), as also suggested in

---

**Fig. 4.** Exocytosis evoked by prerecorded native action potentials (APs). A: \(I_{Ca}\) and \(\Delta C_{m}\) recordings in an apical E12 hair cell consecutive to a single AP, before (black) and after the sequential applications of 10 \(\mu M\) mibefradil (dark gray) and 10 \(\mu M\) nifedipine (light gray) to completely block \(I_{Ca}\) and \(\Delta C_{m}\). B: recording examples as in A, but nifedipine (dark gray) was added first.

**Fig. 5.** Relative contribution of L- and T-type Ca\(^{2+}\)-currents to exocytosis evoked by prerecorded native APs during development. A: mean mibefradil-sensitive \(\Delta C_{m}\) responses measured in E10 (\(n = 9\)), E12 (\(n = 12\)), E16 (\(n = 10\)), and P2 (\(n = 6\)) hair cells. B: mean nifedipine-sensitive \(\Delta C_{m}\) responses measured in E10 (\(n = 9\)), E12 (\(n = 12\)), E16 (\(n = 10\)), and P2 (\(n = 6\)) hair cells. C: Ca\(^{2+}\) efficiency coupling of T- and L-type currents to AP-evoked exocytosis in E10 (\(n = 9\)), E12 (\(n = 12\)), E16 (\(n = 10\)), and P2 (\(n = 6\)) hair cells. D: comparative Ca\(^{2+}\) efficiency of L- and T-type currents using depolarizing steps (100 ms, from -90 to -10 mV) and prerecorded AP.
central synapses (see Neher 2010). A slow recruitment of Ca$^{2+}$/H11001 channels during APs in hair cells could nearly set the rate of vesicle fusion to equilibrium with the rate of site clearance and endocytosis, maximizing therefore exocytosis with Ca$^{2+}$/H11001 influx. On the contrary, an instantaneous recruitment of all Ca$^{2+}$/H11001 channels during voltage steps would instantaneously saturate the process of site clearance, producing then an apparent decrease in Ca$^{2+}$/H11001 efficiency. An alternative explanation for the higher Ca$^{2+}$/H11001 efficiency of exocytosis with the use of AP could also arise from the rate of vesicle replenishment at the sites of release, which could also be a limiting factor. Indeed, since synaptic vesicle reloading at the release sites is also Ca$^{2+}$/H11001 dependent (Levic et al. 2011), the temporal characteristics of Ca$^{2+}$/H11001 influx might also influence exocytosis by affecting the reloading rate of synaptic vesicles at the sites of release.

In mature P2 hair cells, we found that the Ca$^{2+}$/H11001 efficiency of exocytosis during AP stimulation declined, whereas that evoked during voltage steps largely increased, compared with E16 hair cells (see Fig. 5D). Mature P2 hair cells displayed exocytosis with similar Ca$^{2+}$/H11001 efficiency when stimulated either by voltage steps or with APs. One possible mechanism underlying such change in mature hair cells is that the spatial coupling between L-type Ca$^{2+}$/H11001 channels and synaptic vesicles tightens in nanodomains, as previously suggested in mouse cochlear hair cells (Brandt et al. 2005), so that the vesicular Ca$^{2+}$/H11001 sensors are more quickly exposed to higher Ca$^{2+}$/H11001 concentrations. Synaptic release in a nanodomain organization would mainly depend on the open probability of individual Ca$^{2+}$/H11001 channels, rather than the total number of Ca$^{2+}$/H11001 channels activated, and would therefore be less sensitive to the temporal characteristics of Ca$^{2+}$/H11001 diffusion. An alternative or additional explanation to the nanodomain hypothesis could be that the Ca$^{2+}$/H11001 sensors acquire higher Ca$^{2+}$/H11001 sensitivity and can readily detect smaller Ca$^{2+}$/H11001 concentrations during development.

In conclusion, our study shows that chick auditory hair cells use both T-type and L-type Ca$^{2+}$/H11001 channels to initiate synaptic exocytosis during development. The use of low- and high-voltage-activated Ca$^{2+}$/H11001 channels by hair cells may be critical in coding specific patterned information in the central auditory pathway during development.

ACKNOWLEDGMENTS

We thank Christine Petit, Maryline Beurg, and Ebenezer Yemoah for helpful discussion and Yohan Bouleau for skilled technical assistance.

GRANTS

This work was supported by Institut National de la Santé et de la Recherche Médicale, the University of Bordeaux Segalen, and the Fondation Voir et Entendre.
DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.L. and D.D. conception and design of research; S.L. performed experiments; S.L. and D.D. analyzed data; S.L. and D.D. interpreted results of experiments; S.L. and D.D. prepared figures; S.L. and D.D. edited and revised manuscript; S.L. and D.D. approved final version of manuscript; D.D. drafted manuscript.

REFERENCES


Neher E. What is rate-limiting during sustained synaptic activity: vesicle supply or the availability of release sites. Front Synaptic Neurosci 2: 144, 2010.


