Ionic Currents of Isolated Retinal Pacemaker Neurons: Projected Daily Phase Differences and Selective Enhancement by a Phase-Shifting Neurotransmitter

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Barnes, Steven and Jon W. Jacklet. Ionic currents of isolated retinal pacemaker neurons: projected daily phase differences and selective enhancement by a phase-shifting neurotransmitter. J. Neurophysiol. 77: 3075–3084, 1997. The eye of Aplysia expresses a robust circadian rhythm of neuronal activity. We dissociated the retinal cells in primary culture and studied isolated pacemaker neurons to identify ionic currents that may have roles in the circadian clock mechanism. Individual neurons were studied with perpetuated-whole cell recording techniques in current- and voltage-clamp modes. Pacemaker neurons had resting potentials near −40 mV and, if neurites had grown out, produced spontaneous action potentials in darkness at <1 Hz. Depolarizing current injections increased the rate of action potential firing. Hyperpolarizing current injections were followed by slowly decaying (1–3 s) afterhyperpolarizations. Four ionic currents were characterized under voltage-clamp, including a Ca current (I_Ca), a voltage-gated potassium current (I_KV), an A current (I_A), and a hyperpolarization-activated Cl current (I_Cl). I_KV was only seen using Cl–filled electrodes when high concentrations of Cl–diffused from the electrode and is therefore unlikely to be important under physiological conditions. The magnitude of I_KV was significantly larger during the projected zeitgeber predawn phase than during the postdawn phase, whereas the magnitude of I_A was constant at these circadian phases, suggesting that only I_KV is controlled by the circadian clock. Serotonin increased I_KV by 29%, consistent with reports that serotonin suppresses optic nerve activity and phase shifts the circadian rhythm recorded from the intact eye. The enhancement of I_KV likely contributes to membrane hyperpolarization, and it may be required for phase shifting. The phase-dependent changes in I_KV provide evidence that each retinal pacemaker neuron contains a circadian clock, but confirmation must await further recordings made from individual pacemaker neurons that are isolated completely from all other cells in primary culture. From the present experiments, it appears that I_KV is controlled by the circadian clock, in part, and it may be a required element in the pathway that is activated during serotonin-induced phase shifts.

INTRODUCTION

An isolated eye of Aplysia produces a robust and precise circadian rhythm (Jacklet 1969). It contains all of the basic components required for a circadian clock, including the essential oscillator, a photoreceptor to entrain the clock, and an output pathway to communicate the clock information to the central nervous system, where it is used to control rhythmic behaviors and physiological processes. The cellular organization of the Aplysia eye circadian clock is not completely understood, but many important cellular elements have been identified (review in Jacklet 1989).

The pacemaker neurons [also called secondary neurons (Jacklet et al. 1982; Luborsky-Moore and Jacklet 1977) or neurosecretory cells (Herman and Strumwasser 1984)] are 15–23 μm in diameter and monopolar. In the intact retina, their action potentials correspond one-to-one with the optic nerve (ON) compound action potential (CAP) activity that exhibits the circadian rhythm, suggesting that they are the circadian clock output neurons (Jacklet et al. 1982). Pacemaker neurons occur either alone or in small clusters in loose aggregation in the outer layer of the retina (Herman and Strumwasser 1984; Luborsky-Moore and Jacklet 1977) and are confined to the infrapapillary retina near the origins of the ON. They are called pacemaker neurons (Jacklet 1989) because they provide the pacemaker activity that drives the ON CAP activity. Single or clustered pacemaker neurons in primary culture are capable of producing trains of spontaneous action potentials arising from pacemaker activity (Jacklet et al. 1996). However, it has not been directly demonstrated, by either continuous recording from an isolated pacemaker neuron or sampling activity over several circadian cycles, that isolated pacemaker neurons contain a circadian clock. Monopolar neurons, with morphologies identical to the pacemaker neurons used in these experiments, were isolated from the Aplysia retina and placed in primary culture (Strumwasser et al. 1979), but their activities were not reported. The retina also contains several classes of photoreceptors (Herman and Strumwasser 1984).

Previous experiments on the Aplysia eye and Bulla eye, which also expresses a circadian rhythm (Block and Wallace 1982), give some indication of currents likely to be involved in the circadian clock or phase shifting of the clock. Potassium currents are likely to be involved in the circadian clock mechanism because the circadian phase change in membrane conductance reported in isolated Bulla basal retinal neurons is sensitive to tetraethylammonium (TEA) (Michel et al. 1993a). This channel blocker reduced the conductance during the predawn phase when the neurons in the intact eye are inactive and their membrane potentials are hyperpolarized relative to the postdawn phase. Therefore, one would expect potassium currents underlying the resting potential to be larger during the predawn phase than during the postdawn phase. Also, potassium currents dependent on adenosine 3',5'-cyclic monophosphate (cAMP) are thought to mediate the serotonin-induced phase shift of the circadian clock in the Aplysia eye (Colwell et al. 1992) by causing membrane hyperpolarization and a resultant decrease in depolarization.
dependent calcium influx (Colwell et al. 1994). In *Bulla*, calcium influx is necessary for light-induced phase shifts of the circadian clock (Block and McMahon 1984). However, calcium influx is not essential for generation of the circadian rhythm (Khalsa et al. 1993). Chloride currents have been examined but do not seem to be essential for generation of the rhythm because only modest changes in period are caused by complete substitution of extracellular chloride (Khalsa et al. 1990).

We have isolated the *Aplysia* retinal pacemaker neurons, maintained them in primary culture, and made perforated-patch whole cell recordings of membrane potential and current to investigate the ability of the isolated *Aplysia* retinal pacemaker neurons to express all or part of the elements of the circadian clock.

METHODS

Eyes were dissected from *Aplysia californica* obtained from Marinus, Long Beach, CA, or *Aplysia* Resource Center, Miami, FL, and maintained in Calgary at 18°C with equal (12 h) light and dark periods in aquaria. Animals were removed from the aquaria during the early light phase and dissected. Cells were dissociated from >200 retinas using standard techniques (Kleinfield et al. 1990; Schacher and Proshansky 1983). Eyes were treated with 1% protease (type IX, Sigma) in L15 medium for several hours at 33 or 36°C and then rinsed and cleaned of connective tissue. The lens was removed, and the retina was cut along the equator of the eye. The corneal portion of the eye was discarded and the basal portion containing the ON head and the infraroopic retina near the ON head, which contains the majority of the pacemaker neurons (Herman and Strumwasser 1984), was retained. This portion was cut into pieces and then triturated gently in *Aplysia* hemolymph using a 10-µl pipette tip. Dispersed cells were plated on polylysine (Sigma, >500,000 MW) coated Corning 25000 culture dishes. Plated cells were maintained in dim room light (~3 µW/cm²) at room temperature inside the tissue culture hood until recordings were made, usually on the first or second day after isolation. Zeitgeber time (ZT) was assigned to cultures during the recording session according to the projected light-dark schedule experienced by the animals. Zeitgeber time for the projected light phase is ZT 0–12 and for the projected dark phase is ZT 12–24, based on 24-h clock time.

Whole cell tight-seal recordings were made with 1–5 MΩ KCl-, CsCl-, or potassium gluconate-filled electrodes [51.1 mM primary salt, 2 mM MgCl₂, 20 mM K-N-2-hydroxylethylpiperazine-N’-2-ethanesulfonic acid (K-HEPES), 1.5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid, 1.0 mM CaCl₂, pH 7.6] using primarily permeabilized-patch techniques (final nystatin concentration of 150 µg/ml). If cells had been dialysed with high CI containing solution, an inwardly rectifying CI current activating negative to ~30 mV was induced in several minutes, so many recordings were made with gluanic acid.

Cells were superfused with saline containing (in mM) 460 NaCl, 10 KCl, 10 CaCl₂, 50 MgCl₂, 20 Na-HEPES, adjusted to pH 7.8. Bath reference electrode was an agar bridge with a chlorided silver wire immersed in the bridge holder. Neurons were observed on an inverted microscope (Nikon Diaphot) equipped with Hoffman contrast optics. Recordings were made with an Axopatch 200 in either current- or voltage-clamp mode. Data, sampled at 100–2,000 Hz, were written to disk using BASIC-FASTLAB (Sunnyvale, CA) routines. Illumination for recordings was provided by a 100-W halogen microscope lamp, adjusted in intensity (usually 70 µW/cm²). Just before recording cultures were taken from dim room light (~3 µW/cm²) and exposed to white fluorescent room light (23 µW/cm²) before being placed on the microscope stage for recording. Recordings were made at room temperature (19–21°C) and the microscope light was filtered to prevent heating.

RESULTS

Dissociated retinal pacemaker neurons

Isolation of the retinal cells was facilitated by treating the eyes with protease at 33°C before dissociation. To determine if this treatment had effects on the circadian rhythm, the CAP activity of intact eyes was tested after protease treatment. Protease treatment was performed near the end of the zeitgeber light phase, 0–12h. After treatment, the eyes required careful handling because the connective tissue surrounding the neurons was soft and weak. The ON was pulled gently into a tubing electrode and ON CAPs recorded in complete darkness. Figure 1 shows the circadian rhythm of CAP frequency from a pair of eyes from the same animal, one eye was treated and the other eye served as a control. CAP activity of both eyes increased before projected dawn at 0800. This anticipatory activity is typical of isolated eyes (Jacket 1989). The period and phase of the experimental eye rhythm is not different from the control. In four such experiments, the mean difference in phase among treated eyes was 0.3 h, the same as found for untreated eyes. These experiments show that the protease treatment with elevated temperature does not alter the CAP frequency or the period and phase of the circadian rhythm.

The variety of photoreceptors and neurons described in the intact retina by Herman and Strumwasser (1984) were represented in the dissociated cell cultures. Because we were interested in the pacemaker neurons, we reduced the eye to the basal portion near the ON head after dissociation of the retinal cells to enrich the cultures in pacemaker neurons, which are confined to the basal retina near the ON head (Herman and Strumwasser 1984). We have restricted the results presented here to recordings made from isolated pacemaker neurons, which were distinguished by shape, size, appearance, and characteristic activity from the other dissociated retinal cells that were present in the culture dish. Isolated clusters of pacemaker neurons and photoreceptors that also appeared in the cultures have been described...
Figure 2 shows a typical example of an isolated pacemaker neuron. The cell body is spherical and 20 μm in diameter. It lacks pigment but appears to contain tiny dark inclusions as viewed in Hoffman Contrast optics. Within 12 h after plating, pacemaker neurons develop a single outgrowing neurite tipped by a growth cone.

Isolated pacemaker neurons produced spontaneous action potentials (peak-to-peak amplitude of 50–60 mV) under current clamp and had resting potentials near −40 mV, which is within the range (−30 to −60 mV) of membrane potentials recorded from pacemaker neurons in the intact retina (Jacklet et al. 1982). Figure 3A shows an example of the activity of an isolated pacemaker neuron with a noisy resting potential that allowed the cell to periodically exceed threshold, which was near −30 mV. Action potential overshoots typically reached maxima between +10 and +20 mV. As illustrated in Fig. 3, depolarizing current injection caused the pacemaker neuron to spike repetitively, with clear signs of accommodation during the brief burst of induced activity. Hyperpolarizing current injection produced a characteristic voltage signature, consisting of rectification during the current step and a prominent repolarizing phase after the current step. Recovery of the membrane potential to its resting level occurred slowly. Usually membrane potential rose back to the resting potential during a period of 1–2 s. This electrical behavior results from the activation and subsequent inactivation of the transient potassium current, $I_A$, from which inactivation had been removed during the preceding 600-ms hyperpolarization. The properties of $I_A$ that support this activity are described in a following section.

**Ionic currents recorded from pacemaker neurons**

Pacemaker neurons provided an ideal cellular substrate upon which to study the ionic basis of circadian rhythrogenesis due to their compact size and high input resistance. In a typical sample, we found that cells had capacitances of 9.5 ± 3.3 pF (mean ± SD, n = 16) and input resistances of 6.5 ± 6.6 GΩ (n = 16). To describe the ionic currents that underlie the circadian electrical activity of these cells, we voltage-clamped cells using mostly perforated-patch techniques and defined properties of four ionic currents. Although evident in many recordings, we do not report on the properties of sodium currents in any detail, as our focus centered instead upon the currents that might have roles in circadian electrical activities having to do with alteration of the resting potential and pacemaker activity.

**Transient potassium current, $I_A$**

The slow rise back to the resting potential seen in Fig. 3B is a characteristic effect of the activation and inactivation of $I_A$ in other neurons (Hille 1992). Figure 4 summarizes our analysis of this transient outward current under voltage clamp. After a hyperpolarizing command step applied to
FIG. 4. Properties of transient outward current, $I_A$, studied under voltage clamp with a potassium gluconate-filled electrode in a pacemaker neuron. $A$: activation of $I_A$. After inactivation-removing prepulses to $-120$ mV, outward current became evident first during step to $-50$ mV. As 10-mV incremental steps became more positive, outward current increased and peak occurred earlier. $B$: inactivation of $I_A$. Near maximal outward current was elicited at $-30$ mV after a 200-ms prepulse step to $-120$ mV. Prepulse steps to more positive potentials progressively reduced outward current. Time to peak was unchanged. $C$: reversal of $I_A$. $I_A$ was elicited at $-30$ mV, then, near the peak ($\sim 60$ ms), membrane potential was stepped to progressively negative values. Decay of $I_A$ occurred more quickly with increased hyperpolarization, whereas current trajectories became smaller and changed sign between $-70$ and $-80$ mV. $D$: overlap of activation and inactivation curves for $I_A$ shows region of "window current." Inactivation curves, derived from experiments such as that in $B$ and fitted with the Boltzmann function after normalization, had midpoint of $-90 \pm 10$ mV and slope factor of $8 \pm 4$ mV ($n = 37$). Activation curves, derived from experiments similar to that in $A$, were made after division by driving force, normalizing, and fitting with the Boltzmann function, had midpoint of $-40 \pm 10$ mV and slope factor of $5 \pm 2$ mV ($n = 4$). Shaded area between $-90$ and $-60$ mV indicates region of membrane potential where cycling of channels among open, inactivated and closed states could occur, giving rise to a sustained conductance.

remove inactivation, $I_A$ activates with depolarization positive to $-50$ mV. Figure 4A shows that as the depolarizing steps became more positive, outward current increases, and the time-to-peak occurs earlier. As the potential of the conditioning step is made less negative, inactivation of $I_A$ occurs. Figure 4B shows progressive reduction of the outward current, which is not accompanied by a change in the time to peak. To determine its reversal potential, $I_A$ was evoked by stepping the voltage from $-120$ to $-30$ mV, and then, near the time that the outward current peaked, the membrane potential was stepped to negative values to deactivate the channels (Fig. 4C). The reversal of $I_A$ can be seen to occur between $-70$ and $-80$ mV, somewhat positive to the calculated potassium equilibrium potential of $-97$ mV, but a value easily achieved with a $P_{Na}/P_K = 0.034$ calculated from the Goldman-Hodgkin-Katz voltage equation (Hille 1992).

Pharmacologically, $I_A$ in pacemaker neurons resembled previous descriptions of $I_A$ in other cell types, including sensitivity to millimolar concentrations of 4-aminopyridine (4-AP; e.g., complete abolition in 5 mM 4-AP), lower sensitivity to tetraethylammonium, and pronounced block by divalent cations such as Pb$^{2+}$, Cd$^{2+}$, and Zn$^{2+}$ (Agus et al 1991; Dukes and Morad 1991; Hille 1992; Mayer and Sugiyama 1988; S. Barnes, D. E. Kurenny, and J. W. Jacklet, unpublished observations).

Some inactivating ion channels produce "window" currents when the channels have overlapping ranges of activation and inactivation. This overlap results in continuous current flow over a limited range of membrane potentials where some activation occurs but inactivation is not complete. Our analysis in Fig. 4D revealed that $I_A$ in pacemaker neurons shows a region of window current in the range of $-90$ to $-60$ mV. This suggests that steady-state $I_A$ may have a role in setting the resting potential and could be important in responses of these cells near the resting potential. For example, the current-clamp records shown above in Fig. 3B revealed a slow recovery from hyperpolarization that could be accounted for by $I_A$. In that experiment, injections of less current that produced smaller hyperpolarizations failed to remove inactivation as thoroughly as in the example shown and were not followed by the slow repolarization phase. Larger hyperpolarizations caused no greater and no slower repolarization phases, suggesting that the long step to the negative potential shown removed all $I_A$ inactivation. In a few neurons that lacked significant $I_A$, this behavior was not observed under current clamp.
To reveal the voltage-gated Ca channels in pacemaker neurons, it was necessary to replace intracellular K\(^+\) with Cs\(^+\) to reduce potassium currents (see METHODS for description of solutions). After this procedure, which eliminated much but not all outward current, the properties of transient inward currents were assessed. Currents elicited by depolarizing steps from a holding potential of \(-60\) mV, such as those shown in Fig. 5A, could be eliminated readily with 10 mM Cd\(^{2+}\) or Co\(^{2+}\) (not shown). These transient inward currents were much slower activate than the transient sodium currents. At a potential of \(-20\) mV, the inactivation time constant was near 10 ms, as opposed to 2 ms for the sodium currents.

A current-voltage relation of peak inward current at each voltage is shown in Fig. 5B. The currents activate near \(-30\) mV and reach peak amplitude at \(+20\) mV. After division of the I-V relation by the driving force and normalization, the activation curve could be fit with a Boltzmann function having a midpoint at 5 mV and slope factor of 6 mV (Fig. 5C).

When dialysed with the high Cl\(^-\) containing intracellular solutions (K\(^+\) or Cs\(^+\) salts), with either ruptured- or perforated-patch techniques, the cells developed significant "leakage" currents when held at 0 mV. Figure 6 shows an example of a cell undergoing this process after rupture of the patch membrane during recording with a CsCl-filled electrode. Each set of records is plotted without any offset, e.g. 0 current remains fixed at the level of holding current shown in the first two sets. In the first 2 min, only transient inward currents and outward currents were elicited by the depolarizations. As Cs\(^+\) diffused into the cell, outward current was reduced, and the transient inward current appeared to increase (1 min, 50 s). Induction of \(I_{Cl}\) can be detected in this example at 2 min, 53 s by the increasing inward current required to hold the cell at \(-60\) mV. Reversal of the slow current trajectories during depolarizations to membrane potentials just positive to 0 mV, the Cl\(^-\) equilibrium potential, also can be seen superimposed with faster transient inward current trajectories. Voltage-dependent deactivation of the current nearly is complete by the end of the 110-ms steps to 10, 20, 30, and 40 mV.

Properties of the fully induced \(I_{Cl}\) are shown in Fig. 7. A family of inward currents elicited by hyperpolarizing command steps applied from a holding potential of \(-30\) mV are shown in Fig. 7A. Increasingly negative voltage steps between \(-40\) and \(-110\) mV caused appearance of slowly activating inward currents. Activation curves could be derived from the tail currents measured at \(-30\) mV. Figure 7B shows the normalized tail currents from Fig. 7A, fit with a Boltzmann function having a midpoint of \(-70\) mV and a slope factor of 6 mV. Tail current reversal near 0 mV, after steps to \(-120\) mV to activate \(I_{Cl}\), can be seen in Fig. 7C.

Although it was not convenient to test the dependence of the reversal potential of this current on Cl\(^-\) concentration, block of \(I_{Cl}\) with diagnostic agents was performed. Three ions, Zn\(^{2+}\), Ba\(^{2+}\), and Cs\(^+\), have been shown to block inward rectifier Cl\(^-\), K\(^+\), and nonspecific cation (\(I_h\)) currents, respectively (Hille 1992). Although the reversal potential measurement in Fig. 7C already argued against this being a potassium current, the data presented in Fig. 8 rule out also a nonspecific cation current similar to \(I_h\) as Cs\(^+\) was ineffective in suppressing the inward current at all potentials. Zn\(^{2+}\) appears to block the current. The data show that activation of \(I_{Cl}\) is shifted by \(-30\) mV to more negative potentials in the presence of Zn\(^{2+}\), suggesting an open channel block as this is the opposite effect expected from a neutralization of fixed negative membrane surface charges. There was no change in conductance once activation occurred past this membrane potential. When recordings were made with potassium gluconate-filled electrodes, the hyperpolarization-activated current was never observed, providing evidence that the current is elicited when Cl\(^-\) leaked into the cell from the electrode.

Properties of the voltage-gated potassium current, \(I_{Kv}\)

Depolarizations positive to 0 mV evoked large outward currents. Figure 9 shows currents from a cell that had been held at \(-30\) mV to inactivate A current and then was stepped to increasingly positive voltages. Outward currents activated with a time course that could be fitted with a single exponential. Near +10 mV, the time constant for activation was near 50 ms, and after the depolarizing steps, tail currents decayed at \(-30\) mV with

![Fig. 5. Voltage-clamp recordings of \(I_{Ca}\) in a pacemaker neuron obtained with a CsCl-filled electrode. A: currents evoked in response to 110-ms steps to \(-30, -20, -10, 0, 10,\) and \(20\) mV applied from a holding potential of \(-60\) mV. B: current-voltage (I-V) relation of maximal inward currents from experiment in A. Peak inward current was recorded at +20 mV. Use of Cs\(^+\) reduced but did not eliminate outward currents as can be seen in A and B. C: activation curve derived from I-V relation after division by driving force and normalization had midpoint at 5 mV and slope factor of 6 mV.](http://jn.physiology.org/doi/10.1152/jn.1997.278.7.3079)
time constants near 25 ms. The outward currents were sensitive to TEA ion with 45 ± 3% of current remaining in 20 mM TEA
(n = 3). Under no conditions did the I-V relation have a N
shape, which often indicates the presence of a Ca-activated cur-
rent. For this and the reasons described below, we concluded
that these sustained outward currents reflected only voltage-gated
K currents, not Ca-activated, K currents.

$I_{KV}$ activated positive to −20 mV, as can be seen in the
current traces in Fig. 9A and in the I-V relation made from
these data in Fig. 9B. Using the line that fits the data between
+40 and +90 mV as representative of the fully activated
conductance, the activation curve derived from this I-V rela-
tion was fit with a Boltzmann function having a midpoint
at +12 mV and a slope factor of 6.2 mV (Fig. 9C). In
addition, the normalized tail currents seen in Fig. 9A were
used to describe an activation curve and are plotted against
test potential in Fig. 9C. This activation curve was fit with a
Boltzmann function having a midpoint of 13.8 mV and a
slope factor of 6.3 mV. The close agreement between the
good fits to these independently derived activation curves
argues against contaminating currents being present in this
voltage range. The Boltzmann functions also suggest that,
over the voltage range in which these cells normally rest
(−60 to −40 mV), $I_{KV}$ would be activated only with very
low probability (0.02% at −40 mV).

$I_{KV}$ changes according to ZT

We examined the magnitude of $I_{KV}$ and $I_\Lambda$ at ZT 20−23
(4−1 h before projected dawn) and ZT 2−6 (2−6 h after
projected dawn). In intact eyes, during ZT 20−23, there
is no CAP activity and the pacemaker neurons are likely
hyperpolarized. During ZT 2−6, there is high CAP activity
and the pacemaker neurons are likely depolarized. The mag-
nitude of $I_{KV}$ at the end of a 200-ms voltage step to +50
mV from a −30 mV holding potential was measured and
normalized with respect to the capacitance of the neuron.
There was no significant difference in capacitance of the
cells at ZT 20−23 (11.4 ± 0.3 pF, n = 10) compared with
ZT 2−6 (11.5 ± 0.4 pF). Normalized $I_{KV}$ recorded with
nystatin electrodes was significantly larger ($P < 0.05$, n =
10) at ZT 20−23 (day 1) than it was at ZT 2−6 (day 2) as
shown in Fig. 10. The magnitude of $I_{KV}$ increased again at
ZT 20−23 (day 2) to the same value as on day 1 and was
significantly larger than the current magnitude at ZT 2−6
($P < 0.05$), showing that the current was not just declining
with time in culture. The current magnitude for all neurons,
including those studies with ruptured patch recordings, was
larger at ZT 20−23 but this was not significant ($P < 0.10$,
n = 16). $I_\Lambda$ was slightly larger at ZT 20−23 (6.6 ± 0.8 nA,
n = 10) than at ZT 2−6 (5.1 ± 0.9), but the difference was
not significant.

Serotonin enhances $I_{KV}$

The neurotransmitter serotonin is a potent agent for phase
shifting the circadian rhythm of CAP activity in the intact
eye (Corrent et al. 1978). We therefore applied serotonin
to the cultured neurons to see if it altered ionic currents.

![Figure 7](http://jn.physiology.org/Downloaded.png)

**Fig. 7.** Activation of fully induced $I_{KB}$ by hyperpolarization recorded with a CsCl-filled pipette. **A:** from a holding potential of −30 mV, increasingly negative voltage steps between −40 and −110 mV caused appearance of slowly activating inward currents. **B:** activation curve measured from tail currents at −30 mV. Normalized tail currents were fit with Boltzmann function having a midpoint of −70 mV and slope factor of 6 mV. **C:** reversal of tail currents near 0 mV (→) after step to −120 mV to activate $I_{KV}$. Decay of each tail current trajectory speeded up and changed sign as membrane potential increased past 0 mV.
Serotonin increased $I_{KV}$ (Fig. 11), most prominently at positive voltage steps, but there was no change seen in $I_A$ measured after the hyperpolarizing steps as can be seen in the current records at the 300-ms point.

Elicited by a voltage step from −30 to +50 mV, $I_{KV}$ increased 33% in response to serotonin application in the cell shown in Fig. 11. The average increase of 29 ± 7% ($n = 4$) for 10 μM serotonin applied to neurons at ZT 21–23, using nystatin electrodes for recording, was statistically significant at the 0.01 level. Currents recorded from neurons using ruptured-patch techniques did not increase significantly in response to 10 or 50 μM serotonin (1% increase, $n = 3$), suggesting that $I_{KV}$ may be regulated by a diffusible second messenger (e.g. cAMP) that is reduced in concentration after patch rupture. Although it cannot be resolved in the low amplification recording shown in the figure, we measured an increase in outward current in the voltage range between −60 and −30 mV in response to serotonin. We could not determine whether this current increase of 1–2 pA was due to activation of voltage-gated $KV$ channels or $A$ channels or another, uncharacterized $K$ channel. However, the lack of a measurable increase in $I_A$ and the significant increase of $I_{KV}$, in response to serotonin, as seen in Fig. 11, suggest that enhancement of the voltage-gated $KV$ channel activation curve could account for changes in pacemaker neuron resting potential in the −60 to −30 mV range of voltages.

**DISCUSSION**

We investigated the properties of ionic currents that were candidates for having roles in circadian rhythmogenesis. We found that $I_{KV}$ changes during the projected zeitgeber cycle and then found that it was sensitive to serotonin, a transmitter capable of shifting the phase of the circadian rhythm. The general properties of the currents we described, $I_A$, $I_{Ca}$, $I_{Cl}$, and $I_{KV}$, fall within those described previously for molluscan neurons (Adams et al. 1980). The membrane potential of isolated pacemaker cells in darkness is near −40 mV (Fig. 3A) (Jackett and Barnes 1993a; Jackett et al. 1996). Resting potentials of neurons recorded with sharp electrodes in the intact retina are generally more negative than this, ranging between −30 to −60 mV, and they produce spontaneous overshooting action potentials in darkness (Jackett et al. 1982). Because a previous report showed that some isolated pacemaker cells could generate slow, low sensitivity, depolarizing light responses (Jackett and Barnes 1993a), some neurons recorded here under bright microscope illumination may have membrane potentials more depolarized than if the cells had been maintained in complete darkness. Pacemaker neuron membrane potential returned to within a few millivolts of the resting potential after several minutes in microscope light (Jackett and Barnes 1993a). In complete darkness, isolated pacemaker neurons are capable of producing spontaneous action potentials; and pacemaker neurons attached to fragments of retina or in isolated clusters in culture interact to produce robust spontaneous activity, comparable with pacemaker neuron activity recorded in the intact retina (Jackett et al. 1996). Nevertheless, the resting potential of isolated pacemaker neurons is likely more positive than in the intact retina. This suggests that when the pacemaker neurons are isolated in culture, they lack something
that is present in the intact eye but disrupted or reduced by dissociation of the retinal cells. We speculate that it is either electrical coupling mediated by gap junctions that normally exists among the pacemaker neurons or a second messenger system. A similar difference is noted for isolated basal retinal neurons of Bulla (Michel et al. 1993a), which also normally are coupled electrically in the intact retina.

In the related mollusc Bulla, basal retinal neurons have resting potentials of −60 to −70 mV as recorded from the intact eye with sharp electrodes (Jacklet 1988; McMahon et al. 1984). The resting potential changes rhythmically with a circadian period, being −70 mV during the predawn silent phase and approximately −60 mV during the active postdawn phase (McMahon et al. 1984). The membrane potential changes from −70 to −60 mV in anticipation of dawn and subthreshold bumps on the membrane potential increase until overshooting action potentials occur (Jacklet 1988; McMahon et al. 1984). Basal retina neurons isolated in culture showed circadian phase changes in membrane conductance but had resting potentials of −25 to −60 mV that were not rhythmically changing and did not produce spontaneous action potentials when recorded with sharp electrodes (Michel et al. 1993a).

The Aplysia pacemaker neurons are small, electrically compact cells when isolated and even small membrane current changes can lead to large effects on the membrane potential. For example, a current of 1 pA would polarize a cell having an input resistance of 5 GΩ by 5 mV. Our current injection experiments bore this high degree of sensitivity out; for example, 10 pA of injected current altered membrane potential by ±60 mV, and even these voltage excursions always were limited by inward or outward rectification of the membrane. In some cells, we could alter resting potential by ±10 mV with injection of only a few hundreds of femtoamps.

Ion channels not controlled by the circadian clock

$I_{\lambda}$ is a candidate for involvement in circadian rhythmogenesis due to the possibility that $I_{\lambda}$ window current influences membrane potential. Window current is generated over the range of membrane potentials from about −90 to −60 mV. Because the isolated cells used in this study had resting potentials close to −40 mV, we would expect that there would be little window current because inactivation appears to be complete at this potential. However, in the intact eye, the normal resting potential may be more negative (see above), and therefore $I_{\lambda}$ might play a larger role in regulating resting potential. $I_{\lambda}$ did not change when serotonin was applied, so it seems unlikely to be directly involved in seroto-
nin-induced phase shifts. $I_A$ is nonetheless highly sensitive to divalent cations (Agus et al. 1991; Dukes and Morad 1991; Mayer and Sugiyama 1988), some of which have effects on the circadian rhythm (Colwell et al. 1994).

Calcium plays important roles in circadian rhythms. Although a normal gradient of calcium concentration across the membrane of Bulla basal retinal neurons does not seem to be essential for persistence of the ongoing circadian rhythm, external calcium is necessary for light induced phase shifts of the rhythm (Kalsa et al. 1993). A model for phase shifting in Aplysia proposes that serotonin causes phase shifts by membrane hyperpolarization-induced reductions in calcium influx and light causes phase shifts by membrane depolarization-induced increases in calcium influx (Colwell et al. 1994).

The intracellular chloride-induced, hyperpolarization-activated $I_{Cl}$ we have described is consistent with a previous report for Aplysia neurons (Chesnoy-Marchais 1983). Although previous descriptions of modulation of this current by serotonin (Lotshaw and Levitan 1987) suggested its importance, the role for chloride ions in determining neuronal circadian pacemaker period is minimal (Khalsa et al. 1990). Based on our work with nystatin permeabilized patch techniques, where we confirmed that chloride diffusion from KCl-filled electrodes into the cells is responsible for inducing the current, we find it unlikely that $I_{Cl}$ is active in pacemaker cells under physiological conditions.

A voltage-gated potassium current appears to be controlled by the circadian clock

We found that $I_{KV}$ recorded with perforated-patch techniques was significantly larger during predawn zeitgeber phase compared with postdawn phase. The larger current during the predawn phase correlates with the lack of CAP activity in an intact eye during that phase (Jacklet 1969) and the expected hyperpolarization of the pacemaker neurons. However, the increased $I_{KV}$ during the predawn phase may not be the only contributor to the resting potential because it activates most significantly positive to about $-20 \text{ mV}$. A phase-dependent difference in potassium currents also has been reported for isolated cultured basal retinal neurons of Bulla (Michel et al. 1993b), which are nearly equivalent to the pacemaker neurons of Aplysia. Differences in conductance previously had been detected in isolated basal retinal neurons using sharp electrodes (Michel et al. 1993a), and single channel patch recordings have revealed a calcium-independent potassium current that is modulated by the clock in Bulla retinal neurons (Manivannan et al. 1994). Our recordings were made from isolated neurons in cultures containing other retinal cells, and therefore any paracrine interactions that exist among cells could have contributed to the rhythmic changes in current observed. Further experiments are needed to clarify this issue. However, the evidence obtained so far suggests that similar mechanisms are used in the circadian systems of these related mollusks.

Our finding that currents recorded using perforated-patch, but not ruptured-patch, techniques appear to be under circadian control and enhanced by serotonin suggests that a second messenger system may be mediating these effects on membrane potential. A likely candidate is cAMP as this molecule has been shown to mediate phase shifts of the CAP rhythm produced by serotonin (Eskin et al. 1982) and modulate $I_{KV}$ in Aplysia sensory neurons (Sugita et al. 1994). Cyclic nucleotide-gated channels are being found increasingly in a variety of neurons including mollusks (Yau 1994). Further studies using cyclic nucleotide analogues and blocking agents are needed to establish the relationship between cyclic nucleotides and the control of membrane channels and membrane potential via the circadian clock and phase shifting agents.

Serotonin increases the voltage-gated potassium current

Serotonin is a potent agent for phase shifting the circadian rhythm of CAP activity in the intact Aplysia eye. The magnitude and direction of phase shifts are phase dependent, as seen in a phase response curve, revealing that the response of the underlying circadian pacemaker differs according to the phase of the circadian pacemaker. Serotonin at 10 $\mu M$ induces phase advances at ZT 8–16 and phase delays at ZT 18–06 (Corrent et al. 1978). However, it decreases the spontaneous CAP activity during treatment regardless of circadian phase (Corrent et al. 1978). Changes in conductance to $Na^+$, $Cl^-$, or $Ca^{2+}$ do not seem to be involved in phase shifting of the rhythm by serotonin, and because large reductions in external $[Ca^{2+}]$ do not affect phase shifting by serotonin, it appears to be acting directly on circadian pacemaker neurons or electrotonically coupled cells (Corrent and Eskin 1982). Our results are consistent with this view, because isolated pacemaker neurons exposed to serotonin show an increase in $I_{KV}$. This increase could hyperpolarize the pacemaker neurons and thus contribute to the decrease in active CAP activity in the intact eye seen during serotonin treatment. Our description shows $I_{KV}$ in pacemaker neurons activating substantially at potentials greater than $-20 \text{ mV}$. However, we detected small serotonin-induced changes in potassium current near resting membrane potentials that could have induced hyperpolarization of the neurons. These changes were likely dependent on a soluble intracellular constituent, such as cAMP, because ruptured patch recording did not show a serotonin-induced increase in current. This is in agreement with a model proposed for Aplysia in which serotonin appears to phase shift the circadian clock by a hyperpolarization that is mediated by a barium-sensitive, cAMP-dependent potassium conductance (Colwell et al. 1992).

Circadian and diurnal rhythmic changes in photosensitivity are well known in invertebrates and many appear to be mediated by neuromodulators like serotonin. The electroretinogram (ERG) waveform recorded from the Aplysia eye changes rhythmically and maintains a stable phase relationship with the circadian rhythm of CAP activity, and serotonin induces the night waveform (Jacklet 1991). The ERG of the horseshoe crab Limulus changes rhythmically and the change is mediated by a central circadian clock using octopamine as a neuromodulator (Barlow 1983). Diurnal modulation of photoreceptor potassium conductance has been reported for locusts (Cuttle et al. 1995). Apparently this modulation matches photoreceptor membrane properties to the demands of the photic environment. During the day, a slowly activating outward current with little inactivation is predominant. In contrast, a rapidly activating and inactivating current is predominant at night. Serotonin reduces the
slowly activating current and converts membrane properties to the night format. Our finding that a potassium current of pacemaker neurons is modulated by serotonin is consistent with the view that neurons that contain circadian clocks may have circadian phase controlled in part by neuromodulators, such as serotonin, released by the activity of central nervous system neurons. That is, brain activity is capable of resetting the circadian clock as well as environmental stimuli, such as light.

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