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Research report

# Calcium 'leak' through somatic L-type channels has multiple deleterious effects on regulated transmitter release from an invertebrate hair cell

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**Abstract**

Using an identified synapse in the nervous system of the mollusc *Hermissenda*, the influence of somatic calcium accumulation on regulated synaptic transmission was investigated. Hair cells in *Hermissenda* project onto postsynaptic B photoreceptors where they mediate inhibitory postsynaptic potentials (IPSPs). Intracellular recordings in combination with bath perfusion of calcium channel modulators indicated that L-type channels were present on the hair cell soma but not on the terminal branches. In contrast, P/Q and an unidentified channel type (similar to N-type channels) contributed additively to transmitter release from the hair cell. Antibodies raised against rat brain channel proteins detected L- ($\alpha 1_C$) and P/Q-type ($\alpha 1_A$) channels in lysates of the *Hermissenda* nervous system, indicating a homology between the *Hermissenda* channels and their mammalian counterparts. To mimic somatic calcium channel 'leak', hair cells were exposed to the L-type channel agonist ±BAY K 8644. Exposure to ±BAY K 8644 resulted in a rapid (<2 min) increase (40%) in the amplitude of the spike after-hyperpolarization in the hair cell, and was associated with a reduction in evoked firing frequency. This reduction in rate of discharge induced a proportional decrease in the amplitude of compound IPSPs recorded in the postsynaptic B photoreceptors. From Fura-2 emissions we determined that ±BAY K 8644 induced a rapid (<2 min) and persistent increase (70%) in somatic calcium concentration, followed by a slower elevation of calcium in the medial axon (>30 min) and subsequently in the terminal branches (>40 min), suggesting that excessive somatic calcium had diffused or induced a propagation along the axon. Corresponding with a 56% rise in terminal calcium (50–60 min post agonist), postsynaptic potentials declined to 70% of baseline amplitude. These results suggest that prolonged somatic L-channel 'leak' can interfere with regulated transmitter release, both by reducing the rate of presynaptic discharge and by promoting terminal calcium accumulation that may oppose transmitter release. Such effect may have implications for the age-related learning deficits that often accompany somatic calcium 'leak'.


*Keywords:* Calcium; Calcium channel; Calcium buffering; Neurotransmission; Action potential; Aging; Memory

## 1. Introduction

Brain cells in aged animals develop a propensity to 'leak' $Ca^{2+}$ through L-type channels, an effect implicated in age-related learning impairments. For instance, CA1 pyramidal cells in aged rabbits exhibit increased spike afterhyperpolarization (AHP) and greater spike-rate accommodation, effects which are reversed by the L-type $Ca^{2+}$ channel antagonist nimodipine [13,32]. Relatedly, nimodipine treatment of aged rabbits (that exhibit moderate learning deficits) facilitates their acquisition of a classically-conditioned eyeblink response while having no affect on the rate of learning exhibited by young animals [10,11], leading to suggestions that $Ca^{2+}$-induced decreases in cell excitability contribute to age-related learning deficits [12,14]. In addition, the persistent elevation of intracellular $Ca^{2+}$ can impede $Ca^{2+}$ buffering [9,17,21] and may promote neurodegeneration [43], effects characteristic of the aged brain. Moreover, elevated intracellular $Ca^{2+}$ may alter the balance of protein kinase/protein phosphatase interactions [33] and may thus impair $Ca^{2+}$-dependent modifications of synaptic strength such as long-term potentiation (LTP) or depression (LTD) [15,35].

*Abbreviations*: AHP, after-hyperpolarization; ASW, artificial seawater; $Ca^{2+}$, calcium; IPSP, inhibitory postsynaptic potential; $K^+$, potassium; LTD, long-term depression; LTP, long-term potentiation

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While each of the above consequences of $Ca^{2+}$ leak may contribute to age-related learning impairments, somatic $Ca^{2+}$ leak may promote learning impairments as a secondary consequence of its influence on neurotransmitter release. Previous work has indicated that the intracellular $Ca^{2+}$ elevations characteristic of CA1 pyramidal cells in aged animals are correlated with significantly larger amplitude AHPs following single spikes and spike bursts [13,32] as well as a decrease in rate of spontaneous- and depolarization-evoked action potentials [32,41]. Whether the decline in rate of discharge is entirely a consequence of the amplification of the AHP is unclear. Nevertheless, the higher threshold for the elicitation of action potentials as well as the reduced rate of discharge will under many conditions (e.g. during spike trains) induce a commensurate decline in cumulative transmitter release.

In addition to its likely influence on transmitter release as a consequence of its effects on presynaptic discharge, basal $Ca^{2+}$ leak through L-type channels may have a direct impact on the regulation of transmitter exocytosis. Rapid (<1 ms) voltage-dependent $Ca^{2+}$ transients at release sites are the catalyst for transmitter exocytosis [26], but sustained elevation of intracellular $Ca^{2+}$ in these compartments can impede regulated exocytosis by inactivating voltage-dependent $Ca^{2+}$ channels [40] and/or by stimulating spontaneous transmitter vesicle release [7,23]. However, $Ca^{2+}$ channel subtypes are differentially localized in neurons [25,26] in the mammalian brain, and L-type $Ca^{2+}$ channels are commonly expressed at the soma and proximal dendrites of neurons while exocytosis is more typically regulated by combinations of high-voltage activated P/Q-, N-, or R-type $Ca^{2+}$ channels. Thus it is not clear that $Ca^{2+}$ leak through somatic L-type channels would have any *direct* (i.e. $Ca^{2+}$-dependent) influence on transmitter release. Despite the segregation of channel subtypes, the sustained elevation of intracellular $Ca^{2+}$ will in some instances (e.g. in relatively wide retinal rod cells) exceed the capacity of local buffering mechanisms, allowing $Ca^{2+}$ to diffuse away from its point of origin [18]. Moreover, sustained $Ca^{2+}$ elevations may initiate the propagation of slow $Ca^{2+}$ waves by stimulating $Ca^{2+}$ release from intracellular stores [39,42]. Thus it is possible that sustained somatic $Ca^{2+}$ elevation may have consequent effects on $Ca^{2+}$ concentrations and $Ca^{2+}$-dependent processes (e.g. regulated exocytosis) in spatially segregated cell compartments.

In the present experiments, we directly assessed the effects of prolonged somatic $Ca^{2+}$ elevations on regulated transmitter release. Since small elevations in presynaptic $Ca^{2+}$ concentration can in some instances induce an increase in the amplitude of unitary postsynaptic potentials by effectively lowering the threshold for quantal release, we focused our analysis primarily on superthreshold postsynaptic responses, i.e. on compound IPSPs induced by sustained bursts of presynaptic activity. Synaptically-coupled cells in the *Hermissenda* nervous system were identified at which L-type channels were expressed on the presynaptic soma and a combination of P/Q- and possibly N-type $Ca^{2+}$ channels regulated transmitter release. Somatic $Ca^{2+}$ 'leak' was mimicked by inducing L-channel hyperconductance with the L-channel agonist ±BAY K 8644, and intracellular $Ca^{2+}$ accumulation and propagation across subcellular regions were estimated based on the emission of the fluorescent $Ca^{2+}$ indicator Fura-2. In parallel electrophysiological experiments, the effects of this presynaptic $Ca^{2+}$ leak on regulated transmitter release were assessed.

## 2. Procedures

### 2.1. Animals and recording solutions

Adult *Hermissenda* were obtained from Sea Life Supply (Sand City, CA) and were maintained in 14 °C artificial sea water (ASW) according to previous methods [33]. The *Hermissenda* nervous system was surgically excised and secured with stainless steel pins in a glass recording chamber. To facilitate visualization and electrode impalement, isolated nervous systems were treated for 5–8 min at 24 °C with 10 mg/ml protease (Sigma, P6141) in ASW (NaCl 430 mM, $CaCl_2$ 10 mM, $MgCl_2$ 50 mM, KCl 10 mM, Tris buffered to pH 7.4). The nervous systems were then rinsed in 14 °C ASW, and all optical and electrophysiological recordings were conducted in a 14 °C ASW bath supplemented with pharmacological agents.

### 2.2. Pre- and postsynaptic voltage responses during exposure to $Ca^{2+}$ channel modulators

Intracellular recordings were made in *Hermissenda's* vestibular hair cells and in their postsynaptic target, the medial or lateral B photoreceptor. Voltage responses were obtained with glass electrodes (30–55 MΩ, 3.0 M KAc fill) connected in a balanced-bridge circuit to an Axoclamp 2B amplifier (Axon Instruments, Burlingame, CA). Recordings from both the presynaptic hair cell and the postsynaptic photoreceptor were obtained from an imposed resting potential of −60 mV. Little spontaneous spike discharge is observed at this potential, which is ~20 mV more positive than the potential at which hair cell-induced IPSPs in the B cell reverse [37].

The 13 hair cells of the *Hermissenda* statocyst (the vestibular organ) project onto the B photoreceptors where they induce inhibitory postsynaptic potentials (IPSPs) [1,22]. To limit $Ca^{2+}$-induced potentiation of transmitter release from the hair cells that might occur during periods of submaximal transmitter release, super-threshold release was induced with a repetitive and prolonged discharge in all hair cells. For this purpose, hair cells were mechanically stimulated with a glass rod placed on the statocyst that extended from a piezoelectric crystal (Archer 273-073) attached to an audio oscillator (Hewlett-Packard 200AB). When activated (1600 Hz), the vibrating rod induced a rotation of statoconia within the statocyst, depolarizing the hair cells and inducing a compound IPSP in postsynaptic B photoreceptors. The rod was placed on the hair cells in a manner that induced the maximal IPSP (typically 3–8 mV) in the B cell.

To assess the pharmacological sensitivity of transmission from hair cells onto the photoreceptors, IPSPs in the photoreceptor were induced with 2 s of hair cell stimulation before and after introduction of pharmacological agents into the bath. For each cell, the peak hyperpolarization in the B cell during at least three evoked synaptic responses were averaged and served as the data for any single time point. Baseline IPSPs were acquired at 3-min intervals for 9 min, followed by bath perfusion of either T-conotoxin (1 μM), T-agatoxin (300 nM), or nifedipine (10 μM). These concentrations were selected based on work in other systems where they were found to be relatively selective for N-, P/Q-, and L-type $Ca^{2+}$ channels, respectively. Following introduction of the pharmacological agent, IPSPs were collected for 20 min, and IPSPs were again collected for 20 min after drug washout.

The spike afterhyperpolarization (AHP) in the hair cell is dependent on $K^+$ efflux through a $Ca^{2+}$-activated $K^+$ channel and is an indirect index of $Ca^{2+}$ levels near the point of origin of the action potential. As a gross index of whether L-type channels were present on, or proximal to, the soma membrane, we assessed the effects of L-type channel modulators on the hair cell AHP. Depolarizing voltage (0.1–0.6 nA, 300 ms) sufficient to induce a single spike was injected into the hair cell. Following baseline recordings, either nifedipine (10 μM) or the L-type channel agonist ±BAY K 8644 (7 μM) was perfused into the bath. After 3 min, action potentials were again evoked, followed by drug washout and additional recordings for 20 min. For purposes of analysis, three to five spikes were generated at any single time point, and the mean amplitude of the AHP for these spikes served as that data point.

In subsequent experiments we intended to ascertain the effect of somatic $Ca^{2+}$ elevation on evoked transmitter release from the hair cells. For this purpose, $Ca^{2+}$ accumulation would be induced by sustained exposure (60 min) of the hair cells to ±BAY K 8644. Thus it was necessary to determine the effects of prolonged exposure to ±BAY K 8644 on transmitter release-related properties of the hair cell as well as on its general viability. Consequently, in an independent group of hair cells we recorded the resting membrane potential, spike amplitude and duration (of single evoked spikes), and rate of discharge in hair cells before and after 60 min of exposure to ±BAY K 8644. Spike amplitude and duration were assessed based on the average values obtained from four individual spikes (inter-spike interval >10 s), and each spike was evoked during a slow depolarizing current ramp (typically spike thresholds ranging from +0.1 to 0.3 nA) from an imposed resting potential of −60 mV. Amplitude was measured relative to the pre-spike membrane potential, and the duration was calculated at the point on the spike that corresponded to 30% of its peak amplitude. Rate of spike discharge was averaged (in each cell) from two 2-s depolarizing steps (inter-step interval >30 s) sufficient to depolarize the cell to −45 mV from its imposed resting potential of −60 mV. This level of depolarization is comparable to that of the generator potential induced by mechanical stimulation of hair cells (i.e. as in companion experiments reported in Ref. [37]).

In conducting the prior experiment, it became clear that ±BAY K 8644 reduced the rate of spike discharge (but not spike amplitude or duration) during sustained depolarization of the hair cell. Such an effect would be unlikely to influence the amplitude of unitary IPSPs in the B cell, but might reduce the amplitude of the compound IPSP induced during sustained presynaptic depolarization like that used to induce transmitter release in other experiments in this series. Although a likely outcome, it is possible that the rate of discharge in the hair cell is sufficiently high that small decreases in rate will not have a commensurate effect on the IPSP. Moreover, in instances where the probability of vesicle release is high it is sometimes observed that the amplitude of a compound postsynaptic potential can *increase* in response to a reduction in presynaptic rate of firing within a certain limited range. To determine the relationship of hair cell firing frequency to the amplitude of the IPSP in the B cell, we conducted a set of paired intracellular recordings in which the rate of discharge in the hair cells was manipulated (with varying depolarizing current steps) and the IPSP in the postsynaptic B cell recorded. We have previously reported that a typical hair cell depolarizes to approximately −45 mV during mechanical stimulation, and that the associated firing frequency is, on average, ~18 Hz. To encompass a range of discharge rates around this value, hair cells were depolarized to a range of potentials between −50 and −38 mV and the rates of discharge in the hair cell and the IPSP in the postsynaptic B cell were recorded.

To determine the effect of prolonged ±BAY K 8644 exposure on synaptic transmission, IPSPs were recorded in the B cell in response to hair cell stimulation at regular intervals during a 7-min baseline period and during exposure of the hair cells to ±BAY K 8644 (7 μM). Beginning 2 min after introduction of agonist, IPSPs were recorded at 4-min intervals (for 20 min) and at 8–10-min intervals for an additional 40 min. The agonist was then washed from the bath and IPSPs were recorded for an additional 20 min.

As described above, $Ca^{2+}$ accumulation induced by prolonged exposure to ±BAY K 8644 can impinge on transmitter release through multiple mechanisms. First, the amplitude of compound IPSPs might be reduced in response to a reduction in evoked spike rate owing to the prolongation of the presynaptic AHP induced by ±BAY K

8644. Furthermore, $Ca^{2+}$ accumulation in the presynaptic terminal might increase the rate of spontaneous transmitter release [7,23] (and thus reducing the pool of vesicles available for evoked release) and/or may inactivate voltage-dependent $Ca^{2+}$ channels in the presynaptic terminal [40]. Each of these latter two influences would effectively reduce the amplitude of unitary postsynaptic potentials, and could do so independently of any effect of ±BAY K 8644 on evoked spike rate. To assess this possibility, paired intracellular recordings were made from hair cells and postsynaptic B photoreceptors. The amplitude of unitary evoked IPSPs were assessed in the B cell before and for 50 min after the application of ±BAY K 8644, as well as 10 min after the washout of ±BAY K 8644 from the bath.

### *2.3. $Ca^{2+}$ imaging in hair cells*

The tips of glass electrodes (35–45 MΩ) were filled with the $Ca^{2+}$ indicator Fura-2-pentapotassium (40 mM) dissolved in 200 mM KAc, and the electrode barrels were filled with 3.0 M KAc. Fura was iontophoretically injected into a hair cell by applying −1.2 nA through the electrode for 10–12 min. Subsequently, the nervous system was transferred to a $Ca^{2+}$ imaging system (Intracellular Imaging, Cincinnati, OH), and the ratio of fluorescence at 340 and 380 nm was used to estimate intracellular $Ca^{2+}$. Periods of 1–2 s of 340-nm and 170–340 ms of 380-nm stimulation were typical, and ratios were calculated 10–20 times/min throughout a recording session. The decay of the fluorescent signal across a 70-min recording session was less than 5% with these conditions. $Ca^{2+}$ levels were calculated on-line with ratiometric ion analysis software ($InCa^{++}$, Version 2.5; Intracellular Imaging, Cincinnati OH) and a subsample of images were saved for purposes of illustration. For analysis, Fura-2 ratios in three subcellular regions were averaged in 1-min blocks. These regions included the combined soma and hillock (excluding that portion of the soma over the nucleus), a segment of the axon comprising ~10% of its length and located between its midpoint and the terminals, and the field of terminal branches. These regions of interest are illustrated on the image of a hair cell provided in Fig. 4.

### *2.4. Immunoblotting*

*Hermissenda* nervous systems were homogenized in an ice-cold buffer of Tris–HCl (pH 7.5), 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, and 100 μg/ml benzamidine. The sample was heated at 95 °C for 4 min, separated on 7.5% Tris–HCl Ready Gels (Bio-Rad), and transferred to nitrocellulose. Membranes were blocked in 0.2% non-fat dry milk and incubated in polyclonal antibodies against the $\alpha 1_{A,}$ $\alpha 1_{B,}$ $\alpha 1_{C,}$ and $\alpha 1_D$ subunits of rat brain $Ca^{2+}$ channels (Alimone Labs). In independent replications, antibody concentrations ranged from 1:100 to 1:400 and incubation times from 60 min to 6 h. Membranes were subsequently incubated in goat anti-rabbit IgG (H+L)-AP (1:4000) for 70 min and developed in a chemiluminescent substrate (Immun-Star; Bio-Rad).

## 3. Results

### *3.1. $Ca^{2+}$ channel expression and pharmacological analysis of function*

Proteins from the *Hermissenda* nervous system were blotted and incubated in antibodies against the α1 subunit of rat brain P/Q channels ($\alpha 1_A$), N channels ($\alpha 1_B$), and two forms of L channels ($\alpha 1_C$ and $\alpha 1_D$). Antibody binding to two P/Q channel ($\alpha 1_A$) and one L-type channel ($\alpha 1_C$) protein was observed (Fig. 1A). No binding by antibodies against the N-type ($\alpha 1_B$) or the $\alpha 1_D$ form of L-type channels was observed, even when antibody concentrations were increased 4× relative to that which produced strong binding of the $\alpha 1_A$ and $\alpha 1_C$ antibodies. Thus the P/Q- and L-type $Ca^{2+}$ channels that are expressed in the *Hermissenda* nervous system are homologous to their vertebrate counterparts.

### *3.2. $Ca^{2+}$ channel subtypes underlying transmitter release from hair cells*

Compound IPSPs were recorded in B photoreceptors in response to mechanical stimulation of presynaptic hair cells. Typical voltage responses (compound IPSPs) are illustrated in Figs. 3B and 5D. To assess the pharmacological sensitivity of transmitter release from the hair cells, the amplitude of the IPSP was assessed before and after perfusion of various $Ca^{2+}$ channel modulators. The results of these manipulations are summarized in Fig. 1B. After 18 min of exposure, the L-type channel antagonist nifedipine (10 μM; $n=7$) had no effect on the amplitude of the IPSP, $F(2,20)=1.12$, NS. However, the P/Q-type channel antagonist T-agatoxin (300 nM; $n=6$) and the N-type channel antagonist T-conotoxin (1 μM; $n=7$) reduced the IPSP amplitude 43 and 54%, respectively, $F$s $(2,17–21)\geq 6.98$, $P$s $<0.001$. The IPSP recovered following a 20-min washout of agatoxin, while no recovery was observed following washout of conotoxin. In an additional experiment ($n=4$), IPSPs were measured in the presence of conotoxin in both normal ASW (containing 10 mM $Ca^{2+}$) and in ASW in which the $Ca^{2+}$ concentration was increased to 20 mM (data not shown). In normal extracellular $Ca^{2+}$, conotoxin reduced the IPSP amplitude by 58%. When extracellular $Ca^{2+}$ was subsequently raised to 20 mM, the IPSP recovered to within 9% of baseline, indicating that the conotoxin-induced reduction of the IPSP was consequent to the reduced $Ca^{2+}$ influx through at least one class of $Ca^{2+}$ channel, and that this impairment could be compensated for by increased $Ca^{2+}$ flux through conotoxin-insensitive channels. In total, it appears that P/Q and a conotoxin-sensitive (typically N-type) channel contribute additively to transmitter release in the hair cells. Given that the N-type channel antibody $\alpha 1_B$ did not recognize proteins in the *Hermissenda* nervous system, the exact identity of the conotoxin-sensitive channel is unknown, but may be a variant of the N-type channels expressed in mammalian brain.

### 3.3. L-channel modulation of the hair cell AHP

Intracellular recordings from the somatic membrane of hair cells revealed that the L-channel antagonist nifedipine (10 μM) promoted a 40% reduction (within 3 min) of the amplitude of the AHP ($n=6$), an effect that was reversed within 12 min of nifedipine washout, $F(5,17)=13.8$, $P<0.01$ (Fig. 1C, top panel). The racemic form of BAY K 8644 can shift the voltage-dependence of L-type channels to more negative potentials and can simultaneously increase channel open-time, thus promoting $Ca^{2+}$ influx at normal resting potentials as well as during periods of depolarization. As summarized in the bottom panel of Fig. 1C, ±BAY K 8644 (7 μM) increased the amplitude of the hair cell AHP by 31% ($n=5$). This effect of ±BAY K 8644 was reversed following a 20-min washout of the agonist, $F(4,14)=14.3$, $P<0.01$. Action potentials recorded in normal ASW and in ASW containing ±BAY K 8644 are illustrated in Fig. 2C.

The responses of the hair cells to $Ca^{2+}$ channel modulators suggest that L-type channels are localized near the cell soma (where they influence action potential kinetics), while P/Q-type channels (and possibly N-type channels) are localized in terminal regions of the cell where they regulate neurotransmitter release. This apparent distribution of channel subtypes is consistent with prior biophysical characterizations of $Ca^{2+}$ channels on the *Hermissenda* hair cells [45].

### 3.4. Effect of ±BAY K 8644 on neurophysiological properties of the hair cell

In subsequent experiments, it was our intention to assess the effects of prolonged ±BAY K 8644-induced $Ca^{2+}$ elevations on evoked transmitter release from hair cell

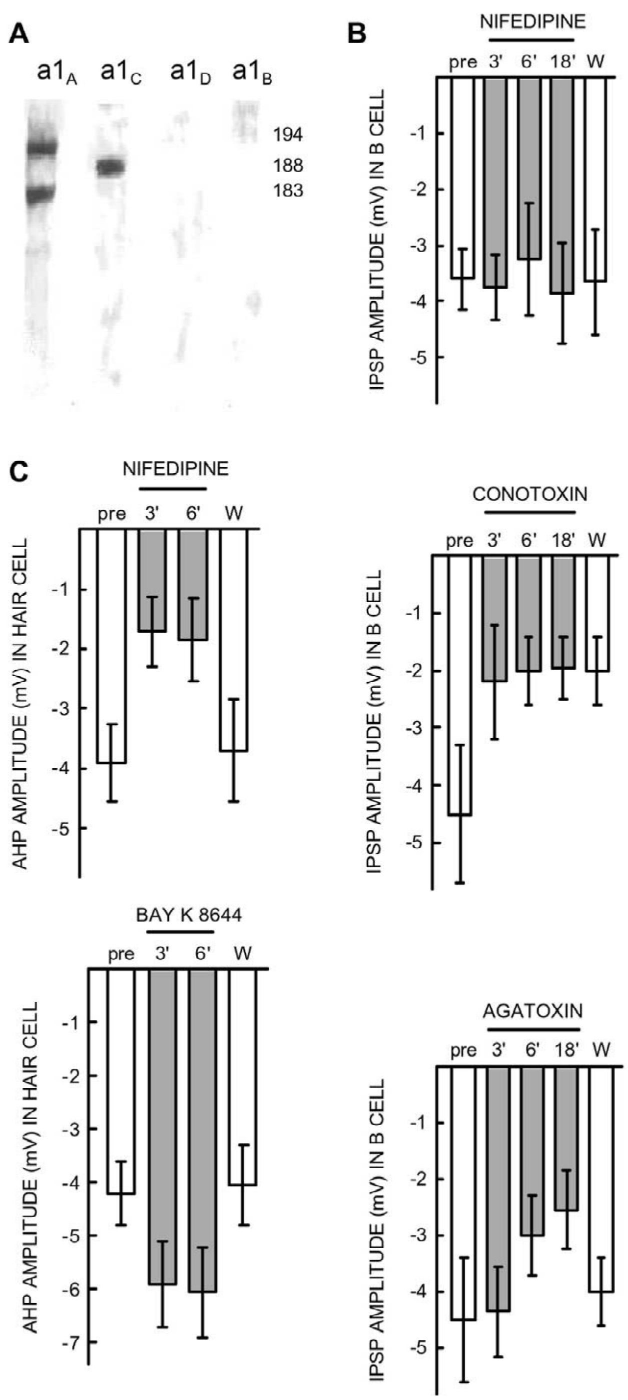


Fig. 1. $Ca^{2+}$ channel subtypes homologous to P/Q- and L-type channels are present in the *Hermissenda* nervous system and have distinct functions in different subcellular regions. (A) Digitized images of immunoblots of the *Hermissenda* nervous system following incubation in polyclonal antibodies against the $\alpha 1_A$ (P/Q), $\alpha 1_C$ (L), $\alpha 1_D$ (L), and $\alpha 1_B$ (N) subunits of rat brain $Ca^{2+}$ channels. Two distinct proteins recognized the $\alpha 1_A$ antibody, and one recognized the $\alpha 1_C$ antibody. No binding of the $\alpha 1_B$ or the $\alpha 1_D$ antibodies could be detected. (B) Summary data ($n=6$–7) of hair cell-induced IPSP amplitudes recorded in B photoreceptors. Inhibition of P/Q-type $Ca^{2+}$ channels by agatoxin (bottom panel) or N-type $Ca^{2+}$ channels by conotoxin (middle panel) each produced partial inhibition of the IPSP, while nifedipine (top panel) had no effect on the response. While the agatoxin-induced inhibition of the IPSP was reversed following washout, the conotoxin-induced inhibition was not. (C) In the hair cell, the L-channel antagonist nifedipine ($n=6$) reduced the amplitude of the $Ca^{2+}$-dependent AHP (top panel) and the L-channel agonist ±BAY K 8644 ($n=5$) increased its amplitude (bottom panel).

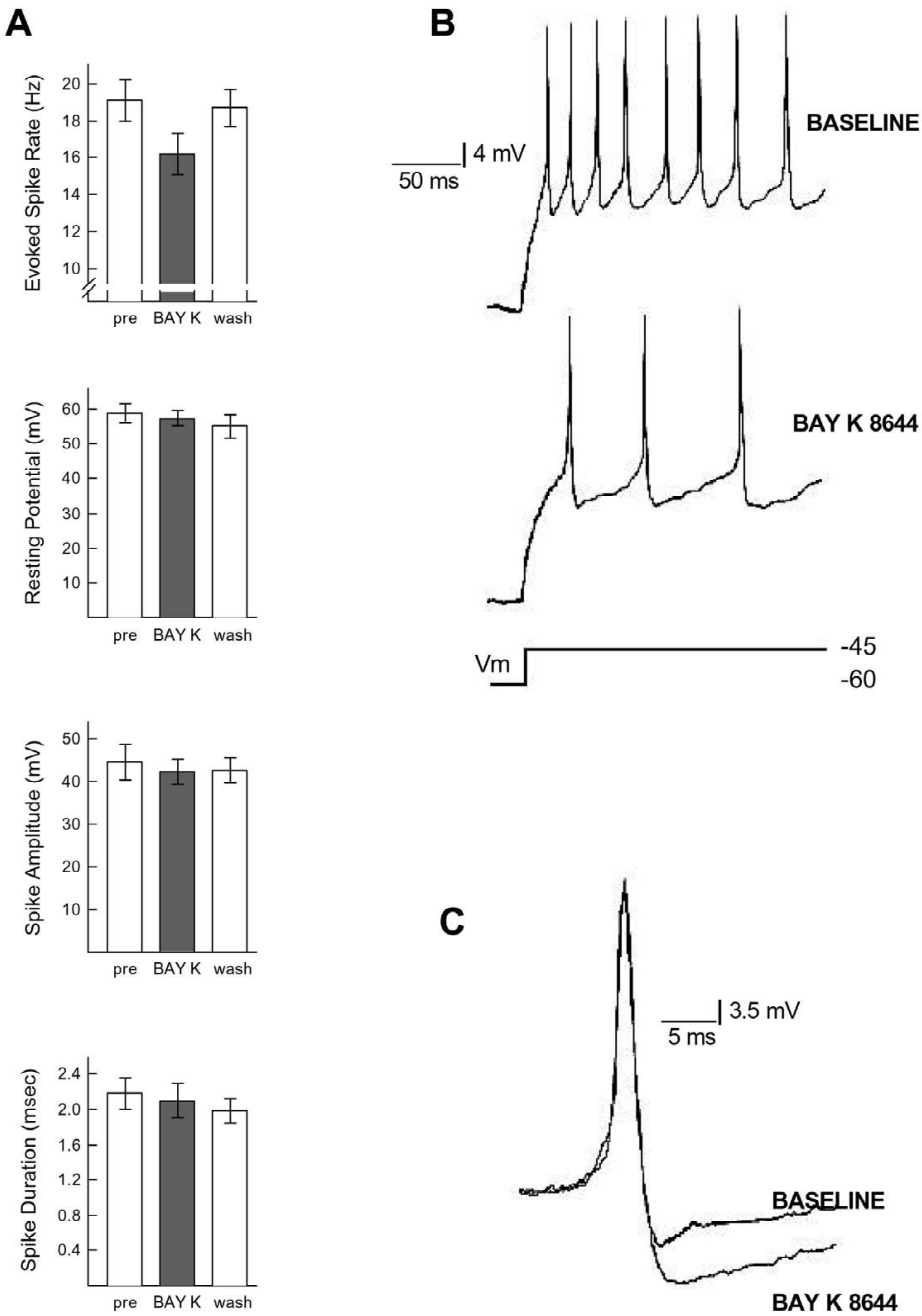


Fig. 2. Intracellular recordings were made from the somatic membrane of hair cells before and 60 min after the introduction of the L-channel agonist ±BAY K 8644 (7 μM) into the intracellular bath. (A) Summary data from hair cells ($n=5$) indicates that ±BAY K 8644 had no effect on the resting membrane potential, spike amplitude, or spike duration. However, ±BAY K 8644 reduced the rate of spike discharge (top panel), and this effect was reversed following washout of the agonist. (B) Representative voltage records obtained during a depolarizing voltage step before and after introduction of ±BAY K 8644 are provided. (C) ±BAY K 8644 increased the amplitude of the post-spike AHP, an effect that likely underlies the reduction in rate of discharge during spike trains. Summary data on the effect of ±BAY K 8644 on the AHP are provided in the bottom panel of Fig. 1C.

terminals, necessitating that we determine the effect of this treatment on basal neurophysiological properties of the hair cells. For this purpose, intracellular recordings from the somatic membrane of hair cells ($n=5$) were made before and after 60 min of exposure of the hair cells to ±BAY K 8644 (7 μM). The effects of ±BAY K 8644 on the rate of discharge during 2 s of current-induced depolarization (analogous to the activity-induced generator potential in the hair cells), the resting membrane potential, and the amplitude and duration of single spikes are summarized in Fig. 2A. After 60 min of exposure, no effect of ±BAY K 8644 on the resting membrane potential, spike amplitude, or spike duration was detected. Repeated-measures ANOVAs found no significant differences between the baseline, drug (60 min), and washout periods on any of these measures, $Fs(4,14)\leq 2.77$, $Ps<0.10$. Although the $Ca^{2+}$-dependent $K^{+}$ current in the *Hermissenda* hair cell has not been characterized, the absence of an effect of ±BAY K 8644 on the amplitude or duration of isolated action potentials (while inducing an increase in the amplitude of the AHP) is likely attributable to the delayed onset and slow rise that is typical of $Ca^{2+}$-dependent $K^{+}$ ($I_{K\text{-}Ca}$) currents. This interpretation is consistent with the known influences of $I_{K\text{-}Ca}$ on the action potential waveform in *Hermissenda* photoreceptors [16,28].

Despite having no effect on the resting potential, spike amplitude, or spike duration, ±BAY K 8644 induced a significant reduction (16%) in the rate of spike discharge during 2 s of depolarization, an effect that was reversed by the washout of the agonist (summarized in Fig. 2A, top panel). This reduction of the evoked spike rate is likely attributable to the amplification of the AHP by ±BAY K 8644, a consequence of which would be to slow the approach to spike threshold and a prolongation of the inter-spike interval. Voltage responses that illustrate the reduction of the rate of discharge induced by ±BAY K 8644 as well as its amplification of the AHP are provided in Fig. 2B and C.

### *3.5. Relationship of hair cell firing frequency to IPSP amplitude*

Paired recordings were made ($n=4$) in which the peak amplitude of the IPSP in the B photoreceptor was recorded in response to bursts of action potentials in presynaptic hair cells. For each hair cell, a distribution of firing frequencies during the activity burst was constructed by alternately depolarizing the hair cell (from an imposed resting potential of −60 mV) to various potentials between −50 and −38 mV. For purpose of analysis, a frequency distribution from 10 to 26 Hz was constructed, and firing frequencies of the hair cells were categorized into four, 4-Hz bins. The amplitudes of the resultant IPSP were recorded for each B cell and constituted a data point for the computation of the mean IPSP corresponding to that firing frequency. As summarized in Fig. 3A (voltage records are illustrated in Fig. 3B), an increase in the hair cell discharge rate from 10 to 25 Hz was accompanied by an approximate doubling of the amplitude of the compound IPSP in the B cell, an effect that is attributable to an increase in the temporal summation of unitary IPSPs concomitant to their frequency of occurrence.

It is important to note that in the presence of ±BAY K 8644, the rate of discharge in the hair cell (at −45 mV) fell from approximately 19 to 16 Hz (Fig. 2A, top panel). On the basis of the present results, we can estimate that this magnitude decrease in firing frequency is apt to result in a 15–20% reduction in the amplitude of the compound IPSP. It should also be noted that an *increase* in the IPSP

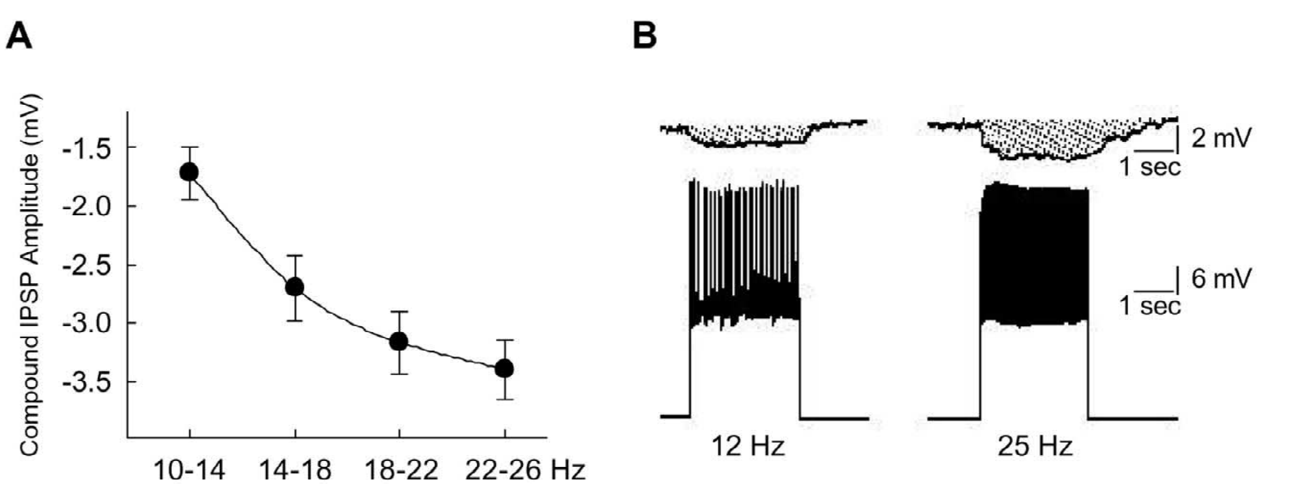


Fig. 3. Paired intracellular recordings from the hair cell and a postsynaptic B photoreceptor ($n=4$) during 3 s of hair cell depolarization. (A) Summary data indicate that the absolute amplitude of the compound IPSP in the B cell is positively related to the firing frequency in the presynaptic hair cell. (B) Representative voltage records illustrating the compound IPSP recorded in the B cell (upper records) in response to 12- or 25-Hz spike trains induced in the presynaptic hair cell.

amplitude was not observed in response to nifedipine (Fig. 1B, top panel), although nifedipine did reduce the amplitude of the hair cell AHP (Fig. 1C, top panel) and might be expected to produce a concomitant *increase* in their rate of discharge. The lack of facilitation of the IPSP by nifedipine is likely attributable to our generation of superthreshold compound IPSPs, i.e. ones that reflect the combined activity induced by the mechanical stimulation of the cluster of hair cells.

### *3.6. $Ca^{2+}$ accumulation and distribution in response to somatic L-channel hyperconductance*

Somatic $Ca^{2+}$ 'leak' through L-type channels was mimicked by prolonged exposure of hair cells to ±BAY K 8644 (7 μM). $Ca^{2+}$ levels were estimated in the soma, intermediate axon, and terminal branches, and pseudocolor $Ca^{2+}$ images acquired for up to 60 min after the introduction of the agonist are provided in Fig. 4. The first image acquired after ±BAY K 8644 introduction (2 min) revealed a marked increase in somatic $Ca^{2+}$ concentration. Within 30 min, $Ca^{2+}$ levels were observed to rise in the intermediate portions of the axon, followed thereafter (>30 min) by a rise in $Ca^{2+}$ concentrations in the terminal regions. This general pattern of results was observed in seven of nine cells (summarized in Fig. 5A). In the remaining two cells (data not shown), a similar fast rise in somatic $Ca^{2+}$ was observed, but no increase in terminal $Ca^{2+}$ was detected after even 60 min of recording. In control experiments ($n=5$), ±BAY K 8644 was omitted from the perfusion vehicle (data not shown). In these cells, $Ca^{2+}$ levels in each of the three cell compartments decreased slightly (average 3–11%) during the 60-min recording. Thus a sustained elevation of somatic $Ca^{2+}$ precipitates $Ca^{2+}$ elevations in distal cell regions.

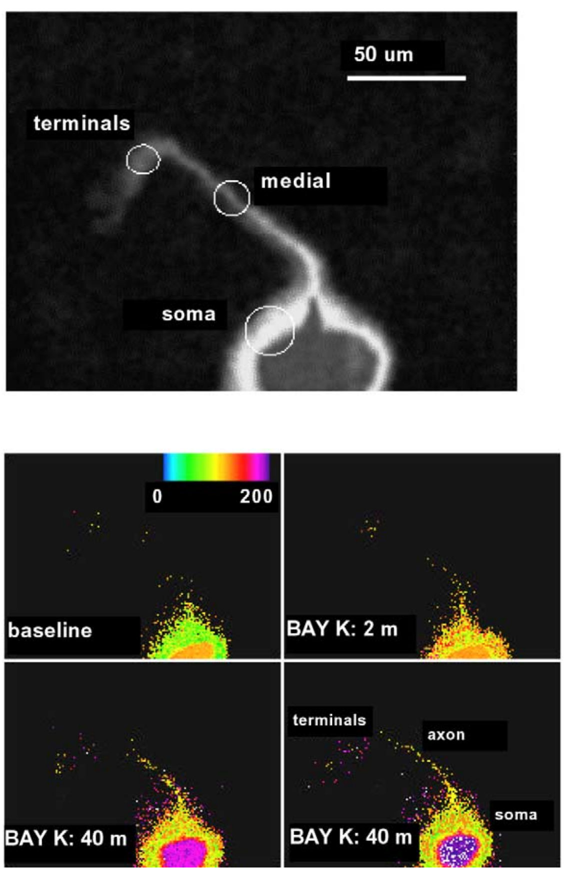


Fig. 4. Pseudocolor Fura-2 images of a hair cell exposed to ±BAY K 8644 (7 μM). After baseline recording, ±BAY K 8644 was introduced into the bath and images were acquired at 2, 40, and 60 min. A significant increase in somatic $Ca^{2+}$ was observed 2 min after ±BAY K 8644 introduction that persisted throughout the remaining 60 min. A distinct rise in $Ca^{2+}$ concentration can be detected in the medial axon 40 min after ±BAY K 8644 introduction, and a similar rise is evident in the terminal regions after 60 min. The region of the soma over the nucleus was excluded from all analyses.

### *3.7. Transmitter release during periods of somatic $Ca^{2+}$ accumulation and propagation*

Compound IPSPs in B photoreceptors induced by 2 s of mechanical hair cell stimulation were measured preceding and for 60 min following exposure of the hair cells ($n=7$) to ±BAY K 8644. This data is summarized in Fig. 5B, and representative voltage records of compound IPSPs are provided in Fig. 5D. The amplitude of the synaptic response decreased ~70% within 60 min of ±BAY K 8644 introduction, i.e. at the time at which terminal $Ca^{2+}$ levels were elevated 56% relative to baseline. During the washout of ±BAY K 8644, we could maintain electrode impalements in only five of the seven cells from the larger sample, and the summary data from those five cells are provided in Fig. 5C. Within 20 min of ±BAY K 8644 washout, the IPSP recovered to within ~17% of its pre-agonist baseline. The mean IPSP amplitudes of these five cells during baseline, 60 min after introduction of ±BAY K 8644, and following washout were compared by repeated-measures ANOVA and were found to differ significantly, $F(3, 11)=5.08$, $P<0.05$.

Paired of intracellular recordings were obtained from hair cells and postsynaptic B photoreceptors before and after bath application of ±BAY K 8644. Evoked unitary IPSPs were obtained in four pairs of cells before, 2 and 50 min after ±BAY K 8644 application, as well as 10 min after washout of the agonist. Voltage traces and summary data for the four pairs of recordings are provided in Fig. 6. The amplitude of the evoked IPSP was initially unaffected by ±BAY K 8644, but was found to decline in amplitude within 50 min of application. A partial recovery of the IPSP was observed within 10 min of the washout of the agonist.

The results from this experiment indicate that the sustained elevation of somatic $Ca^{2+}$ promotes an accumulation of $Ca^{2+}$ in distal cell compartments, and that the eventual elevation of $Ca^{2+}$ in the terminal branches was closely related to a decline in the amplitude of regulated

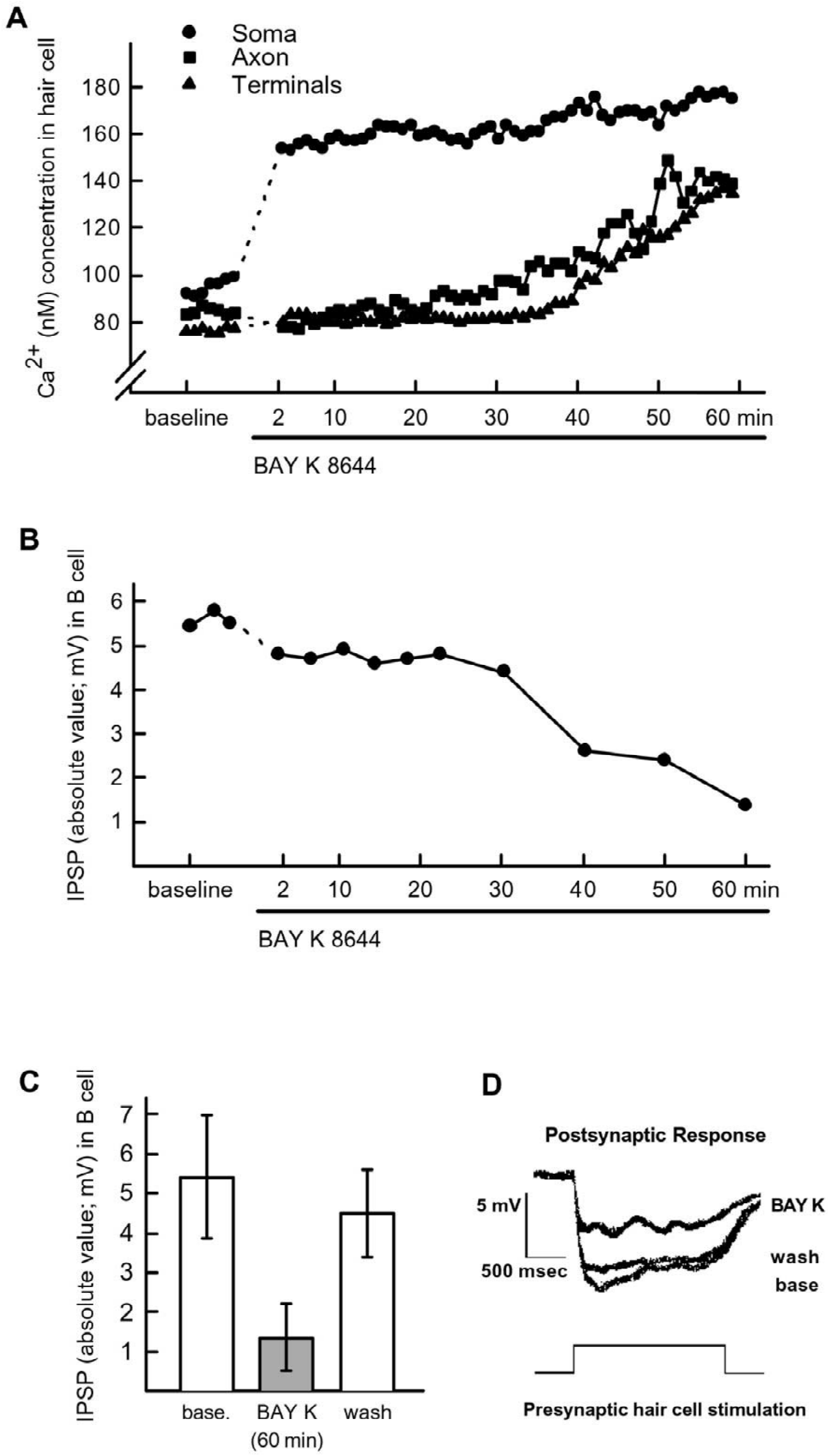


Fig. 5. (A) The mean ($n=7$) concentration of intracellular $Ca^{2+}$ in the soma, medial axon, and terminal branches of hair cells before and for 60 min after exposure to ±BAY K 8644. (B) IPSPs in B photoreceptors were evoked in response to hair cell stimulation, and the mean ($n=7$) IPSP amplitude is plotted before and after exposure of the hair cells to ±BAY K 8644. The amplitude of synaptic responses fell to less than 50% of baseline within 50 min of ±BAY K 8644 exposure, i.e. at the time when terminal $Ca^{2+}$ levels were significantly elevated. (C) Mean ($n=5$) IPSP amplitudes during baseline recordings, after 60 min of exposure to ±BAY K 8644, and again after a 20-min washout period. (D) Representative voltage records of evoked IPSPs in the B cell during baseline recording, after 60 min of exposure to ±BAY K 8644, and again after washout of the agonist.

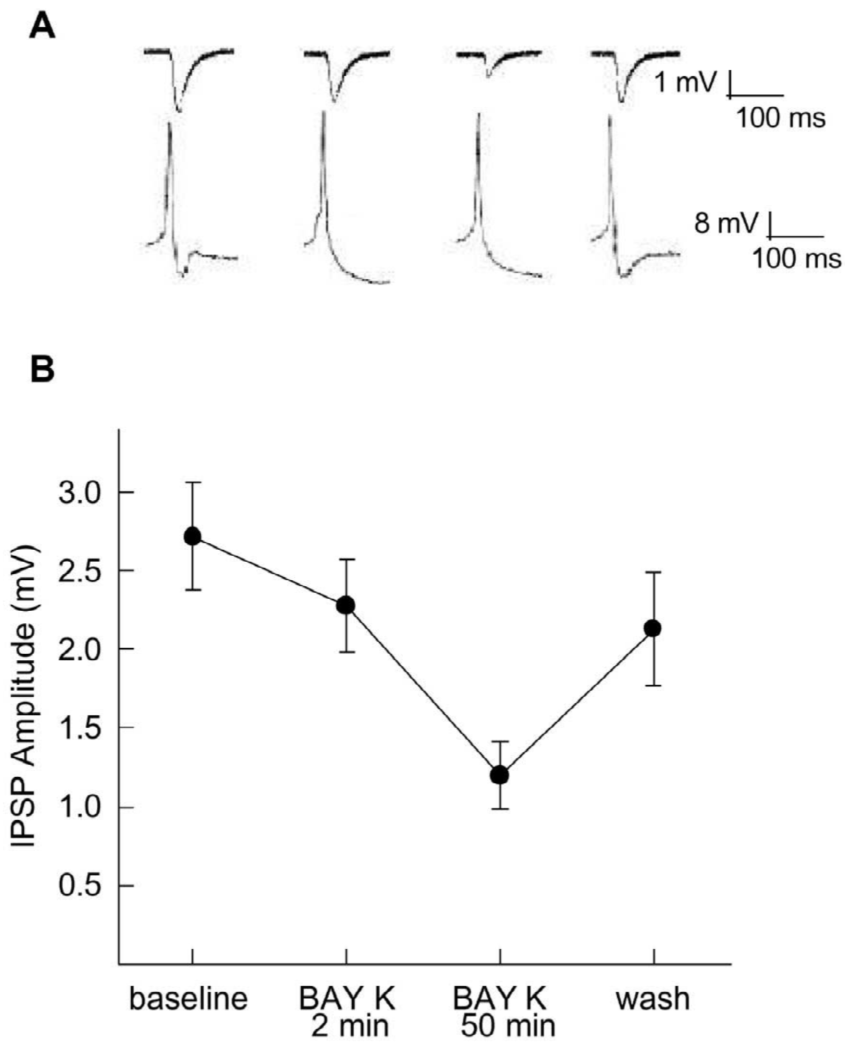


Fig. 6. (A) Paired intracellular recordings were made from a hair cell (lower records) and a postsynaptic B photoreceptor (upper records) before, 2 and 50 min after bath application of ±BAY K 8644, and again after a 10-min washout of the agonist. (B) Summary data obtained from four pairs of recordings (means±S.E.) illustrating the IPSP generated in the B photoreceptor as described in (A).

postsynaptic potentials. This decline in postsynaptic responses is not obviously attributable to a $Ca^{2+}$-induced rundown of cell health, as the hair cells maintained normal resting potentials and supported normal action potentials throughout the 60-min exposure to ±BAY K 8644 (Fig. 2A). Moreover, the recovery of the evoked IPSP following washout of ±BAY K 8644 (Figs. 5C and 6) indicates that the agonist itself (and its associated elevation of intracellular $Ca^{2+}$) was not acutely toxic. The overall pattern of results obtained in these experiments suggests that exposure to ±BAY K 8644 has multiple effects on transmitter release. A rapid decline in the amplitude of compound IPSPs was associated with a ±BAY K 8644-induced decline in evoked spike rate, while after longer exposure, unitary IPSPs were found to decline in amplitude. These results indicate that somatic $Ca^{2+}$ accumulation can have distinct deleterious effects on transmitter release as a function of the duration of the $Ca^{2+}$ elevation.

## 4. Discussion

In this series of experiments we observed that hyperconductance of somatic L-type $Ca^{2+}$ channels can result in a fast and persistent elevation of somatic $Ca^{2+}$ that is associated with a slow accumulation of $Ca^{2+}$ along the cell's axon and eventually in its terminal branches. Although L-type channels did not regulate transmitter release at these terminals, the increase in terminal $Ca^{2+}$ was correlated with a significant impairment of evoked transmission onto postsynaptic targets. This impairment of transmitter release reflected multiple influences of somatic $Ca^{2+}$ accumulation. Initially, somatic $Ca^{2+}$ elevations were associated with an increase in the amplitude of the post-spike hyperpolarization and a consequent decrease in evoked-spike frequency, resulting in a decrease in the amplitude of compound postsynaptic potentials. The subsequent accumulation of $Ca^{2+}$ into the terminal branches of the cell's axon had an additional deleterious effect on the transmitter release evoked in response to single spikes and which was independent of spike rate.

The impairment of regulated exocytosis by terminal $Ca^{2+}$ elevation might reflect a depletion of available transmitter vesicles arising from a $Ca^{2+}$-induced increase in spontaneous vesicle release [23] or a $Ca^{2+}$-dependent inactivation of voltage-dependent P/Q- or N-type $Ca^{2+}$ channels [38]. These possibilities cannot at present be distinguished. Likewise, we cannot determine whether the proliferation of $Ca^{2+}$ from the soma to the terminal regions reflects an active $Ca^{2+}$-induced release of $Ca^{2+}$ from intracellular stores [30,42] or the passive diffusion of unbuffered $Ca^{2+}$ down the axon [18]. This later point is particularly relevant to any attempt to extrapolate these results beyond the current preparation, given that the axons of these sensory cells (and invertebrate cells generally) are relatively broad and short ($<100$ μm).

There is often a cooperative relationship between resting intracellular $Ca^{2+}$ and the influx of $Ca^{2+}$ that accompanies depolarization [2] such that moderate elevations of resting $Ca^{2+}$ can potentiate regulated transmitter release [44]. Our failure to observe such an effect during the early phases of terminal $Ca^{2+}$ elevation (when levels were elevated but relatively low) is likely attributable to our having generated super-threshold compound IPSPs. (It is noted that such an effect might have been observed in the IPSPs evoked by single spikes as reported in Fig. 6. However, for technical reasons we were unable to record IPSPs in the interval between 2 and 50 min following the application of ±BAY K 8644.) During the compound IPSP, presynaptic $Ca^{2+}$ influx during stimulation is likely to far exceed that required for the initiation of exocytosis and thus might not be susceptible to potentiation by elevated resting $Ca^{2+}$. In addition, models of $Ca^{2+}$-induced facilitation of transmitter release assume that the binding kinetics of $Ca^{2+}$ buffers are rapid relative to the rate of $Ca^{2+}$ diffusion [4,34], a requirement that is violated during prolonged periods of $Ca^{2+}$ elevation. Thus our data are consistent with the observation that prolonged release of $Ca^{2+}$ from intracellular stores inactivates voltage-dependent $Ca^{2+}$ channels (and thus regulated transmitter release) [38] and that spike bursts facilitate transmitter release while longer spike trains do not [3]. These patterns of results suggest that accumulation of $Ca^{2+}$ in presynaptic terminals can either enhance or impair synaptic transmission depending on the basal probability of release as well as the duration of the $Ca^{2+}$ signal.

Similar to previous work [13,31], we find that hyperconductance through somatic L-type channels increases the amplitude (and consequently the rate of decay) of spike afterhyperpolarization and results in the inhibition of spike discharge. In addition to the consequent effect on transmitter release during spike trains, prolonged $Ca^{2+}$ flux through somatic L-type channels directly precipitated an attenuation of regulated transmitter exocytosis at release sites distal to the soma membrane. Because activity-dependent forms of neuronal/synaptic plasticity [5,20,29] and related forms of associative learning [6,8,19,27] are directly influenced by basal synaptic efficacy, any impairment of synaptic integration by somatic $Ca^{2+}$ leak is likely to impair learning. Likewise, a deterioration of sensory function has been implicated in age-related cognitive decline [24,36], an effect that may in part reflect an impairment of normal synaptic transmission.

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