Protein Kinase A Mediates Voltage-Dependent Facilitation of Ca\(^{2+}\) Current in Presynaptic Hair Cells in *Hermissenda crassicornis*

Catherine T. Tamse, Yanfang Xu, Haitao Song, Liping Nie, and Ebenezer N. Yamoah. Protein kinase A mediates voltage-dependent facilitation of Ca\(^{2+}\) current in presynaptic hair cells in *Hermissenda crassicornis*. J Neurophysiol 89: 1718 –1726, 2003; 10.1152/jn.00766.2002. The simplest cellular model for classical conditioning in the nudibranch mollusk, *Hermissenda crassicornis*, involves the presynaptic hair cells and postsynaptic photoreceptors. Whereas the cellular mechanisms for postsynaptic photoreceptors have been studied extensively, the presynaptic mechanisms remain uncertain. Here, we show that the phenotype of the voltage-dependent Ca\(^{2+}\) current in the presynaptic hair cells that may be directly involved in changes in synaptic efficacy during classical conditioning. The Ca\(^{2+}\) current can be classified as a P-type current because its activation voltage under seawater recording conditions is approximately −30 mV, it showed slow inactivation, and it is reversibly blocked by ω-agatoxin-IVA. The steady-state activation and inactivation curves revealed a window current, and the single-channel conductance is approximately 20 pS. The P-type current was enhanced by cAMP analogs (approximately 1.3-fold), and by forskolin, an activator of adenylyl cyclase (approximately 1.25-fold). In addition, the P-type current showed voltage-dependent facilitation, which is mediated by protein kinase A (PKA). Specifically, the PKA inhibitor peptide [PKI(6–22)amide] blocked the enhancement of the Ca\(^{2+}\) current produced by conditioning depolarization prepulses. Because neurotransmitter release is mediated by Ca\(^{2+}\) influx via voltage-gated Ca\(^{2+}\) channels, and because of the nonlinear relationship between the Ca\(^{2+}\) influx and neurotransmitter release, we propose that voltage-dependent facilitation of the P-type current in hair cells would produce a robust change in synaptic efficacy.

**INTRODUCTION**

Classical conditioning in the mollusk, *Hermissenda crassicornis*, involves the presentation of light as the conditioned stimulus and turbulence as the unconditioned stimulus. The result is a change not only in this animal’s phototactic behavior (Crow and Alkon 1978; Farley and Alkon 1982) but also in its inherent cellular characteristics. One of these cellular correlates identified after associative learning is found in the ocular photoreceptors. Following conditioning, the excitability of type B photoreceptors and their response to light are enhanced (Alkon et al. 1982, 1985; Blackwell 2002; Crow 1985) while the type A cells exhibit a decrease in light-evoked response (Farley and Han 1997; Richards et al. 1984). The learning-induced photoresponse in these cells is intrinsic (Crow and Alkon 1980; Farley and Alkon 1982; McPhie et al. 1993), with a corresponding increase in input resistance of type B cells (Crow and Alkon 1980). Furthermore, voltage-clamp studies have linked the enhanced excitability of type B photoreceptors in conditioned animals to a reduction in the amplitude of two K\(^{+}\) currents, the transient (I\(_{\lambda}\)) and Ca\(^{2+}\)-activated K\(^{+}\) (I\(_{K,Ca}\)) currents (Alkon et al. 1985).

Extensive studies on neuronal plasticity have also been done in other model systems, using both vertebrates and other invertebrates. In *Aplysia californica*, studies of its defensive siphon and tail withdrawal reflex have demonstrated the role of presynaptic facilitation and a Ca\(^{2+}\)-dependent postsynaptic enhancement (Bao et al. 1998; Hawkins et al. 1983; Murphy and Glanzman 1996), as well as the involvement of activity-dependent plasticity after associative conditioning (Antonov et al. 2001). Moreover, the associative changes in membrane properties and in the synaptic strength of neurons such as in long-term potentiation (LTP) of *Aplysia*’s sensorimotor neurons, are similar to those in hippocampal cells (Lynch et al. 1990; Staubli and Rogers 1994; Williams et al. 1989) and cerebellar cells (Linden and Ahn 1999).

Studies have demonstrated that learning-induced changes involve the influx of Ca\(^{2+}\), activation of Ca\(^{2+}\)-dependent protein kinases, and the resultant phosphorylation of transmembrane ionic channels. However, such cellular changes as observed in *Hermissenda* have most been studied in the cell body of photoreceptors (Crow and Alkon 1980; Yamoah and Crow 1994, 1995; but see Tamse and Yamoah 2002). Among the diverse ionic currents that have been identified in B photoreceptors is depolarization-induced inward rectifier current (I\(_{b}\)) that is activated by membrane hyperpolarization (Yamoah et al. 1998). Thus inhibitory inputs from the *Hermissenda* hair cells can be manifested as excitatory output in the B photoreceptors. Several ionic currents in hair cells were described previously (Yamoah 1997). However, it still remains unclear what modifications ensue in the hair cells during conditioning. We hypothesize that activity-dependent modification of the biophysical properties of hair cells can increase the strength of the hair cell-photoreceptor synapse.

Here, we show that *Hermissenda* hair cells express a P-type Ca\(^{2+}\) current that exhibits voltage-dependent facilitation. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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single-channel conductance of the P-type channel in hair cells is approximately 20 pS. The current is enhanced by cAMP analogs, and by an activator of adenylyl cyclase, forskolin. We demonstrate that the voltage-dependent facilitation of the P-type current is dependent on the activation of protein kinase A (PKA) using specific inhibitors of the enzyme. These results suggest that repetitive stimulation can accentuate Ca\(^{2+}\) influx into hair cells through the P-type channels. Because of the nonlinear relation between Ca\(^{2+}\) influx and neurotransmitter release (Katz and Miledi 1967), we expect that voltage-dependent facilitation of the P-type current in hair cells would produce a robust change in synaptic efficacy.

**METHODS**

**Hair cell isolation**

The mollusk, *Hermisenda crassicornis*, was obtained from Sea Life Supply (Sand City, CA). Animals were held in modified 50-ml culture bottles in seawater (ASW) kept at 12–14°C. *Hermisenda* were fed scallops and a 12/12-h light/dark cycle was instituted. Dissection protocols of the CNS were followed as described previously (Yamoah 1997; Yamoah and Crow 1994; Yamoah et al. 1994). Briefly, the CNSs were dissected in ASW and allowed to stabilize at 4°C for 10 min. These were then treated with an enzyme cocktail consisting of protease XXIV (Sigma, St. Louis, MO) and dispase II (Boehringer Mannheim, Mannheim, Germany) at 1 mg/ml ASW and 3 mg/ml ASW, respectively. Isolated CNSs were digested for 15–20 min at 4°C and for another 10 min at room temperature. ASW wash was done several times at 4°C, for a total of 10–15 min. The statocyst was then excised from the CNS, and the transparent capsule around the statocyst torn gently apart to expose the underlying hair cells.

**Solutions**

All chemicals were obtained from Sigma unless indicated otherwise. ASW used for dissection and CNS wash was comprised of the following (in mM): 400 NaCl, 10 KCl, 10 CaCl\(_2\), 50 MgCl\(_2\), and 15 HEPES. The solution was sterile-filtered and the pH adjusted to 7.8 with 1N NaOH. The extracellular or bath solution during recording of whole cell Ca\(^{2+}\) currents consisted of (in mM) 300 chloride, 50 MgCl\(_2\), 20 CaCl\(_2\), 10 glucose, 5 4-aminopyridine (4-AP), 100 tetraethylammonium chloride (TEA-Cl), and 15 HEPES, sterile-filtered and adjusted to pH 7.7 with 1 M TEA-OH. The pipette solution was made up of the following (in mM): 400 CsCl, 20 NaCl, 2 MgCl\(_2\), 5 EGTA, 20 TEA-Cl, 10 reduced glutathione, and 40 HEPES. This was also sterile-filtered and adjusted to pH 7.4 with 1 M TEA-Cl. Stock solutions of Ca\(^{2+}\) channel blockers Cd\(^{2+}\) (100 mM), Co\(^{2+}\) (100 mM), nimodipine (100 mM in DMSO), and 0.001% Triton X-100 were made, and final concentrations of 1 mM, 5 mM, 0.001–0.100 mM, and 0.001–1.000 µM, respectively, were used. Stock solutions of the synthetic peptide inhibitor of PKA [PKAI(6–22)] (500 µM in distilled water, Gibco BRL, Rockville, MD) were made and stored at −20°C before use. For experiments in which DMSO was used as the solvent for pharmacological agents, the final concentration of the solvent was approximately 0.001%. The corresponding control experiments were performed using similar concentrations of DMSO (0.001%). For single-channel recordings, the bath solution contained (in mM) 300 KCl, 100 TEA-Cl, 50 MgCl\(_2\), 10 d-glucose, 10 CaCl\(_2\), 5 4-AP, and 10 HEPES, and was adjusted to pH 7.4 with TEA-OH, to shift the resting potential to approximately 0 mV. Patch electrodes were filled with (in mM) 250 Ba\(^{2+}\), 100 TEA-Cl, 5 4-AP, and 10 HEPES at pH 7.4 (adjusted with TEA-OH). For all recording solutions, TEA-Cl was used to maintain an osmolarity of approximately 1,000 mosmol.

**Electrophysiology**

**Whole cell Ca\(^{2+}\) current recordings.** Whole cell recordings were performed using standard patch-clamp recordings with the Axopatch 200B amplifier (Axon Instruments, Foster City, CA) (Hamill et al. 1981). A horizontal electrode puller (Sutter Instrument Model P-97) was used to make patch pipettes from borosilicate glass capillaries (1.5 mm OD and 1 mm ID; World Precision Instruments, Sarasota, FL). Pipette tips were then fire-polished using a micro-force (MF-830, Narishige, Tokyo, Japan) to obtain tip diameters approximately 1 µm. Using pipette solutions with ion strength equivalent to seawater (internal solution), the pipette resistances were approximately 1 MΩ, and only cells in experiments with seal resistances >1.2 Ω were accepted for analysis. A 3% agar bridge with 3 M KCl was used for reference electrode. Currents were digitized through an A/D converter (Digit/data 1200, Axon Instruments). Data collection was controlled through pClamp software (version 8.0, Axon Instruments), and experiments were carried out at room temperature. Data analysis of recorded currents was carried out using Clampfit 8.1 (Axon Instruments) and Origin 6.0 (Microcal Software, Northampton, MA).

**Single-channel recording.** The cell-attached configuration was used. Patch pipettes were made from borosilicate glass with 2.0 mm OD and 1 mm ID. The tips of electrodes were fire-polished, and to reduce the capacitance of electrodes, regions close to the tips (approximately 10 µm) were coated with Sylgard (Dow Corning, Midland, MI). Patch pipettes filled with single-channel recording solution had resistances of 1.1 ± 0.7 MΩ (n = 51). Single-channel currents were filtered at 1–2 kHz using a low-pass Bessel filter, sampled at 10–40 kHz, and stored in a personal computer. The channels were activated at a frequency of 0.2 Hz. Analysis was carried out using custom-written software, which was linked to Origin software (Microcal, Northampton, MA). Leak and capacitative currents were corrected off-line by fitting smooth templates to null traces and subtracting it from active traces. Open-close transitions were detected using half-height threshold analysis criteria. Idealized records were used to determine the unitary conductance. Single-channel current-voltage relations were fitted by a linear least-square regression line, and single-channel conductance obtained from the slope of the regression line. Experiments were carried out at room temperature (approximately 21°C). Where appropriate, pooled data were presented as means ± SD. Significant differences between groups were tested using Student’s t-test and the statistical significance was set at P < 0.05.

**RESULTS**

**Whole cell Ca\(^{2+}\) currents in *Hermisenda* hair cells**

The outward K\(^+\) currents responsible for the repolarizing phase of membrane action potentials of hair cells in the statocysts of *Hermisenda* have been described in detail (Yamoah 1997). In contrast, phenotypes of the inward currents that shape the depolarization phase of the membrane action potential are unknown. Here, we first studied the inward Ca\(^{2+}\) current in hair cells of *Hermisenda* and determined the properties of the current that may contribute toward presynaptic mechanisms of associative conditioning in this organism. Outward K\(^+\) cur-
The instantaneous tail $I-V$ shows a family of instantaneous tail currents elicited using and peaked at approximately 20 mV (Fig. 1B). Figure 1B shows that the current activated at approximately 100 mV and stepped to test voltages from −50 to 60 mV. For clarity, a few of the current traces were not plotted. $D$: reversal potential of the Ca$^{2+}$ current was measured by clamping back to voltages ranging from −70 to +130 mV after stepping at 10 mV for approximately 25 ms. In $n = 9$ cells, the mean reversal potential was estimated from the regression line to be 100 ± 3 mV, which is close to that of Ca$^{2+}$, indicating Ca$^{2+}$ as the key charge carrier.

The half-blocking concentration of $\text{Cd}^{2+}$ estimated from the dose response curve was approximately 0.5 μM (Fig. 1A). The effects of $\text{Cd}^{2+}$ and $\text{Co}^{2+}$ were reversible (data not shown).

To identify the Ca$^{2+}$ current subtype that is expressed in hair cells, we used known organic Ca$^{2+}$ channel blockers. Nimodipine, at micromolar concentrations (5–20 μM), produced no effect on whole cell Ca$^{2+}$ currents (at a test potential of 10 mV, control current, 0.44 ± 0.05 nA; 10 μM nimodipine, 0.43 ± 0.07 nA; $P = 0.2$, $n = 4$). In contrast, $\omega$-agatoxin-IVA blocked the Ca$^{2+}$ current (Fig. 2A). Although the washout of $\omega$-agatoxin-IVA appeared incomplete (Fig. 2A), this is likely due to the unavoidable rundown of the whole cell current over time. The half-blocking concentration of $\omega$-agatoxin-IVA estimated from the dose response curve was approximately 0.5 μM (Fig. 2B). Thus the pharmacology of the current suggests that it may belong to the P/Q-subtype.

The steady-state activation and inactivation properties of the current were examined using standard voltage protocols (Yamoah and Crow 1994; Yamoah et al. 1994). Normalized, steady-state activation and inactivation curves for the Ca$^{2+}$ current are presented in Fig. 3. Using the Boltzmann distribution to fit the two curves, the estimated half-activation voltage ($V_{1/2}$) and the maximum slope, $k_{m}$, were 1.2 ± 0.6 and 7.6 ± 20 C. T. TAMSE, Y. XU, H. SONG, L. NIE, AND E. N. YAMOAH

![Figure 1](http://jn.physiology.org/)

**FIG. 1.** Whole cell Ca$^{2+}$ currents recorded from hair cells of *Hermissenda*. $A$: representative traces for a family of Ca$^{2+}$ currents obtained from a holding potential of −80 mV and stepped ≤60 mV. Outward potassium currents were inhibited with bath TEA, 4-AP, and pipette Cs$^+$, and inward sodium current was removed by substituting choline for sodium ions. $B$: current-voltage curve obtained from 15 hair cells (mean ± SD). $C$: representative tail current traces and the corresponding protocol used to elicit the current. For clarity, some of the current traces were not plotted. $D$: reversal potential of the Ca$^{2+}$ current was measured by clamping back to voltages ranging from −70 to +130 mV after stepping at 10 mV for approximately 25 ms. In $n = 9$ cells, the mean reversal potential was estimated from the regression line to be 100 ± 3 mV, which is close to that of Ca$^{2+}$, indicating Ca$^{2+}$ as the key charge carrier.

rents were suppressed using external TEA-Cl, 4-AP, and internal Cs$^+$, and inward Na$^+$ currents were eliminated by replacing the external sodium with choline ions (Yamoah et al. 1994). Figure 1A shows a family of inward current traces elicited from a holding potential of −30 mV and peaked at approximately 20 mV (Fig. 1B). Figure 1C shows a family of instantaneous tail currents elicited using two-pulse voltage-clamp protocol. The instantaneous tail $I-V$ curve could be fitted with a regression line, with zero current at approximately 100 mV (Fig. 1D). By method of exclusion and reversal potential of the current, we inferred that the current was carried predominantly by Ca$^{2+}$ ions. Furthermore, we established that the inward current was a Ca$^{2+}$ current by examining its sensitivity toward Cd$^{2+}$ and Co$^{2+}$. Whereas 1 mM Cd$^{2+}$ was sufficient to completely block approximately 97% of the current (at a test potential of 10 mV, control current, 0.46 ± 0.11 nA; Cd$^{2+}$, 0.013 ± 0.008 nA; $P < 0.05$, $n = 7$), a higher dosage of Co$^{2+}$ (5 mM) was required to block approximately 95% of the current (at a test potential of 10 mV, control current, 0.43 ± 0.16 nA; Co$^{2+}$, 0.02 ± 0.01 nA; $P < 0.05$, $n = 5$). The effects of Cd$^{2+}$ and Co$^{2+}$ were reversible (data not shown).

To identify the Ca$^{2+}$ current subtype that is expressed in hair cells, we used known organic Ca$^{2+}$ channel blockers. Nimodipine, at micromolar concentrations (5–20 μM), produced no effect on whole cell Ca$^{2+}$ currents (at a test potential of 10 mV, control current, 0.44 ± 0.05 nA; 10 μM nimodipine, 0.43 ± 0.07 nA; $P = 0.2$, $n = 4$). In contrast, $\omega$-agatoxin-IVA blocked the Ca$^{2+}$ current (Fig. 2A). Although the washout of $\omega$-agatoxin-IVA appeared incomplete (Fig. 2A), this is likely due to the unavoidable rundown of the whole cell current over time. The half-blocking concentration of $\omega$-agatoxin-IVA estimated from the dose response curve was approximately 0.5 μM (Fig. 2B). Thus the pharmacology of the current suggests that it may belong to the P/Q-subtype.

The steady-state activation and inactivation properties of the current were examined using standard voltage protocols (Yamoah and Crow 1994; Yamoah et al. 1994). Normalized, steady-state activation and inactivation curves for the Ca$^{2+}$ current are presented in Fig. 3. Using the Boltzmann distribution to fit the two curves, the estimated half-activation voltage ($V_{1/2}$) and the maximum slope, $k_{m}$, were 1.2 ± 0.6 and 7.6 ±
0.6 mV \((n = 8)\), respectively. The inactivation curve generated from currents at a test potential of 10 mV, and at several conditioning potentials was sigmoidal in shape, and the calculated half-inactivation voltage \((V_{1/2})\) and the slope factor, \(k_m\), were \(-14.0 \pm 3.0\) and \(13.2 \pm 1.6\) mV \((n = 7)\), respectively. The presence of a window current from approximately \(-20\) to 10 mV suggest that the Ca\(^{2+}\) current may contribute substantially toward the action potential of hair cells.

**Single-channel currents in hair cells**

To further identify Ca\(^{2+}\) currents in *Hermissenda* hair cells, we measured the unitary current from cell-attached patches. Figure 4A shows a family of single-channel currents traces carried by 250 mM Ba\(^{2+}\). The amplitude histograms of the unitary current obtained at a test potential of 15 mV is shown (Fig. 4B). Illustrated in Fig. 4C is the unitary current amplitude plotted against the test potentials. The slope conductance of the channel was approximately 20 pS.

**Ca\(^{2+}\) currents show voltage-dependent facilitation**

Figure 5A shows Ca\(^{2+}\) current traces generated using a test potential of 0 mV from a holding potential of \(-80\) mV. In Fig. 5B, however, the initial test pulse was followed by a conditioning depolarization pulse to 70 and 90 mV for approximately 700 ms with a gap of approximately 5 ms, followed by a second test potential to 0 mV. The Ca\(^{2+}\) current was visibly enhanced following the conditioning depolarization pulses, increasing by approximately 1.2- and 1.5-fold using 70 and 90 mV 700-ms conditioning depolarization pulses, respectively (Fig. 5C). For example, control current at preconditioning test potential of 0 mV = 0.35 \pm 0.07 nA; postconditioning (90 mV) current was at 0.52 \pm 0.10 nA \((P < 0.05, n = 7)\). Voltage-dependent facilitation of the Ca\(^{2+}\) current was influenced by the duration of the conditioning pulse. As shown in Fig. 5D, the magnitude of the test currents increased with the duration of the conditioning pulses.

Aside from voltage-dependent facilitation of the Ca\(^{2+}\) current, the current was enhanced on application of dibutyryl-cAMP (500 μM; Fig. 6, A and C). Analysis of the group data of the peak current, as elicited by a voltage step to 20 mV from a holding potential of \(-80\) mV, revealed that the cAMP analog resulted in a significant enhancement of the Ca\(^{2+}\) current (control mean = 0.60 \pm 0.03 nA; cAMP = 0.84 \pm 0.05 nA;
The effects of forskolin, an activator of adenyl cyclase, suggest that the increase in Ca\(^{2+}\) current was mediated by PKA (Fig. 6, B and C). In the presence of forskolin, the Ca\(^{2+}\) current elicited by a voltage step to 20 mV from −80 mV was increased (control mean = 0.50 ± 0.09 nA; cAMP = 0.80 ± 0.08 nA; \(P < 0.05\), \(n = 6\)). Cells that were dialyzed with the PKA inhibitor, PKI, produced a substantial reduction of the Ca\(^{2+}\) current (data not shown, but see Fig. 7C). Likewise, administration of H-89 produced a significant reduction of the current, suggesting PKA was constitutively active in the hair cells (control mean = 0.51 ± 0.04 nA; H-89 = 0.29 ± 0.10 nA; \(P < 0.05\), \(n = 7\)).

Previous studies have shown that voltage-dependent facilitation of the L-type Ca\(^{2+}\) currents resulted from PKA phosphorylation of the channels (Dolphin 1998; Kamp et al. 2000; Sculptoreanu et al. 1993). We examined the effects of dibutyryl-cAMP on Ca\(^{2+}\) current pre- and postdepolarizing conditioning pulses. Consistent with the data shown in Fig. 6, the cAMP analog increased the Ca\(^{2+}\) current (data not shown, but see Fig. 7C). Likewise, administration of H-89 produced a significant reduction of the current, suggesting PKA was constitutively active in the hair cells (control mean = 0.51 ± 0.04 nA; H-89 = 0.29 ± 0.10 nA; \(P < 0.05\), \(n = 7\)).

The neuronal network that confers associative conditioning-induced plasticity in *Hermissenda* involves polysynaptic connections (Crow and Tian 2002). However, until recently, only the monosynaptic connection between the photoreceptors and hair cells have been examined (Alkon and Fuortes 1972; Crow and Tian 2000; Frysztak and Crow 1994, 1997). However, the roles of the presynaptic hair cells remain most uncertain. The study was motivated, in part, by the established role of presynaptic neurons in associative conditioning-induced plasticity (Schuman and Clark 1994). This is the first detailed description of the voltage-dependent Ca\(^{2+}\) currents in the presynaptic hair cells, and we found that the currents exhibit a voltage-dependent facilitation. This characteristic could potentially facilitate Ca\(^{2+}\) influx, increasing neurotransmitter release as well as activating a variety of Ca\(^{2+}\)-dependent mechanisms that may be required for the cellular mechanisms associated with classical conditioning in *Hermissenda*. Similar to the modulation of ionic currents and presynaptic mechanisms of neuronal plasticity in *Aplysia* sensory neurons, facilitation of Ca\(^{2+}\) currents in *Hermissenda* hair cells is mediated by PKA activation. However, it appears that in hair cells, the Ca\(^{2+}\) current is modulated directly by PKA, which is different from *Aplysia* sensory neurons wherein K\(^{+}\) currents are the main targets for PKA modulation (Baxter and Byrne 1990; Byrne and Kandel 1996; Goldsmith and Abrams 1992; Sugita et al. 1997).
Identity of the Ca\(^{2+}\) current in Hermissenda hair cells

Studies of Ca\(^{2+}\) channels in neurons and other cells in vertebrates have led to the classification of at least six subtypes of Ca\(^{2+}\) channels, namely, T-, N-, L-, P-, Q-, and R-type channels (Randall and Tsien 1995; Tottene et al. 1996; Tsien et al. 1988). The heterogeneity of the channels results from the expression of distinct \(\alpha_1\)-subunits (Snutch and Reiner 1992). Molecular cloning techniques have been used to identify at least six types of \(\alpha_1\)-subunits, which are classified as A, B, C/D, E, and H and correspond to the P/Q-, N-, cardiac and neuronal L-, R-, and T-type channels, respectively (Catterall 2000). There are striking similarities between the Ca\(^{2+}\) currents described in presynaptic hair cells of Hermissenda to that of the P-type currents, the latter of which are expressed in presynaptic terminals (Mulligan et al. 2001). The whole cell Ca\(^{2+}\) current in Hermissenda hair cells was insensitive to nimodipine and \(\omega\)-conotoxin GVIA (data not shown), ruling out the presence of L- and N-type currents, respectively. However, the Ca\(^{2+}\) current was blocked markedly by \(\omega\)-agatoxin-IVA, which suggest strongly that hair cells express the P-type current. Typically, the activation-voltage and the peak P-current in mammalian neurons occur at approximately −50 and 0 mV, respectively (Regehr and Atluri 1995; Tsujimoto et al. 2002) compared with that observed in Hermissenda hair cells, i.e., approximately −30 and 20 mV, respectively. However, we expect a shift in the voltage-dependent activation, arising from surface charge effects imposed by the high (approximately 60 mM) divalent cations in the seawater recording conditions (Rodriguez-Contreras et al. 2002). Taken together, we can infer that the P-type channel carries the predominant Ca\(^{2+}\) current in Hermissenda hair cells.

Mechanisms of voltage-dependent facilitation in P-type current in hair cells

Whereas voltage-dependent facilitation of Ca\(^{2+}\) current has been observed in several preparations (Dolphin 1996, 1998), and in recombinant Ca\(^{2+}\) channels expressed in HEK-293 cells (Kamp et al. 2000), others have failed to detect voltage-dependent facilitation (Meza and Adams 1998; Zong et al. 1995). The diverse outcomes of these studies suggest that voltage-dependent facilitation of Ca\(^{2+}\) currents may be regulated by a variety of mechanisms (Kamp et al. 2000). Still, there are some uncertainties regarding a given mechanism. For example, there are contrasting reports on whether PKA-mediated phosphory-
lation of Ca\(^{2+}\) channels is necessary for voltage-dependent facilitation (Eisfeld et al. 1996; Kamp et al. 2000; Sculptoreanu et al. 1993). However, a common feature for voltage-dependent facilitation is that it is prevalent in the L-type Ca\(^{2+}\) currents. In contrast, the Ca\(^{2+}\) current observed in hair cells was of the P-type, and showed voltage-dependent facilitation.

![Figure 6](image1.png)

**FIG. 6.** Modulation of Ca\(^{2+}\) currents in *Hermissenda* hair cells using dibutyryl-cAMP. **A:** Ca\(^{2+}\) current traces were generated by holding the membrane at -80 mV and stepping to voltages from -80 to 40 mV. Some of the traces were removed for clarity. The experiments were conducted before and after application of 500 \(\mu\)M dibutyryl-cAMP. Dibutyryl-cAMP produced a noticeable increase in the magnitude of the current. **B:** similarly, forskolin enhanced the Ca\(^{2+}\) current as shown. **C:** current-voltage relationships before (○) and after addition of cAMP (●), showing increased current amplitude in the presence of this 2nd messenger (n = 7). The group data showing the effects of forskolin before (□) and after (●) application indicate a significant enhancement of the current (n = 6).

![Figure 7](image2.png)

**FIG. 7.** Voltage-dependent facilitation is mediated by cAMP. **A:** Ca\(^{2+}\) current trace elicited from a holding potential of -80 mV to a step potential of 0 mV for approximately 4 ms. The current protocols were similar to that described in Fig. 5B. Note the different time scale for the test pulse to 0 mV and the conditioning depolarizing pulse to 80 mV. **B:** cAMP enhanced the current, postconditioning. On application of 500 \(\mu\)M dibutyryl-cAMP, the voltage-dependent facilitation of the current was further enhanced. **C:** in the presence of 5 \(\mu\)M PKI in the recording pipette, voltage-dependent facilitation was completely abolished. **D:** normalized group data, expressed as percentage facilitation from 6 cells in each set of experiments, are plotted.
that is mediated by PKA activation (Fig. 7). Another distinct feature of the P-type current facilitation in hair cells is that its kinetics can be described by a monophasic time constant (approximately 0.4–1.2 s), which is relatively slow compared with the more rapid voltage-dependent facilitation reported for the L-type current (approximately 10 ms: Kamp et al. 2000). Thus the slow kinetics of the voltage-dependent facilitation of Ca\(^{2+}\) currents in *Hermissenda* hair cells would be consistent with the requirement for PKA activation. Facilitation of the P/Q-type Ca\(^{2+}\) current has also been well documented (Glanzman 1995). The missesenda serve as one of the mechanisms of synaptic plasticity in the calyx of Held synapse in vertebrates, with a relatively fast time constant (approximately 15 ms). Moreover, in that system, neuronal calcium sensor 1 mediates activity-dependent facilitation of the current (Tsujimoto et al. 2002). In addition, the P/Q-type current in vertebrate neurons exhibits G-protein-mediated inhibition that is relieved by strong depolarizations, producing an apparent voltage-dependent facilitation (Jones and Elmslie 1997).

**Functional implication for synaptic plasticity in *Hermissenda***

Since neurotransmitter release is mediated by Ca\(^{2+}\) influx via voltage-gated Ca\(^{2+}\) channels and because of the nonlinear relation between Ca\(^{2+}\) influx and neurotransmitter release (Katz and Miledi 1967), we predict that voltage-dependent facilitation of the P-type current in hair cells would produce robust change in synaptic efficacy. Thus prolonged stimulation of hair cells can enhance the efficacy of the hair cell-photoreceptor synapse. Although synaptic plasticity is one of the hallmarks for mechanisms of learning and memory, it remains unclear how the present observation could be incorporated in the mechanisms of classical conditioning in *Hermissenda*. However, it is also conceivable that inhibitory inputs from hair cells can be manifested as excitatory output in the B photoreceptors. Moreover, this can be mediated through activation of a depolarization-induced inward rectifier current (I\(_k\)) that is activated by membrane hyperpolarization in type B cells (Yamouh et al. 1998). The prediction would rely on the temporal synchrony between inhibitory inputs from hair cells, [unconditioned stimulus (US)] and activation of I\(_k\) in the B photoreceptors, which receive the conditioned stimulus, CS. Nonetheless, voltage-dependent potentiation of Ca\(^{2+}\) currents may serve as one of the mechanisms of synaptic plasticity in *Hermissenda* neurons.

Modulation of synaptic connections between neurons that are involved in both associative and nonassociative conditioning has also been well documented (Glanzman 1995). The mechanisms underlying associative and nonassociative learning in the gill and siphon withdrawal reflexes involve changes in synaptic efficacy, mediated in part, by 5-HT–mediated facilitation of the sensory neurons (Byrne and Kandel 1996; Clark et al. 1994) and a subsequent enhancement of the release of glutamate at the sensory–motor neuron synapse (Chin et al., 2001; Chitwood et al. 2001; Levenson et al. 2000; Marinesco and Carew 2002). These studies have shown that synaptic facilitation results in activation of adenyl cyclase, resulting in increased levels of cAMP, activation of PKA, which in turn, leads to the phosphorylation of proteins (Klein 1993; Wu et al. 1995). The enhanced cAMP levels and the ensuing PKA activation and phosphorylation of ion channels result in the reduction of outward K\(^{+}\) currents, leading to the broadening of action potential duration, and enhancement of Ca\(^{2+}\) influx (Bao et al. 1998; Kandel 2001; Sugita et al. 1997). The aforementioned studies reported that K\(^{+}\) currents are the primary ionic currents subject to PKA modification. However, our present findings demonstrate that modulation of the P-type Ca\(^{2+}\) current in *Hermissenda* hair cells may contribute toward neuronal plasticity associated with learning and memory. Thus neuronal plasticity in these two invertebrate systems may share common features, which involves the requirement for enhanced Ca\(^{2+}\) influx.

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