Hyperpolarization-Activated Currents Regulate Excitability in Stellate Cells of the Mammalian Ventral Cochlear Nucleus

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ABSTRACT

The differing biophysical properties of neurons whose axons form the different pathways from the ventral cochlear nucleus (VCN) determine what acoustic information they can convey. T stellate cells, excitatory neurons whose axons project to locally and to the inferior colliculus, and D stellate cells, inhibitory neurons whose axons project to the ipsi- and contralateral cochlear nuclei, fire tonically when they are depolarized and, unlike other cell types in the VCN, their firing rates are sensitive to small changes in resting currents. In both types of neurons the hyperpolarization-activated current (Ih) reversed at –40 mV, was activated at voltages negative to –60 mV and half-activated at ~–88 mV; maximum hyperpolarization-activated conductances (gh max) were 19.1 ± 2.3 nS in T and 30.3 ± 2.6 nS in D stellate cells. Activation and deactivation were slower in T than in D stellate cells. In both types of stellate cells 50 µM ZD7288 and 2 mM Cs⁺ blocked a six- to ten-fold greater conductance than the voltage-dependent gh determined from Boltzmann analyses at –62 mV. The voltage-insensitive, ZD7288-sensitive conductance was proportional to gh max and ginput. 8-Br-cAMP shifted the voltage-dependence of Ih in the depolarizing direction, increased the rate of activation and slowed its deactivation in both T and D stellate cells. Reduction in temperature did not change the voltage-dependence but reduced the maximal gh with a Q10 of 1.3 and slowed the kinetics with a Q10 of 3.3.
INTRODUCTION

Hyperpolarization-activated cation currents ($I_h$) perform a wide range of functions in excitable cells. In many neurons $I_h$ contributes to setting the resting potential (Banks et al. 1993; Pape 1996; Doan and Kunze 1999; Bal and Oertel 2000) and to controlling the rate of spontaneous and evoked firing (Spain et al. 1987; McCormick and Pape 1990b; Maccaferri and McBain 1996). In hippocampal cells $I_h$ contributes to synaptic integration in dendrites (Magee 1998; Williams and Stuart 2000). These functions can be modulated through cAMP-mediated signaling pathways (McCormick and Pape 1990a; Tokimasa and Akasu 1990; DiFrancesco and Tortora 1991; van Ginneken and Giles 1991; Pedarzani and Storm 1995; Ingram and Williams 1996; Ludwig et al. 1998; Cathala and Paupardin-Tritsch 1999; Vargas and Lucero 1999; Saitow and Konishi, 2000).

The shapes, projections and biophysical characteristics of the four groups of principal cells of the VCN, bushy, octopus, T stellate and D stellate cells, are distinct. Octopus cells occupy the most caudal and dorsal VCN in a sharply delineated area (Golding et al. 1995). The remainder of the VCN contains intermingled bushy and stellate cells with bushy cells being more common anteriorly and stellate cells more common posteriorly. The low input resistances that result from the activation of $g_h$ and a low-voltage activated K$^+$ conductance, $g_{KL}$, make responses to synaptic currents in octopus and bushy cells fast and precisely timed, and responses to current pulses transient (Manis and Marx 1991; Oertel et al. 2000; Bal and Oertel 2000; Cuttle et al. 2001). The absence (or near absence) of $g_{KL}$ in stellate cells, allows them to fire tonically when they are depolarized; their rates of firing are sensitive to small changes in resting currents (Oertel et al. 1990; Fujino and Oertel 2001; Ferragamo and Oertel, 2002). The responses of VCN cells to sound have been shown in vivo to be sensitive to neuromodulation (Kossel and Vater 1989).
Two types of stellate cells have been distinguished and named on the basis of several differences. In slices from mice they were named for their projection patterns. Both types project locally in the ventral and dorsal cochlear nuclei but the main axon projects through the Trapezoid body in T stellate cells but dorsalward through the intermediate acoustic stria in D stellate cells (Oertel et al. 1990). Other authors have distinguished T and D stellate cells on the basis of other differences: “type 1” and “type 2” vary in the degree of somatic inputs in electron micrographs (Cant 1981), responses to tones are “chopper” or more transient “onset-chopper” (Smith and Rhode 1989; Blackburn and Sachs 1989), the dendrites of “planar” stellate cells are aligned with auditory nerve fibers whereas those of “radial” stellate cells are not (Doucet and Ryugo 1997). T stellate cells are glutamatergic and form a major excitatory pathway to the contralateral inferior colliculus (Adams and Warr 1976) whereas D stellate cells are glycinergic and inhibitory (Ferragamo et al. 1998, Needham and Paolini 2003). Many, but probably not all, D stellate cells project to the contralateral cochlear nuclei (Cant and Gaston 1982; Needham and Paolini 2003; Arnott et al. 2004). T stellate cells are narrowly tuned and fire tonically in responses to tones whereas D stellate cells are more broadly tuned and fire transiently at the onset of tones (Smith and Rhode, 1989; Blackburn and Sachs 1989; Nelken and Young 1994; Jiang et al. 1996; Palmer and Winter 1996; Palmer et al. 1996; Arnott et al. 2004; Smith et al. 2005).

The present experiments show that \( g_h \) regulates the firing of stellate cells through its influence on the resting potential and input resistance. The sensitivity of \( g_h \) to cAMP levels indicates that firing in both types of stellate cells can be regulated neuronally. We were surprised to discover in the course of our analysis that ZD7288 and extracellular Cs\(^+\), drugs that are commonly used to block \( g_h \), block more than the voltage-sensitive \( g_h \).
METHODS

Preparation of slices

All experiments were done in accordance with the guidelines of the Research Animal Resources Center at the University of Wisconsin-Madison. Parasagittal slices were prepared from the cochlear nuclei of ICR mice between 16 and 18 days old. During the surgery the auditory nerve was cut where it exits the internal auditory meatus. A block of brain tissue containing the cochlear nucleus was trimmed and glued onto a Teflon-coated specimen disc. The slices (200 µm thick) were prepared with a VT1000S oscillating tissue slicer (Leica, Nussloch, Germany) using a sapphire blade (Delaware Diamonds Knives, Wilmington, DE). The tissue was kept submerged in physiological saline of the following composition (mM): 127 NaCl, 3 KCl, 1.2 KH₂PO₄, 2.4 CaCl₂, 1.3 MgSO₄, 3 HEPES, 20 NaHCO₃, 10 glucose, pH 7.4 when saturated with 95% O₂-5% CO₂ at 26°C. The osmolality, measured with a 3D3 Osmometer (Advanced Instruments Inc, Norwood, MA), was 302 mosm/kg. Slices were transferred to a recording chamber (~0.6 ml) and superfused continually at 5 to 6 ml/min. The temperature of the inflowing perfusate was monitored with a small thermistor, IT-23, and a Thermalert thermometer TH-5 (Physitemp, Clifton, NJ) and kept at 33 ± 0.5°C in most of the experiments by a custom-built, feedback-controlled heater. The recording chamber was placed on the stage of an Axioskop 2FS microscope (Zeiss, Oberkochen, Germany) and individual neurons were visualized using differential interference contrast optics (63x/0.9 w water immersion lens).

Electrophysiological Recordings

Patch pipettes were made from borosilicate glass capillaries (1B120F-4, World Precision Instruments, USA) and had resistances of between 3.8 and 5 MΩ when filled with a solution containing (mM): 110 potassium gluconate, 9 HEPES, 9 EGTA, 4.5 MgCl₂, 14 phosphocreatinine, 0.3 GTP, 4 Na₂ATP, pH 7.3 (KOH), 293 mosm/kg. In normal extracellular saline, this solution yielded a
junction potential of −12 mV that was added to all voltages. Whole-cell current- and voltage-clamp recordings were performed with an Axopatch 200B amplifier controlled by a PC computer through a Digidata 1200 computer interface and pClamp 8.0 software (Axon Instruments, Foster City, CA). The access resistance ($R_a$) was monitored throughout all experiments and ranged from 9 to 17 MΩ. $R_a$ was partially compensated online between (60 to 75 %) with a 20 µs lag time. For analysis of the voltage dependence of activation, the voltage drop caused by the uncompensated $R_a$ was subtracted offline from the applied voltage command to yield a precise value of transmembrane voltage (Rothman and Manis 2003). Voltage responses to current injection and current responses to voltage steps were recorded at 40 kHz and 5 kHz and filtered at 10 and 1 kHz, respectively.

Data analysis

The input resistance ($R_{input}$) was determined in a subset of cells from slope of the relationship between the mean voltage responses in three consecutive recordings to the last 50 ms of 300-ms current pulses from −60 to +60 pA in 10 pA-steps. To achieve the greatest possible accuracy in these measurements, the voltage drop across the access resistance was subtracted (bridge was balanced) off line in the following way. The 0.2 ms after the end of the current pulse that was contaminated by electrode artifacts was eliminated and reconstructed by fitting the time course of decay after the offset with a single exponential and extrapolating back to the offset of the current pulse. Activation and deactivation time constants of $I_h$ were determined by fitting the current evoked during an activating or deactivating pulse to double exponential functions of the form: $I_h(t) = I_{SS} + A_f e^{-t/\tau_f} + A_s e^{-t/\tau_s}$, where $I_h(t)$ is the current at time $t$, $I_{SS}$ is the steady state current, $A_f$ and $A_s$ are the initial amplitudes of the fast ($\tau_f$) and slow ($\tau_s$) exponential components, respectively. To avoid contamination by the capacitative artifacts, the first 12 to 15 ms of the recorded current were not included in the fit. The voltage sensitivity of $g_h$ was measured from tail currents. The amplitude of individual tail currents, measured
12 ms after the end of the conditioning step (I), was normalized as a function of maximal and minimal tail currents as \( I(V) = \frac{(I - I_{\text{min}})}{(I_{\text{max}} - I_{\text{min}})} \), and plotted as a function of the voltage of the preceding step (V), and reflects the relative conductance (g). Each of the measurements from individual cells was fitted by a Boltzmann function of the form: \( g(V) = \frac{1}{1 + e^{(V-V_{0.5})/k}} \) from which the value of half-maximal activation voltage (\( V_{0.5} \)) and the slope factor (k) were derived. The maximal conductance (\( g_{\text{max}} \)) was determined from tail currents using the Ohm’s law \( g = \frac{I_{\text{max}} - I_{\text{min}}}{(V - V_{\text{rev}})} \), where \( I_{\text{max}} \) was the tail current after a fully activating voltage step, \( I_{\text{min}} \) was the tail current after a step to -52 mV, V was the voltage at which the tail currents were measured (-77 mV), and \( V_{\text{rev}} \) the reversal potential of \( I_h \). The \( Q_{10} \) for the change in \( g_{\text{max}} \) and kinetics of activation was determined from the relationship: \( Q_{10} = (Q_{10})^\frac{\Delta T}{10} \). Data are expressed as mean ± standard error of the mean (S.E.M.). Statistical significance was determined using Student’s t test; data were considered significant when the probability of the null hypothesis was < 0.05. Measurements were initially analyzed with Clampfit 9.0 (Axon Instruments) and all the plots, fits and statistics were performed with Origin 7.5 (Microcal, Northampton, MA) software.

**Chemicals**

All measurements of \( I_h \) under voltage-clamp were made after blocking other currents pharmacologically. Glycinergic and glutamatergic synaptic currents were blocked with 0.5 µM strychnine and 40 µM 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX). Voltage-sensitive Na⁺ and K⁺ currents were blocked with 1 µM tetrodotoxin (TTX) and 2 mM 4-aminopyridine (4-AP). All drugs were added to the saline solution and applied in the bath. In some other recordings \( I_h \) was blocked with 50 µM 4(N-ethyl-N-phenylamino)1,2-dimethyl-6-(methylamino) pyridinium chloride (ZD7288) or...
with 2 mM CsCl. Most drugs and reagents were purchased from Sigma (St. Louis, MO), ZD7288 was obtained from Tocris (Bristol, UK) and TTX from Alomone Labs (Jerusalem, Israel).

**RESULTS**

The following results are based on whole-cell patch-clamp recordings from 120 T and 15 D stellate cells in the VCN in which the access resistance, input resistance and resting potential remained stable throughout the recording. Our sample of recordings from T stellate cells is larger than that of D stellate cells because there are more of them in the VCN. The electrophysiological distinctions between T and D stellate cells are illustrated in Figure 1. While both T and D stellate cells responded to depolarizing current pulses with sustained firing, the repolarization after action potentials in responses to depolarizing current and the rectification of responses to hyperpolarizing current were subtly different (Oertel et al. 1990; Ferragamo et al. 1998; Fujino and Oertel 2001). In both groups of cells, repolarizing currents associated with the action potential caused a fast and pronounced voltage undershoot (Fig. 1, *). Unlike T stellate cells, repolarization in D stellate cells exhibits an additional slower and more variable undershoot (Fig. 1, lower panel, ▲). In response to injection of hyperpolarizing current, voltages fell to a hyperpolarizing peak and then sagged back toward rest. Experiments described below indicate that the sag results from the activation of $I_h$. The sag was slower and smaller in T stellate than in D stellate cells (Fujino and Oertel 2001). Rebound action potentials after the end of a hyperpolarizing current step were common in both types of cells.

The resting potentials and input resistances of T and D stellate neurons in the absence of any ion channel blockers were not significantly different. The resting potentials were $-64.4 \pm 0.8$ mV (n=22) in T stellate and $-64.8 \pm 0.4$ mV (n=13) in D stellate cells. In a subset of cells, responses were measured to small (10 pA) steps of current in triplicate as described in the Methods. In this sample of
cells, the input resistances around rest \( R_{\text{input}} \) had a mean of \( 74 \pm 11 \, \text{M\Omega} \) (n=12) in T stellate and \( 60 \pm 5 \, \text{M\Omega} \) (n=11) in D stellate cells.

\textit{Isolation and estimation of } I_h

To examine \( I_h \), recordings were made in voltage-clamp. It is unlikely that stellate cells are isopotential under voltage clamp because they have long, tapering dendrites and long segments of axons in slices (Oertel et al. 1990). In clamping these cells at depolarized potentials, escaped action potentials were often observed in the absence of \( \text{Na}^+ \) channels blockers. Outward currents exceeded 12 nA at +10 mV, but were unlikely to be clamped well because plots of the instantaneous component of the tail current versus the voltage were not linear and in some cells repeated voltage steps evoked inconsistent current responses. Responses to hyperpolarizing voltages were, however, generally stable under voltage-clamp. One interpretation of these observations is that the majority of \( g_h \) is located electrically near the recording site and that depolarization-activated \( g_K \) and \( g_{\text{Na}} \) are often located more distantly on axons or other fine processes.

After recording the responses to current pulses in current-clamp mode in the absence and presence of synaptic blockers, 1 \( \mu \text{M} \) TTX was added to the physiological saline to block \( \text{Na}^+ \) currents. Figure 2 shows voltage-clamp recordings of \( I_h \) in a T and a D stellate cell when the voltage was stepped from a holding potential of –62 mV to voltages between –57 and –117 mV. Shifts in voltage elicited an instantaneous current \( (I_{\text{inst}}) \) followed by a slowly developing \( I_h \) (Fig. 2A). The capacitative current at the onset of a voltage step lasted 6 to 10 msec. To avoid distortion of \( I_{\text{inst}} \) by the capacitative artifact, \( I_{\text{inst}} \) was measured as the mean value of the current over 1 ms centered 12 ms after the onset of steps to between –57 to –77 mV. For larger hyperpolarizing steps the first 12 ms were excluded and \( I_{\text{inst}} \) was measured by fitting the activation of the current with a double exponential function and extrapolating back to the onset of the step. To isolate \( I_h \), recordings were made in the presence of 2
mM 4-AP. Figure 2B shows that 2 mM 4-AP reduced $I_{\text{inst}}$, perhaps as a result of blocking a small, low-voltage activated $K^+$ current ($I_{\text{KL}}$) (Rothman and Manis, 2003). In some cells, the plot of $I_{\text{inst}}$ versus the applied voltage deviated from linearity in the absence of 4-AP causing measurements of the input conductance ($g_{\text{input}}$) (slope) to depend on the range of voltages over which the slope was fit. The $g_{\text{input}}$ at $-62$ mV was therefore estimated from linear fits of $I_{\text{inst}}$-V plots between $-57$ and $-77$ mV (Fig. 2C).

Adding 2 mM 4-AP decreased the slope and made $I_{\text{inst}}$ a more linear function of voltage. In the T and D stellate neurons shown in Figure 2C, the $g_{\text{input}}$ decreased from 12.9 to 10.4 nS and from 17.7 to 15.8 nS, respectively. On average, in the presence of 2 mM of 4-AP, the $g_{\text{input}}$ was 12.0 ± 1.3 nS (n=16) in T stellate cells and 12.4 ± 1.2 nS (n=6) in D stellate cells. Adding 4-AP reduced $g_{\text{input}}$ by 19.4 ± 4.6 % (n=6) in T stellate and by 17.8 ± 3.8 % (n=5) in D stellate cells. To determine whether 4-AP affects $I_h$, $I_h$ was first separated from other currents by subtracting $I_{\text{inst}}$ from the current evoked by a voltage step to between $-62$ and $-92$ mV (not shown). $I_h$ was reduced by 18.4 % (n=8) on average in T stellate and by 14.4% (n=3) in D stellate cells by 2 mM 4-AP.

Reversal potential of $I_h$

The reversal potentials were measured from the intersection in $I_{\text{inst}}$-V plots after conditioning pulses to three different voltages. Figure 3 illustrates how the measurement of the reversal potential for $I_h$ was made in a D stellate cell. $I_h$ was activated by hyperpolarizing, conditioning pulses to three different voltages (Fig. 3A). Pulses were longer for the smaller hyperpolarizations to allow currents to approach the steady state. Plots of $I_{\text{inst}}$ 12 ms after the end of the conditioning pulses were linear as a function of the voltage, with the slopes reflecting the magnitude of the previous, steady state conductance (Fig. 3B). At the reversal potential, no current flows through $g_h$ so that $I_h$ is equal (0 nA) under all conditions. The reversal potential was measured as the point at which the regression lines, fitted to the $I_{\text{inst}}$-V relationships, intersect. Reversal potentials in T stellate cells were $-40 \pm 2$ mV...
(n=6), those in D stellate cells were $-41 \pm 4$ mV (n=4) (Fig. 3C). The $I_{\text{inst}}$-V relationships were linear and intersected at one point, indicating that $g_h$ was reasonably well space-clamped.

**Voltage-sensitivity and maximum amplitude of $g_h$**

Tail currents were used to measure the voltage-sensitivity and maximum amplitude of $g_h$. The steady-state activation of $g_h$ is reflected in the amplitude of tail currents on return to $-77$ mV after steps to voltages between $-52$ to $-132$ mV (Fig. 4). The tail currents measured at the test voltage ($-77$ mV) result from the activation or deactivation of $I_h$ after the end of the conditioning pulse. Over the depolarizing voltage range where little or no $g_h$ was activated by the conditioning pulse, the step to $-77$ mV caused activation of $g_h$; when the variable voltage pulse was strongly hyperpolarizing, the step to $-77$ mV caused $g_h$ to be deactivated (Fig. 4A). The relative amplitudes of tail currents between the two extremes are a measure of the voltage-sensitivity of $g_h$. The relationship of the normalized conductance as a function of voltage is shown for 7 T stellate and 8 D stellate cells in Figure 4B. Half-maximal activation of $g_h$ occurred at $-88.9 \pm 0.7$ mV in T stellate and $-86.8 \pm 1.2$ mV in D stellate cells and were not significantly different. The slope factors ($k$) that reflect the steepness of the relation between voltage and fractional activation of $I_h$, were $7.8 \pm 0.6$ mV and $6.6 \pm 0.2$ mV in T and D stellate cells respectively, and were not significantly different. The Boltzmann relationships indicate that only a small fraction of $g_{h\text{ max}}$ is activated at the resting potential, on average 4.1 % and 4.5 % of the maximal $g_h$ in T and D stellate neurons respectively (Fig. 4B). To test whether measurements of the voltage-sensitivity might have been distorted by the failure of conditioning pulses to allow the activation of currents to reach the steady state, $g_h$-V curves generated from tail currents after 3-sec pulses and from responses to pulses that varied from 3 to 6.2 sec in a T stellate cell were compared. Differences were minimal (Fig. 4C). The slope factor differed significantly in T stellate cells when measurements were made with 2 and 3 sec pulses, however (data not shown).
Pharmacological block by ZD7288 and Cs+

In other cells ZD7288 (BoSmith et al. 1993; Harris and Constanti, 1995; Gasparini and DiFrancesco 1997) and Cs⁺ (McCormick and Pape 1990b; Magee 1998; Harris and Constanti 1995) have been shown to block Iₜ. We examined the sensitivity of the currents activated from a holding potential of −62 mV to 50 µM ZD7288. In both T and D stellate cells, ZD7288 blocked Iₜ almost completely and it significantly reduced Iᵢₙ (Fig. 5A and B). In responses to strongly hyperpolarizing voltages, some unblocking of ZD7288 occurred with time, as has also been observed in other types of neurons (Harris and Constanti 1995; Shin et al. 2001). The Iᵢₙ-V plot shows that gᵢₙ at −62 mV in the T stellate cell was reduced from 10.4 to 4.4 nS and in the D stellate cell from 10.1 to 6.1 nS (Fig. 5C). On average, 50 µM ZD7288 reduced the gᵢₙ by 49.4 ± 3.1 % (n=11) in T and by 46.2 ± 3.1 % (n=3) in D stellate neurons. The sensitivity to Cs⁺ was determined in a similar way. Figure 5 illustrates that 2 mM Cs⁺ blocked a substantial proportion of Iₜ in a T stellate cell. Strong hyperpolarizing pulses revealed that a small, hyperpolarization-activated current remained in the presence of Cs⁺, however, indicating that the block by 2 mM Cs⁺ was not complete. The gᵢₙ at −62 mV in this cell was reduced from 10.0 to 8.2 nS. On average 2 mM Cs⁺ reduced gᵢₙ in T stellate cells by 13.2 ± 3.5 % (n=7). The block by 2 mM Cs⁺ in T stellate cells was significantly smaller than that by 50 µM ZD7288.

Voltage-insensitive, ZD7288-sensitive conductance

A goal of the present experiments was to understand how gₜ contributes to regulating the excitability of stellate cells and how it contributes to the setting of the resting potential. The large differences, even within an individual cell, of estimates of gₜ near rest made from the Boltzmann function, and sensitivity of the input conductances near rest to ZD7288 and Cs⁺ prompted a quantitative analysis of conductances near rest.
Measurements were made of $g_{h\text{ max}}$ and $g_{\text{input}}$ as described above. Maximal $g_{h}$ was $19.1 \pm 2.3$ nS ($n=16$) in T and $30.3 \pm 2.6$ nS ($n=6$) in D stellate cells (Fig. 6A) and were significantly different. All recordings that form the basis of this analysis of conductances at $-62$ mV were made in the presence of 2 mM 4-AP. Correcting for the fraction of $g_{h}$ that was blocked by 4-AP, $g_{h\text{ max}}$ were 23.4 nS for T and 35.4 nS for D stellate cells.

There was considerable variability in the $g_{\text{input}}$ and $g_{h\text{ max}}$ among recorded neurons. This might be expected if recordings were made from stellate cells whose processes were cut to varying degrees in the making of slices. We therefore plotted $g_{h\text{ max}}$ as a function of $g_{\text{input}}$ and found that they covaried, that the T and D stellate cells with the largest $g_{\text{input}}$ also had the largest $g_{h\text{ max}}$ (Fig. 6A).

Figure 6B shows a comparison of the estimates of $g_{h}$ active at $-62$ mV derived from the Boltzmann functions and those derived from pharmacological sensitivity to $50 \mu$M ZD7288 and 2 mM Cs$^+$. In every T and D stellate cell for which measurements were made, the measurement of the voltage-sensitive $g_{h}$ was smaller than the conductances blocked by ZD7288 and Cs$^+$ at $-62$ mV. Distortions in the Boltzmann function are unlikely to have been large enough to account for these differences. One explanation for these results is that ZD7288 and Cs$^+$ are not specific for $g_{h}$ and that each blocks other, unidentified conductances. Such an explanation cannot be excluded but there is a more interesting possibility.

It has recently been demonstrated that in an expression system, the expression of $g_{h}$ is associated with a voltage-insensitive conductance, an instantaneous current (Proenza et al., 2002; Macri and Accili 2004). If the expression of a voltage-sensitive $g_{h}$ is associated with a voltage-insensitive conductance in T and D stellate cells, then the magnitude of the voltage-insensitive, ZD7288-sensitive conductance would be expected to be related to the voltage-sensitive conductance. Figure 6C shows that this was the case in T stellate cells and perhaps also in D stellate cells, that the
magnitude of the voltage-insensitive ZD7288-sensitive instantaneous conductance is proportional to the $g_{\text{h max}}$ in stellate cells. Whether the voltage-insensitive Cs$^+$-sensitive conductance follows the same pattern was unclear (because the spread of $g_{\text{h max}}$ in our sample of recorded neurons was not wide enough). However, Figure 6A implies that the voltage-insensitive, ZD7288-sensitive conductance is also proportional to $g_{\text{input}}$ and that its expression may simply be related to the surface area of the cell.

**Kinetics of $I_h$ activation and deactivation**

The activation and deactivation kinetics of $I_h$ in T and D stellate cells are compared in Figure 7. $I_h$ activated more rapidly in D than in T stellate cells (Fig. 7A). The time course of the activation of currents was well described by the sum of two exponentials, whose fast and slow time constants, $\tau_f$ and $\tau_s$, were voltage-dependent and in the tens and hundreds of ms respectively. Not only were each of the time constants significantly faster but also the relative contribution of the fast time constant was greater in D than in T stellate cells. For instance, in response to a voltage step to $-97$ mV, $\tau_f$ was $137 \pm 16$ ms in T stellate cells (n=8) and $75 \pm 9$ ms in D stellate cells (n=7); in the same cells $\tau_s$ was $966 \pm 89$ ms and $567 \pm 45$ ms in T and D stellate cells. The time constants became shorter with increasing hyperpolarization (Fig. 7B). The deactivation of $I_h$ was examined by fitting double exponential functions to the decay of the tail currents. After pulses to $-122$ mV that were long enough for the current to reach steady state, steps to between $-62$ and $-82$ mV caused $I_h$ to deactivate. Deactivation kinetics were voltage-dependent and were significantly faster in D stellate than T stellate neurons. When the voltage was returned to near rest ($-67$ mV), the fast components ($\tau_f$) were $148 \pm 17$ ms and $56 \pm 7$ and slow components ($\tau_s$) were $866 \pm 93$ ms and $356 \pm 57$ in T (n=6) and D (n=4) stellate neurons, respectively, and the fast components were more dominant in D than T stellate neurons.
To gain an understanding of how ZD7288- and Cs⁺-sensitive currents affect the excitability of stellate cells, we compared the T and D stellate cells’ behavior in current clamp in the absence and presence of these blockers. 50 µM ZD7288 and 2 mM Cs⁺ hyperpolarized T and D stellate cells, increased their input resistance, abolished the sag in the voltage response, and prevented action potentials at the end of hyperpolarizing current steps (Fig. 8). Figure 8A shows that application of 50 µM ZD7288 to a T stellate cell increased the input resistance from 45 to 124 MΩ and induced a hyperpolarization of 10 mV; injecting 40 pA of positive current brought the resting potential at the cell body to its original level. Figure 8B shows that 2 mM Cs⁺ increased the input resistance from 70 to 102 MΩ in a D stellate cell; the 3 mV-hyperpolarization of the resting potential could be compensated by 10 pA. On average, 50 µM ZD7288 increased the input resistance in T stellate cells from 69 ± 5 to 130 ± 12 MΩ and hyperpolarized the resting potential by 8 ± 2 mV (n=5). In D stellate cells ZD7288 increased the input resistance from 96 ± 10 to 167 ± 12 MΩ and hyperpolarized the resting potential by 4 ± 1 mV (n=3). In T stellate cells 2 mM Cs⁺ increased the input resistance from 86 ± 5 to 131 ± 17 MΩ and hyperpolarized the resting potential by 3.2 ± 0.7 mV (n=3). Both ZD7288 and Cs⁺ hyperpolarize the resting potential, indicate that they block a net depolarizing current; ZD7288 hyperpolarizes the resting potential significantly more than Cs⁺.

The shapes of action potentials were altered by 50 µM ZD7288 or 2 mM Cs⁺ (Fig. 8A, B). Less current was required to bring cells to threshold in the presence of both blockers. Action potentials were broader and the first, fast undershoot following the action potential was less deep when Ih was blocked. In the presence of 50 µM ZD7288, large depolarizing currents generated a few action potentials at the onset of the pulse whose peaks became progressively lower and eventually cells ceased firing (Fig.
8A). In the presence of Cs⁺, failure to generate action potentials was less pronounced than in ZD7288, but was evident when cells fired at high frequencies.

In the presence of blockers of ZD7288 and Cs⁺, two repolarizing components were clearly visible after action potentials, much like those illustrated for a D stellate cell in Figure 1. Presumably the increased input resistance in the presence of ion channel blockers made voltage changes by small currents more prominent. Under control conditions, the voltage always rises monotonically after an action potential in T stellate cells whereas double undershoots are observed in D stellate cells, characteristics that have served to distinguish these two types of cells (Fig. 1) (Oertel et al. 1990; Fujino and Oertel 2001). These results show that differences in the repolarization of action potentials in T and D stellate cells arise from differences in the magnitudes of the currents rather than from their presence or absence.

Modulation of \( I_h \)

One of the properties of \( I_h \) that makes it biologically interesting is that it can be modulated by changes in intracellular levels of cAMP (Robinson and Siegelbaum, 2003). The observation that the voltage range of activation of \( I_h \) in octopus cells, cells that lie immediately adjacent to stellate cells, was not affected by cyclic nucleotides (Bal and Oertel, 2000) raised the question whether \( I_h \) in stellate cells is modulated by cAMP. As in the previous study, modulation of \( I_h \) in stellate neurons was examined by perfusing a membrane-permeable analog of cAMP, 8-Br-cAMP, in the bath. Both the voltage-sensitivity and the kinetics of \( I_h \) in stellate cells were affected by 8-Br-cAMP.

In every T and D stellate cell tested (n=8) the voltage-activation curve shifted in the depolarizing direction upon addition of 8-Br-cAMP. Figure 9 shows an example of the action of 500 \( \mu \)M 8-Br-cAMP on a T stellate cell and 100 \( \mu \)M 8-Br-cAMP on a D stellate cell. The negative shift in the holding current at \(-62 \text{ mV}\) reveals the activation of a depolarizing current. At 100 \( \mu \)M, 8-Br-cAMP
caused a shift in the half-activation voltage \( (V_{0.5}) \) of \( I_h \) of 4.2 ± 1.0 mV (n=3) in T stellate cells and 6.7 ± 0.4 mV (n=2) in D stellate cells. The effect of 8-Br-cAMP was concentration-dependent; in T stellate cells 500 µM 8-Br-cAMP shifted \( V_{0.5} \) by 11.7 ± 1.4 mV (n=4).

In the presence of 8-Br-cAMP, activation of \( I_h \) was accelerated and deactivation was slowed. Changes in the kinetics of \( I_h \) that were observed in all T and D stellate cells tested are illustrated in a D stellate cell in the presence of 500 µM 8-Br-cAMP in Figure 10. Activation by a pulse to –97 mV proceeded with time constants \( \tau_s = 1050 \) ms and \( \tau_f = 148 \) ms in control conditions and \( \tau_s = 784 \) and \( \tau_f = 116 \) ms in the presence of 8-Br-cAMP with the fast component being more prominent (Fig. 10B). Deactivation time constants slowed and the slower component increased in prominence in the presence of 8-Br-cAMP (Fig. 10C). In this cell, the voltage range of activation of \( I_h \) shifted by 11.2 mV in the presence of 8-Br-cAMP (Fig. 10D).

**Influence of temperature on \( I_h \)**

Changes in temperature affected the kinetics and the magnitude of \( I_h \) in stellate neurons (Fig. 11). Lowering the temperature from 33 to 27 °C reduced the amplitude of the evoked inward current as well as the rates of activation and deactivation. The changes in amplitude of \( I_h \) in this cell, as in all others tested, were stable over the 12 minutes over which it was monitored. The reduction in temperature did not alter the voltage-dependence of activation; in T stellate cells \( V_{0.5} \) was –91 ± 1.6 at 33°C and –90 ± 2.0 at 27 °C (n=5). In T stellate cells \( g_{h_{\text{max}}} \) was reduced with a \( Q_{10} 1.3 \pm 0.05 \) and the fast activation time constant was slowed with a \( Q_{10} 3.3 \pm 0.3 \) when the temperature was reduced from 33 to 27 °C (n=6).
DISCUSSION

Hyperpolarization-activated conductances are common in neurons even though the voltage range of activation spans membrane potentials that are largely beyond the physiological range. The present experiments show that \( g_h \), whose voltage-sensitivity is regulated through cAMP, affects the excitability of stellate neurons by its influence on the resting potential and on the input conductance. In making measurements in T and D stellate cells that were sufficiently detailed to illustrate how consistent certain features of \( g_h \) are among neurons and also how other features of \( g_h \) seem to be tailored to suit different types of neurons in the VCN, we found unexpectedly that these cells also have a voltage-insensitive, ZD7288-sensitive and Cs\(^+\)-sensitive conductances that are five to ten times larger than \( g_h \) at –62 mV and whose magnitude is proportional to these cells’ maximum \( g_h \).

Characteristics of \( I_h \) in neurons from the VCN

\( I_h \) reverses at approximately –40 mV in both T and D stellate cells. In other cells, too, reversal potentials of this mixed cation current generally lie between –30 and –44 mV (Spain et al. 1987; Banks et al. 1993; Chen 1997; Maccaferri and McBain 1996; McCormick and Pape 1990b; Mo and Davis 1997; Bal and Oertel 2000; Saitow and Konishi 2000). This reversal potential reflects a permeability ratio \( P_{Na}/P_K \) of about 0.3 (Wollmuth and Hille 1992). The \( g_{h\max} \) of stellate cells, ~20 nS in T and ~30 nS in D stellate cells, was considerably smaller than that in octopus cells (~150 nS) and the half-activation-voltages around –88 mV were more hyperpolarized than that measured in octopus cells (~–65 mV) (Bal and Oertel 2000). The differing maximal conductances and voltage ranges of activation result in large differences in the resting \( g_h \). On average only about 4% \( g_{h\max} \), is activated in T and D stellate cells at rest whereas 62 nS \( g_h \), 41% of \( g_{h\max} \), is activated in octopus cells (Bal and Oertel 2000). The kinetics of \( I_h \) is more rapid in D stellate than in T stellate cells and both are slower than in octopus cells. In each of the cells \( \tau_{\text{fast}} \) dominates the time course of the activation of \( I_h \). Currents
evoked by steps from \(-62 \text{ mV}\) to the voltage where \(g_h\) was about half-maximally activated had a \(\tau_{\text{fast}}\) of 50 ms (\(-67 \text{ mV}\)) in octopus cells, 140 ms (\(-97 \text{ mV}\)) in T stellate, and 75 ms (\(-97 \text{ mV}\)) in D stellate cells. For the same voltage steps, \(\tau_{\text{slow}}\) was 190 ms in octopus, 960 ms in T stellate, and 560 ms in D stellate cells. Bushy cells express \(g_h\) with characteristics generally similar to those described here for stellate neurons; measurements in bushy cells are not directly comparable because they were made from younger animals and at lower temperatures (Cuttle et al. 2001); the expression and possibly the voltage dependence of \(I_h\) are developmentally regulated in bushy cells as in other cell types (Cuttle et al. 2001; Tanaka et al. 2003).

A characteristic feature of \(g_h\) is that its biophysical properties are regulated by cAMP. In T and D stellate cells, 8-Br-cAMP shifted its voltage dependence in the depolarizing direction. Increases in intracellular cAMP shift the voltage range of activation in the depolarizing direction with little or no change in \(g_{h\text{max}}\) in many cell types including bushy cells (McCormick and Pape 1990b; Tokimasa and Akasu 1990; DiFrancesco and Tortora, 1991; Banks et al. 1993; Ingram and Williams 1996; Ludwig et al. 1998; Santoro et al. 1998; Cathala and Paupardin-Tritsch 1999; Santoro and Tibbs 1999; Vargas and Lucero 1999; Saitow and Konishi 2000; Chen et al. 2001; Cuttle et al. 2001). The maximal shift is related to the type of channels that form \(I_h\) and also to the amount of basal modulation by the resting levels of cAMP (Chen et al. 2001). In octopus cells, \(g_h\) seems not to be modulated in similar experiments (Bal and Oertel 2000) suggesting that in these cells, \(I_h\) channels are either fully modulated or that they are formed largely from subunits whose sensitivity to cAMP is small. In octopus cells the magnitude of \(I_h\) also changes with temperature and then adapts over tens of minutes (Cao and Oertel, 2005). We did not observe such adaptation in T or D stellate cells.
**Possible molecular identity of \( I_h \) in neurons from the VCN**

\( I_h \) is mediated through hyperpolarization-activated cyclic nucleotide-gated (HCN) channels. Four subunits, HCN1-HCN4, form these channels (Santoro et al. 1997; Ludwig et al. 1998; Santoro et al. 1998; Santoro and Tibbs 1999). Homomeric channels expressed in heterologous expression systems have differing voltage-sensitivity, kinetics and sensitivity to cyclic nucleotides. Channels of HCN1 subunits are fastest, HCN2 channels activate and deactivate at intermediate rates, and HCN4 channels are the slowest (Moosmang et al. 2001). HCN1 are less sensitive to cyclic nucleotide modulation than the others (Santoro et al. 1998; Ulens and Tytgat 2001) whereas HCN2 is more sensitive to cAMP (Chen et al. 2001). HCN subunits also combine to form heteromeric channels (Ulens and Tytgat 2001; Chen et al. 2001; Altomare et al. 2003).

The protein of HCN1 and HCN2 subunits has been detected in the octopus cell area and in adjacent regions where T and D stellate cells are located (Koch et al. 2004). The observed properties indicate that stellate neurons seem preferentially to express the relatively slow and modulatable HCN2 subunits. On the other hand, the fast kinetics, depolarized voltage range of activation, and apparent insensitivity to cAMP indicate that HCN1 is the most prevalent subunit expressed in octopus cells. Measurements of the levels of mRNA expression in the VCN also indicate that HCN1 and HCN2 are the most commonly expressed subunits (Santoro et al. 2000).

**Assessment of three methods for isolating \( I_h \)**

In our experiments \( I_h \) was isolated by widely used electrophysiological and pharmacological techniques, its voltage-sensitivity, ZD7288-sensitivity and Cs\(^+\)-sensitivity. It was surprising that the ZD7288-sensitive and Cs\(^+\)-sensitive conductances were so much greater than the estimate of \( g_h \) from the Boltzmann relationship (Fig. 6B).
It has long been known that $I_h$ is sensitive to extracellular $Cs^+$ (Spain et al. 1987; McCormick and Pape 1990b; Maccaferri et al. 1993). In the present experiments, 2 mM Cs$^+$ blocked a conductance that was about six-fold greater than the voltage-sensitive $g_h$ but about half as great as that blocked by 50 $\mu$M ZD7288 at $-62$ mV in T stellate cells. In the MNTB Cs$^+$ also blocked a larger conductance at rest than expected from the Boltzmann analysis, (Banks et al. 1993). The block may not have been complete or entirely specific. The block of $I_h$ by Cs$^+$ is known to be more complete at hyperpolarized than at depolarized voltages; for example, at $-62$ mV, only 60% of heterologously expressed HCN2 channels were blocked by 5 mM Cs$^+$ (DiFrancesco 1982; Moroni et al. 2000). There is also the possibility that Cs$^+$ is not entirely specific for inwardly rectifying mixed cation channels; it could, for example, affect inwardly rectifying K$^+$ channels (Lesage et al. 1995; Mermelstein et al. 1998). In stellate cells extracellular Cs$^+$ at 2 mM seems to block primarily $g_h$ because its application causes the resting potentials to hyperpolarize. The present results are consistent with the conclusion that Cs$^+$ blocks the voltage-sensitive $g_h$ as well as a voltage-insensitive conductance but that the block is incomplete.

ZD7288 has been widely used to test the action of $I_h$ because it is considered to block this current nearly completely and with considerable specificity (BoSmith et al. 1993). This drug has been used to assay $I_h$ in octopus cells of the VCN (Bal and Oertel 2000), substantia nigra neurons (Harris and Constanti 1995), CA1 hippocampal pyramidal cells (Macaferri and McBain 1996; Gasparini and DiFrancesco 1997), thalamic neurons (Luthi et al. 1998), and cerebellar basket cells (Saitow and Konishi 2000). In hippocampal CA1 neurons, the half-maximal blocking of $I_h$ required 10.5 $\mu$M and at 50 $\mu$M, the concentration used in the present experiments, the block was nearly saturated (Gasparini and DiFrancesco 1997). At 50 $\mu$M, ZD7288 has been reported to block glutamate receptors of both AMPA and NMDA subtypes (Chen 2004) and a low-voltage activated Ca$^{2+}$ conductance (Felix et al.
but these conductances were blocked by DNQX and by voltage respectively in our experiments. In hippocampal pyramidal cells and in thalamic relay neurons, neither the Na$^+$-dependent or Ca$^{2+}$-dependent action potentials were affected by ZD7288 (Maccaferri and McBain 1996; Gasparini and DiFrancesco 1997; Luthi et al. 1998). Furthermore, the removal of Ca$^{2+}$ does not affect the resting potential or the shape of action potentials in T stellate cells (Wickesberg and Oertel 1989), indicating that voltage-sensitive Ca$^{2+}$ conductances are small. It thus seems unlikely that the block of these conductances accounts for the unexpectedly large conductance block by ZD7288 near rest.

An interesting possibility is that ZD7288 blocks a voltage-insensitive current that is mediated through HCN channels. Accili and his colleagues have suggested that the expression of HCN2 channels is associated with the expression of an “instantaneous current” that is voltage-insensitive over the tested voltage range and whose magnitude is directly proportional to the amount of heterologously expressed HCN protein and that increased on coexpression with the regulatory subunit MiRP (Proenza et al. 2002; Macri and Accili 2004). These authors concluded that HCN channels mediate a voltage-insensitive, instantaneous current through leaky channels. In contrast to the present results, the instantaneous current was insensitive to extracellular Cs$^+$ but its sensitivity to ZD7288 was not reported. In hippocampal pyramidal cells ZD7288, at 10–20 $\mu$M, blocked about 18% of the resting conductance (Gasparini and DiFrancesco 1997). Other authors, however, did not observe an instantaneous, ZD7288-sensitive conductance in thalamic relay neurons (Luthi et al. 1998) and with coexpression of HCN with the MiRP1 protein in cardiac sinoatrial cells (Qu et al. 2004).

Boltzmann analyses of conductances activated and deactivated by voltage are also subject to errors. First, if the conditioning pulses are too short, activation can be underestimated, especially for voltage pulses that activate the conductance slowly. Our tests suggest this was not a significant problem when the pulses were 3 sec or longer. Second, the voltage range over which $g_{ih}$-V curves were
measured should be wide enough for activation and deactivation to be saturated. The range of
activation used, –50 and –125 mV, was generally wide enough to include saturation (Fig. 4). Third,
with imperfect space-clamping, the slope of the \( g_h \)-V function can appear less steep than it is. One
indication that \( g_h \) was reasonably well space-clamped is that plots of the chord conductances were
linear and intersected at one point (Fig. 3). It is unlikely that the small errors in the \( g_h \)-V curves in our
measurements can underly the large difference in resting activation of \( g_h \) and resting ZD7288-and Cs\(^+\)-
sensitivity, indicating that 50 \( \mu \)M ZD7288 and 2 mM Cs\(^+\) block a voltage-insensitive conductance in
addition to \( g_h \).

Factors that determine the excitability of T and D stellate cells

The electrical excitability of T and D stellate cells is sensitive to small changes in resting
potential and resting conductance. The resting potentials of the T and D stellate cells recorded here in
parasagittal slices were on average 5 mV more negative and the input resistances lower by more than a
factor of two than those recorded from coronal slices (Fujino and Oertel 2001). These results suggest
that processes of stellate cells have a greater tendency to be cut in coronal than parasagittal slices, a
conclusion consistent with the morphology of these cells (Oertel et al. 1990). These results also
suggest that the resting potential at the cell body is hyperpolarized by the presence of these processes.

Several conductances have been identified that contribute to setting the resting potential of
stellate cells. Of the resting conductances, about 15 nS in both types of stellate cells, about 20% is
blocked by 2 mM 4-AP. 4-AP blocks low-voltage-activated K\(^+\) conductances (Manis and Marx 1991;
Brew and Forsythe 1995; Banks et al. 1993; Rathouz and Trussell 1998; Wu 1999; Bal and Oertel
2001; Rothman and Manis 2003) and a small proportion of \( g_h \) (present experiments, Bal and Oertel
2000). The present experiments show that the voltage-sensitive \( g_h \) accounts for about 10% of the input
conductance and that the voltage-insensitive, ZD7288-sensitive conductance contributes roughly
another 20%. A muscarine-sensitive, voltage-insensitive conductance of about 1 nS affects T stellate, but not D stellate, cells (Fujino and Oertel, 2001).

The present experiments also reveal the importance of the input conductance in the generation of action potentials. Increasing the input resistance with ZD7288 or Cs⁺ weakens or prevents firing. In the presence of these blockers, action potentials become broader and their peaks become lower with repeated firing, presumably because slower depolarization results in greater inactivation of Na⁺ currents. By increasing the input conductance at rest, ZD7288- and Cs⁺-sensitive conductances promote rapid firing in stellate cells.

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FIGURE LEGENDS

**Figure 1**- Responses to somatic current injection in a T and a D stellate neuron. Current pulses evoked hyperpolarizations that were initially large and then sagged back toward rest. In T stellate neurons the sag was slower and of smaller magnitude than in D stellate cells. Depolarizing current pulses evoked suprathreshold depolarizations that caused steady firing in both types of neurons. The input resistances were 63 MΩ in the T and 68 MΩ in the D stellate neuron. Differences in the shapes of action potentials that contributed to the identification of neurons are illustrated at an expanded time scale in the *insets* (position in the train indicated). In T stellate cells recovery from the undershoots after action potentials (*), was monotonically rising whereas in D stellate neurons some action potentials were followed by two distinct hyperpolarizations (*▲*). As is typical, occasional spontaneous synaptic events were observed in both types of neurons; their pharmacological blockade did not affect the observations of undershoots. Recordings were made in normal physiological saline.

**Figure 2**- Sensitivity of stellate neurons to 4-AP. **A.** Voltage-clamp recordings in a T (upper panel) and a D (bottom) stellate neuron. From −62 mV, voltage pulses to between −57 and −117 in 5 mV steps elicited an instantaneous and a slowly activating, inward current, I_{h}. In control recordings 40 µM DNQX, 0.5 µM strychnine and 1 µM TTX were added to the bath. To visualize the instantaneous currents, only the first 2 sec of responses to 3-sec voltage pulses are illustrated. **B.** Adding 2 mM 4-AP reduced the amplitude of the instantaneous and steady-state currents. **C.** Plots show the instantaneous current (open symbols), measured 12 ms after the beginning of the voltage pulses (−57 to −72 mV) or by extrapolating the exponential fits to t = 0 (−77 to −117 mV), and steady state current (filled symbols), measured at the end of the pulse. The slopes of the relationships between instantaneous
current and voltage are a measure of the cells’ input conductance. 4-AP reduced the input conductance at –62 mV from 12.9 to 10.4 nS in the T stellate cell and from 17.7 to 15.8 nS in the D stellate cell.

**Figure 3** - Estimate of the reversal potential of $I_h$ in a D stellate cell. **A.** Conditioning pulses long enough for currents to reach steady state levels to three conditioning potentials (–92, –107, and –122 mV) activated $I_h$. The instantaneous current after the end of the conditioning pulses (symbols) reflected a voltage-dependent $g_h$ and a voltage-independent leakage conductance. **B.** Instantaneous currents following the conditioning pulses are plotted as a function of the test potential. For each conditioning voltage, the instantaneous currents were linear functions of voltage whose slopes reflected the conductance at the end of the conditioning pulse. At the intersection of the three regression lines (dotted line), $I_h$ is equal to zero under each of the three conditions, and thus is the reversal potential of $I_h$. **C.** Reversal potentials were measured for 6 T stellate and 4 D stellate cells (open symbols). On average the reversal potentials of $I_h$ lay at –40 mV in both T and D stellate cells (solid symbols).

**Figure 4** - Voltage-sensitivity of $g_h$ in T stellate and D stellate neurons. **A.** The voltage was held at –62 mV, then stepped to conditioning pulses between –52 to –132 mV for 3 seconds, and taken to the test voltage, –77 mV, where the driving force for $K^+$ was small and where voltage-dependent inward currents are minimally activated. **B.** Plots of the normalized tail currents (measured 12 ms after the beginning of the test pulse) as a function of the voltage of the conditioning pulse. The voltage was corrected for uncompensated access resistance and therefore differs slightly from the command voltage shown in A. Each cell’s (7 T stellate, 7 D stellate cells) measurements were fit with a Boltzmann distribution. At the resting potential ($V_r$) $g_h$ was activated to 4.5 % in T stellate and 4.1 % in D stellate cells. **C.** To test whether $g_h$-$V$ relationships were distorted because slowly activating currents had not reached steady state in a T stellate cell, $g_h$-$V$ curves determined from constant 3-sec pulses (left panel)
were compared with measurements when the duration of pulses was varied so that the slowest pulses were the longest (middle panel). The $g_h$-V plots were almost identical (right panel) indicating that the duration of pulses was long enough to obtain a good representation of the voltage sensitivity of $g_h$. At the resting potential of this cell (–67 mV) the fractional activation of $g_h$ was 0.047 for the longer pulses, and 0.040 for the shorter pulses.

**Figure 5**- Effects of ZD7288 and Cs$^+$ on the instantaneous and time-dependent currents in T and D stellate cells. **A.** Voltage-clamp recordings of $I_h$ in T (upper and lower panels) and a D (middle panel) stellate neurons. From –62 mV, voltage pulses elicited an instantaneous and a slowly activating, inward current, $I_h$. In control recordings 2 mM 4-AP, 40 µM DNQX, 0.5 µM strychnine and 1 µM TTX were added to the bath. To visualize the instantaneous currents, only the first 2 sec of responses to 3-sec voltage pulses are illustrated. **B.** Adding 50 µM ZD7288 or 2 mM Cs$^+$ reduced the amplitude of the instantaneous current and reduced $I_h$. In the presence of ZD7288 there was some unblocking at strongly hyperpolarized voltages. In the presence of 2 mM Cs$^+$ some activation of $I_h$ was observed, indicating that the block was incomplete. **C.** Plots show the relationship between voltage and the instantaneous (open symbols) and steady state currents (filled symbols). In the T stellate cell, whose maximum $g_h$ was 23.2 nS, the input conductance at –62 mV was reduced from 10.4 to 4.4 nS by ZD7288. In the D stellate cell, whose maximum $g_h$ was 30.2 nS, the input conductance was reduced from 10.1 to 6.1 nS, by ZD7288. The bottom panel shows that Cs$^+$ reduced the input conductance from 10.0 to 8.2 nS in the T stellate neuron whose maximum $g_h$ was 16.8 nS.

**Figure 6**- Relationship of the voltage-dependent $g_h$ and the conductances sensitive to ZD7288 or Cs$^+$ at –62 mV in T and D stellate cells. All the measurements were made in the presence of 2 mM 4-AP **A.** Plots of $g_{h\max}$ as a function of $g_{\text{input}}$ indicate that the two variables are linearly associated. Filled
symbols indicate the mean ± SEM. B. The percentage of \( g_{h \text{ max}} \) active at –62 mV (clear bars) was determined as illustrated in Figure 4. The magnitude of the conductance at –62 mV that is sensitive to ZD7288 or Cs\(^+\) is expressed as percentage of \( g_{h \text{ max}} \) (hatched bars) in the same sample of cells. In both T and D stellate cells, ZD7288 and Cs\(^+\) block a significantly (*) larger conductance than the proportion of \( g_{h \text{ max}} \) than is expected to be active at this voltage from the analysis of the voltage-dependence of \( g_h \). The ZD7288-sensitive conductance is also significantly larger than the Cs\(^+\)-sensitive conductance (#). C. The magnitude of the voltage-insensitive, ZD7288-sensitive conductance (the ZD7288-sensitive conductance minus \( g_h \) at –62 mV) was plotted as a function of \( g_{h \text{ max}} \) for 10 T stellate (Δ) and 4 D stellate cells (○). A regression line fit to the T stellate cell data indicates a positive correlation in the data set (coefficient  \( r = 0.77 \), \( p=0.0023 \)).

**Figure 7**- Activation and deactivation kinetics of \( I_h \) in stellate neurons are voltage-dependent. A. Activation of \( I_h \) (continuous lines) is shown in responses to steps between –97 and –117 mV from a holding potential of –62 mV. Deactivation of \( I_h \) is illustrated in the tail currents after the voltage was returned from a fully activating pulse to –62, –67, or –72 mV. Superimposed on these recordings in a T stellate (top) and a D stellate cell (bottom) are fits with the sum of two exponential functions (small circles). The first 12 to 15 ms of the recorded current were excluded to avoid contamination of the fits by the capacitative currents. B. Slow (\( \tau_s \), top) and fast (\( \tau_f \), middle panel) time constants for the activation (mean ± SE: n=8 T stellate cells, open circles; n=7 D stellate cells, filled circles) and deactivation of \( I_h \) (n=6 T stellate cells, n=4 D stellate cells). Bottom panel shows that all time constants are voltage-dependent, are faster in D stellate than T stellate cells, and that the faster time constant (A\( \tau_f \)) accounted for the majority of amplitude.
Figure 8- ZD7288 and Cs+ alter the firing patterns and the shapes of action potentials in stellate cells.

A. In the presence of blockers of synaptic inputs, the T stellate neuron had a resting potential of –67 mV, fired a train of action potentials that adapted slightly (top left). Application of ZD7288 hyperpolarized the cell to –76 mV, eliminated the sag in responses to hyperpolarization (bottom left), and increased its input resistance (plot at right). The membrane potential was returned to –67 mV with +40 pA DC current. Depolarizing pulses evoked firing only at the onset of the pulse with action potentials that were followed by double undershoots; stronger current pulses did not overcome the firing block. Inset at right shows action potentials with and without ZD7288; both were the first in a train elicited by a current step of +120 pA. Action potentials were broader and undershoots shallower in the presence of ZD7288 (thick trace).

B. In a D stellate neuron Cs+ shifted the resting potential from –65 to –68 mV that was compensated with +10 pA of DC current (bottom left). Slope of voltage-current relationship document the increase in input resistance caused by 2 mM Cs+ that it is not altered by returning the resting potential to its original level (plot at right). First action potentials in a train evoked by +120 pA current steps before (thin) and after (thick) addition of Cs+ (traces at bottom right). In the presence of Cs+, action potentials were taller, had smaller fast undershoots and larger slow undershoots. All experiments were done in the presence of DNQX and strychnine to block spontaneous synaptic inputs.

Figure 9- 8-Br-cAMP modulates the voltage dependence of gh in stellate cells. Voltage-clamp recordings were made of Ih in T stellate (A) or in a D stellate neuron (B) under control conditions (left panels) and after 8-Br-cAMP was added to the bath (middle panels). Neurons were held at –62 mV, then stepped to conditioning voltages between –52 and –132 mV for 3 seconds, and finally stepped to –77 mV. The plots (right panels) compare the voltage-dependence of gh, derived from tail currents as
in Figure 4, in the absence (open symbols) and presence (filled symbols) of 8-Br-cAMP. Lines are fits to the Boltzmann equation. 500 µM 8-Br-cAMP shifted the $V_{0.5}$ from $-94.2$ (k=10.5 mV) to $-79.6$ mV (k=9.7 mV) in a T stellate cell (A). 100 µM 8-Br-cAMP shifted the $V_{0.5}$ from $-85.9$ mV (k=7.1 mV) to $-77.8$ mV (k=7.2 mV) in a D stellate cell.

**Figure 10-** 8-Br-cAMP modulates the kinetics of activation and deactivation of $I_h$. **A.** Activation and deactivation were compared in the presence and absence of 500 µM 8-Br-cAMP in a D stellate cell. From a holding potential of $-62$ mV, a pulse to $-122$ mV activated $I_h$ fully and then deactivation was induced to voltages between $-62$ and $-77$ mV (left panels). The time course of activation was compared with averaged currents at $-122$ mV normalized to the steady state current at an expanded time scale (upper right). Time course of deactivation was compared after the voltage was stepped from $-122$ to $-62$ mV (lower right). Superimposed on these currents are fits of the sum of two exponential functions (small circles). **B.** In the presence of 8-Br-cAMP (●), $I_h$ activated faster and the contribution of the fast component was larger than under control conditions (○). **C.** In the presence of 8-Br-cAMP (●), deactivation slowed; the fast time constant was slower and its contribution was smaller. **D.** 8-Br-cAMP shifted the voltage range of activation by 11 mV in the depolarizing direction. Boltzmann fits show that the $V_{0.5}$ shifted from $-90.7$ to $-79.5$ mV; slope factors were 8.9 mV, control and 9.3 mV, presence of 8-Br-cAMP.

**Figure 11-** Temperature affects the amplitude, kinetics and voltage-dependence of $I_h$. A T stellate cell was held at $-62$, stepped to between $-62$ and $-127$ mV and then returned to $-77$ mV. Currents were first recorded at the normal temperature, 33°C (top left). The temperature was then reduced to 27°C and $I_h$ was recorded about 8 min later (top right). Currents in responses to a step to $-122$ mV at the two
temperatures were fitted with double exponential functions *(small circles)*. \( \tau_f \) and \( \tau_s \), represent the fast and slow exponential components and \( g_h \) the conductance at the steady state *(bottom left)*. The tail currents shown in A and B were used to compare the voltage dependence of \( g_h \) at the two temperatures. Fits to the Boltzmann function showed that there was no difference between them: at 33\(^\circ\)C, \( V_{0.5} = -89.4 \text{ mV} \) and \( k = 7.3 \text{ mV} \) and at 27\(^\circ\)C \( V_{0.5} = -90 \text{ mV} \) and \( k = 7.2 \text{ mV} \) *(bottom right)*.
Figure 1
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Figure 3
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Figure 10
Figure 11