Hyperpolarization-Activated Currents in the Growth Cone and Soma of Neonatal Rat Dorsal Root Ganglion Neurons in Culture

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Wang, Z., R. J. Van Den Berg, and D. L. Ypey. Hyperpolarization-activated currents in the growth cone and soma of neonatal rat dorsal root ganglion neurons in culture. J. Neurophysiol. 78: 177–186, 1997. Dissociated dorsal root ganglion neuron growth cones and somata from neonatal rats were voltage and current clamped with the use of the perforated-patch whole cell configuration to study the occurrence and properties of slow hyperpolarization-activated currents (Ih) at both regions. Under voltage-clamp conditions Ih, blockable by 2 mM extracellular CsCl, was present in 33% of the growth cones tested. Its steady-state activation as a function of voltage could be fitted with a single Boltzmann function with a midpoint potential of −97 mV. The time course of current activation could be best described by a double-exponential function. The magnitude of the fully activated conductance was 3.5 nS and the reversal potential amounted to −29 mV. At the soma, Ih was found in 80% of the somata tested, which is much higher than occurrence at the growth cone. The steady-state activation curve of Ih at the soma, fitted with a single Boltzmann function, had a midpoint potential of −92 mV, which was more positive than that in the growth cone. The double-exponential activation of the current was faster than in the growth cone. The fully activated conductance of 5.1 nS and the reversal potential of −27 mV were not significantly different from the values obtained at the growth cone. Membrane hyperpolarization by current-clamp pulses elicited depolarizing sags in 30% and 78% of the tested growth cones and somata, respectively, which is in agreement with our voltage-clamp findings. Termination of the hyperpolarizing current pulse evoked a transient membrane depolarization or an action potential at both sites. Application of 2 mM extracellular CsCl hyperpolarized the membrane potential reversibly by −5 mV and blocked the depolarizing sags and action potentials following the current injections at these regions. Thus Ih contributes to the resting membrane potential and modulates the excitability of both the growth cone and the soma. Intracellular perfusion with the second messenger adenosine 3′,5′-cyclic monophosphate (cAMP) was only possible at the soma by the use of the conventional whole cell configuration. Addition of 100 μM cAMP to the pipette solution shifted the midpoint potential of the Ih activation curve from −108 to −78 mV. The current activation time course was also accelerated. The reversal potential and the fully activated conductance underlying Ih were not changed by cAMP. These results imply that cAMP primarily affects the gating kinetics of Ih. Our results show for the first time quantitative differences in Ih properties and occurrence at the growth cone and soma membrane. These differences may reflect differences in intracellular cAMP concentration and in the expression of Ih.

INTRODUCTION

Neuron somata are able to grow new neurites in culture after mechanical dissociation from the intact tissue. The mechanism of nerve regeneration and neurite outgrowth is poorly understood. Neurite formation and outgrowth can be manipulated by both blockers and openers of voltage-dependent Na⁺, K⁺, and Ca²⁺ channels (Berdan and Easaw 1992). In neuroblastoma cells it has been found that the inwardly rectifying K⁺ current I_K was essential for neuron growth, because blockage of this current completely and reversibly suppressed neurite outgrowth (Arcangeli et al. 1994). Thus evidence is growing that ion channels are functionally involved in the process of neurite outgrowth. In addition, second messengers, like adenosine 3′,5′-cyclic monophosphate (cAMP), influence neuron regeneration probably through actions on ion channels (Lankford and Letourneau 1991). It is, therefore, of interest to study the role of cAMP-dependent ionic conductances in neuron regeneration. For example, the possible involvement of the cAMP-modulated and hyperpolarization-activated current Ih in neurite outgrowth has not yet been studied.

The presence of Ih has been described in different neuronal somata (Destexhe et al. 1993; Halliwell and Adams 1982; Maccacarri et al. 1993; McCormick and Pape 1990; Solomon and Nerbonne 1993; Tokimasa and Akasu 1990), including dorsal root ganglion (DRG) neurons of the mouse (Mayer and Westbrook 1983). From these studies it appeared that both the resting membrane potential and excitability are influenced by Ih. Because we have shown that at different regions of regenerating neurons, the membrane properties may vary considerably (Wang et al. 1994a), we wondered whether Ih might be differentially expressed in the neuron soma and growth cone. To gain insight into this, we have investigated the presence of Ih in isolated growth cones and in neuron somata without processes. Up to now, the occurrence of Ih and its properties in growth cones have not been studied yet. Because the modulation of Ih by cAMP in rat DRG neurons has not been examined either, we also investigated the effect of cAMP on Ih in the neuron soma.

Our results show that Ih was less frequently found in the growth cone than in the soma. On the other hand, an inwardly rectifying current, similar to I_K (Arcangeli et al. 1994), occurred more frequently in the growth cone than in the soma. The time course of activation of Ih in the growth cone was slower than that in the soma, probably related to modulating actions by intracellular cAMP. Some of these results have been published in abstract form (Wang et al. 1994b).

METHODS

DRG neurons were mechanically dissociated from 1-day-old neonatal Wistar rats and cultured on poly-L-lysine-coated (Sigma, St. Louis, MO) coverslips in Dulbecco’s modified Eagle’s medium...
enriched with 10% fetal calf serum (Wang et al. 1994a) or F14 medium (Imperial Lab) with 10% horse serum (Gibco) (Wood et al. 1988).

Both perforated-patch (Horn and Marty 1988) and conventional whole cell patch-clamp configurations (Hamill et al. 1981) were used. The first technique allowed us to study I_k at undisturbed intracellular conditions, whereas the second technique permitted us to vary intracellular concentrations of, e.g., cAMP. Growth cones with neurite stumps ≤ 100 μm were separated from 1-day (24–36 h)-old cells. This was achieved by placing a patch pipette on the neurite at a site 50–100 μm from the growth cone and aspirating the neurite (cf. Mayers 1993). Another technique was to destroy the soma in the whole cell mode by applying high pressure through the pipette. Somata studied were either isolated freshly (4–5 h) or after 1 day in culture, when they had no processes. Recordings were accepted only from growth cones and somata that had a steady resting membrane potential for >20 min and could fire action potentials on stimulation. A series of depolarizing current pulses, consisting of 15 pulses of 200 ms in steps of 20 pA, was applied to determine the firing threshold. Hyperpolarizing current injections, consisting of 15 current pulses of 200 ms to 1 s in steps of 20 or 50 pA, were used to measure membrane resistance and to record membrane potential responses. Under voltage-clamp conditions the holding potential was set at −70 or −80 mV. Hyperpolarizing pulses from −65 to −145 mV and lasting 1–3 s were applied to activate I_k. Small patch pipettes with a resistance of ~10–20 MΩ were used to patch the growth cone and large patch pipettes with a resistance of ~2 MΩ were used to patch the soma. The series resistances were compensated for 50–80%.

Because usually the magnitude of I_k was < 1 nA, the residual series resistance could give rise to small voltage errors only (<5 mV). A reasonable control of voltage of the growth cone extension can be expected, because the space constant of our neurites is estimated to be ~1 mm (Wang et al. 1994a).

Patch pipettes were filled with an intracellular-like solution (ICS) composed of (in mM) 140 KCl, 10 NaCl, 1.0 CaCl_2, 2.0 MgCl_2, 10 ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, and 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)/KOH, pH 7.2. To achieve the perforated-patch configuration, pipettes were filled with ICS with addition of 250 μg/ml of the antibiotic nystatin (Sigma). The effect of cAMP was studied by adding 100 μM cAMP and 5 mM ATP (Sigma) to the ICS, or the membrane-permeable dibutyryl-cAMP (1 mM) was used in an extracellular-like solution (ECs) containing (in mM) 140 NaCl, 5.0 KCl, 1.0 CaCl_2, 1.0 MgCl_2, 6.0 glucose, and 10 HEPES/NaOH, pH 7.2. To minimize the breakdown of dibutyryl-cAMP, the solution containing the drug was freshly made and kept at light exposure was minimized by covering the tube with aluminium foil. Neurons were incubated with this drug for 10–20 min. Glass coverslips with attached DRG neurons were mounted in an open-bottom Teflon culture dish, which was placed on the stage of an inverted microscope (Zeiss). To block I_k and to measure the leakage current, 2 mM CsCl was added in the ECS (Cs-ECS). The inward rectifier I_k was blocked by 2 mM BaCl_2 (Rudy 1988).

For stimulus protocols and data acquisition, the software package pClamp (version 5.6 and 6.01, Axon Instruments, Burlingame, CA) was used. Recordings were made with the use of an EPC-7 amplifier (List Electronic, Darmstadt, Germany) and stored after A/D conversion (TL-100). The data were analyzed with the software packages pClamp and FigP (version 6.0, Biosoft, Cambridge, UK) and the results are presented as means ± SE n, with n being the number of cells investigated. The Student’s t-test (Asyntest, version 1.10) and χ² test on 2 × 2 table, with Yates correction (SPSS/PC+, version 5.0), were used to compare values and percentages, respectively, obtained under different conditions. The F test for the residual sum of squares (RSS) described by Ljung (1987) was used to compare the different mathematical models, fitted to our experimental data. The F ratio, given by \[ \frac{(RSS_r - RSS_0)/(df_r - df_0))}{(RSS_0)/(df_0 - df_0)} \]

where df = N – p, with N being the number of data points and p the number of free parameters, and the subscripts r and 0 representing the simple and more complex models, respectively, was compared with a table of critical values for the F distribution with (df_r – df_0), df_0 degrees of freedom. The asymptotic information criterion for the RSS was also used for model discrimination. The significance level was chosen as P = 0.05.

**RESULTS**

**Perforated-patch current-clamp measurements on the isolated growth cone**

Patch growth cones were difficult because their lamellipodia were flat (<2 μm) and small (<20 μm), resulting in a success rate of <10%. Accepted growth cones exhibited resting membrane potentials of ~57 ± 1 mV and membrane resistances of 921 ± 80 MΩ (n = 16) as measured close (~10 mV) to the resting potential. Injection of depolarizing current pulses evoked action potentials in all growth cones tested (n = 16) and 63% of them fired repetitively. The firing threshold was equal to ~20 ± 2 mV, the action potential duration at half-height (half-width) was 10 ± 1 ms, and the overshoot was 33 ± 3 mV (n = 16). On small (~20-pA) hyperpolarizing current pulses, the voltage transients [\( \Delta V(t) \)] were fitted with a two-exponential function (Wang et al. 1994a; cf. Rall 1969)

\[ \Delta V(t) = C_0 e^{-t/\tau_m} + C_1 e^{-t/\tau_1} \]

where \( \Delta V(t) \) is the membrane potential transient (~10 mV) due to the current step, \( C_0 \) and \( C_1 \) are constants, \( \tau_m \) is the passive membrane time constant of the growth cone, and \( \tau_1 \) is the first equalizing time constant due to the charging of the axonal compartment. However, curve fitting showed that our growth cone responses were best described by a single-exponential function, indicating a negligible contribution of the neurite stump. The value of \( \tau_m \) was found to be 32 ± 3 ms (n = 16). The membrane capacitance, calculated from \( \tau_m/R_m \), where \( R_m \) is the steady-state membrane resistance, amounted to 35 ± 4 pF (n = 16).

On larger (>100-pA) hyperpolarizing current injections, the membrane potential exponentially relaxed toward a new steady-state level (not shown) in 11 of 16 growth cones tested. However, in five growth cones (31%) the initial membrane hyperpolarization was followed by a slow depolarizing sag of the membrane potential to a less hyperpolarized level (Fig. 1A). Interestingly, just after the applied current pulses, in particular in the smaller cones, the membrane potential first decayed toward a depolarized subthreshold level and then back to the resting membrane potential (Fig. 1A). In response to larger hyperpolarizing current injections, the amplitude of the membrane afterdepolarizations increased from subthreshold to suprathreshold levels, resulting in action potentials (Fig. 1A). The appearance of the depolarizing sag suggested the presence of a slow hyperpolarization-activated conductance. Indeed, extracellular application of 2 mM CsCl reversibly blocked the depolarizing sags and also the action potentials following the hyperpolarizing current injections (Fig. 1, B and C). In the presence of Cs⁺ the membrane resistance was significantly increased.
from 754 ± 67 MΩ to 1,034 ± 89 MΩ \((P = 0.02, n = 5)\) in a reversible manner and the resting membrane potential was reversibly hyperpolarized by \(\sim 7 \pm 2\) mV \((P = 0.01)\). This indicates that Cs\(^+\) blocked a conductance, associated with a reversal potential that is positive with respect to the resting potential and not an inward rectifier type of K\(^+\) conductance. Apparently, the blocked conductance underlies the hyperpolarization-activated current \(I_h\).

In conclusion, two types of membrane responses were found in the growth cone on hyperpolarizing current injections: exponential relaxations (69%) and hyperpolarizations followed by depolarizing sags (31%). Thus about one-third of the investigated growth cones possessed a hyperpolarization-activated conductance, contributing to the resting membrane potential. Blockage of action potentials by Cs\(^+\) strongly suggested that the hyperpolarization-activated conductance is able to modulate the excitability of these growth cones.

**Perforated-patch voltage-clamp measurements on the isolated growth cone**

In response to a series of hyperpolarizing voltage pulses in the range from \(-80\) to \(-140\) mV, three distinct types of current responses could be observed in individual growth cones (Fig. 2). First was a slowly activating current, which did not show inactivation (Fig. 2A), probably identical to the hyperpolarization-activated current \(I_h\) (Maccaferri et al. 1993; Mayer and Westbrook 1983; Solomon and Nerbonne 1993). This current was found in 33% of the growth cones tested \((n = 18)\). Second, a fast-activating and subsequently partly inactivating current (Fig. 2B), similar to the inward rectifier K\(^+\) current \(I_{ki}\) (cf. Arcangeli et al. 1994), was present in 39% of the growth cones. Finally, the remaining 28% of the growth cones exhibited a leakage or small background current (Fig. 2C). The properties of the hyperpolarization-activated currents will be further examined in this paper.

First, the stability of \(I_h\) during 20 min was tested by applying a hyperpolarizing pulse from \(-70\) to \(-120\) mV every 2 min. The amplitude of \(I_h\) is plotted as a function of time in Fig. 2D. Linear regression of pooled amplitudes on time yielded regression coefficients of 0.6 ± 0.7 pA/min \((n = 5)\), which is not significantly different from zero. Thus under our experimental conditions no significant current rundown occurred during the observation period. The leak currents \((I_L)\) were measured in the presence of Cs-ECS as a function of voltage \(V\). The \(I_L-V\) relation could be fitted by a straight line according to \(I_L = g_L(V - V_L)\), where \(g_L\) is the leakage conductance and \(V_L\) is the reversal potential of the leakage current with \(g_L = 5 \pm 2\) nS and \(V_L = -41 \pm 4\) mV \((n = 6)\), respectively. Steady-state activation of the hyperpolarization-activated current as a function of membrane voltage was determined from the peak amplitude of the inward tail current at \(-70\) mV following a series of hyperpolarizing prepulses \((V_pp)\) ranging from \(-70\) to \(-140\) mV (Fig. 3A). To remove capacitive and leakage currents from the instantaneous component of the tail current, we either subtracted the currents in Cs-ECS from the currents in ECS or we corrected for these currents digitally, yielding the Cs\(^+\)-sensitive tail current \(I_h\). \(I_h\) was normalized with respect to the maximal tail current \(I_{tmax}\), obtained after the most negative prepulses \((-130\) to \(-145\) mV). The relationship between the normalized tail currents \((I/I_{tmax})\) and \(V_pp\) could be described by a Boltzmann equation (Fig. 3B)

\[
I/I_{tmax} = 1/[1 + \exp((V_{pp} - V_{0.5})/k)]
\]

where \(V_{0.5}\) is the midpoint potential at which the conductance \((g_h)\) underlying the hyperpolarization-activated current \(I_h\) is half-activated and \(k\) is the slope factor. Curve fitting according to Eq. 2 yielded the parameters \(V_{0.5}\) and \(k\), which amounted to \(-97 \pm 3\) mV and \(12 \pm 3\) mV \((n = 6)\), respectively.

To measure the fully activated amplitude and the reversal potential \((V_{rev})\) of \(I_h\), a voltage protocol, shown in Fig. 3D, was applied after \(I_h\) was activated at \(-140\) mV for 1 s. The tail currents were recorded at the moment when the membrane potential was stepped to test voltages \((V)\) ranging from \(-120\) to \(-70\) mV. This voltage range was chosen to avoid the activation of depolarization-activated conductances. The value of the instantaneous component of the tail...
FIG. 2. Voltage-clamp recordings of hyperpolarization-activated currents from isolated growth cones in perforated-patch configuration. A–C were obtained from different growth cones; applied voltage protocol is shown in C. Three types of currents were observed in growth cones on hyperpolarizing voltage steps. A: slowly activating $I_h$ was observed in 33% of growth cones tested. B: fast-activating current similar to the inwardly rectifying $K^+$ current $I_{K_i}$ was observed in 39% of growth cones tested. C: remaining 28% of growth cones exhibited leakage or small background currents. At the most negative steps there were probably dielectric breakdowns of membrane. D: plot of mean amplitude of $I_h$ as a function of time since start of perforated patch.

current $I_t$ at a test potential is the fully activated $I_h$ at that potential. The $I_t$-$V$ relation could be described by a straight line according to

$$I_t = g_{h,\text{max}}(V - V_{\text{rev}})$$  \hspace{1cm} (3)

where $g_{h,\text{max}}$ is the maximal conductance underlying $I_h$, attained at fully activated current levels (Fig. 3E). Fitting Eq. 3 to the data points yielded $g_{h,\text{max}} = 3.5 \pm 0.7$ nS and $V_{\text{rev}} = -29 \pm 4$ mV ($n = 5$).

The activation time course of $I_h$ was clearly voltage dependent. Either one- or multiexponential functions were fitted to the measured currents to decide which function yielded the best fit. A two-exponential function fitted better than a single-exponential one in every case (F test for RSS: $P \approx 0.02$, cf. Fig. 4, A and B). A three-exponential function resulted in poor fits (F test for RSS: $P > 0.06$). Thus the current traces were best fitted with a double-exponential function, given by

$$I(t) = I_0 + I_{h,f}(1 - \exp(-t/\tau_f)) + I_{h,s}(1 - \exp(-t/\tau_s))$$  \hspace{1cm} (4)

where $I_0$ is the instantaneous value of $I_h$, and $I_{h,f}$ and $I_{h,s}$ are the steady-state levels of the fast and slow components, respectively, with $\tau_f$ and $\tau_s$ as activation time constants. In Fig. 4C it is shown that both $\tau_f$ and $\tau_s$ were dependent on membrane voltage $V$, with $\tau_f = 221 \pm 43$ ms and $\tau_s = 952 \pm 98$ ms at $-100$ mV, and $\tau_f = 88 \pm 15$ ms and $\tau_s = 428 \pm 74$ ms at $-130$ mV ($n = 6$). The magnitudes of $\tau_f$ and $\tau_s$ were significantly decreased ($P \approx 0.03$) at larger hyperpolarizing voltages. The amplitude of the fast component $I_{h,f}$ was, as expected, voltage dependent, with a value of $-249 \pm 63$ pA at $-100$ mV and with an increased value of $-466 \pm 106$ pA at $-130$ mV ($P = 0.01, n = 6$). Contrary to our expectation, the amplitude of the slow component $I_{h,s}$ was not voltage dependent, with a value of about $-173 \pm 40$ pA ($n = 6$) in the voltage range of $-130$ to $-100$ mV (Fig. 4D). This unusual finding has also been reported for rat auditory brain stem cells with the use of the same kinetic model (Banks et al. 1993).

In summary, we demonstrated for the first time that $I_h$ was present in 33% of the investigated growth cones. This percentage is close to that found under current clamp (31%), for neurons exhibiting depolarizing sags in response to hy-
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Perforated-patch current-clamp measurements at the soma

Under control conditions, the resting membrane potential and membrane resistance of somata were found to be $-60 \pm 5 \text{ mV}$ and $645 \pm 56 \text{ M}\Omega$ ($n = 14$), respectively. Action potentials could be evoked in all the somata investigated ($n = 14$) on suprathreshold current injections, and $21\%$ ($n = 3$) of the somata fired repetitively. The firing threshold was $-28 \pm 5 \text{ mV}$, half-width was $4 \pm 1 \text{ ms}$, and overshoot was $40 \pm 8 \text{ mV}$ ($n = 14$). On small (≤20-pA) hyperpolarizing current pulses, the voltage transients (≤10 mV) were fitted with Eq. 1. Here too, curve fitting showed that a single-exponential function fitted the traces best (cf. Wang et al. 1994a). The values of $\tau_m$ and membrane capacitance ($C_{m} / R_{m}$) were found to be $26 \pm 3 \text{ ms}$ and $40 \pm 8 \text{ pF}$ ($n = 14$), respectively.

On larger (>100-pA) hyperpolarizing current injections, the membrane potential exponentially relaxed toward a new steady-state level in 3 of 14 somata tested (not shown). However, in the remaining 11 somata (79%) slow depolarizing sags of membrane potential were found to be present. On termination of the first relatively small current pulses, the membrane potential first increased toward a depolarized level and then back to the resting membrane potential. On larger hyperpolarizing current injections, the amplitude of these membrane depolarizations was increased from sub-threshold to suprathreshold levels, resulting in action potentials similar to those in the growth cones, as shown in Fig. 1A. The appearance of the depolarizing sag was consistent with the presence of a slow hyperpolarization-activated conductance. Application of extracellular 2 mM CsCl reversibly blocked the depolarizing sags and action potentials following the current injections (cf. Fig. 1A). Furthermore, the membrane potential was reversibly hyperpolarized by $-5 \pm 3 \text{ mV}$ ($P = 0.02$) and the membrane resistance was increased to $861 \pm 89 \text{ M}\Omega$ ($P = 0.03$). These findings indicated that Cs$^+$ blocked a conductance in series with a reversal potential that is positive with respect to the resting potential. Thus the blocked conductance is not of the inward rectifier type.

Fig. 3. Properties of $I_h$ at isolated growth cone and soma under conditions of perforated-patch configuration. A: growth cone tail currents at $-70 \text{ mV}$ on a series of hyperpolarizing prepulses from $-70$ to $-140 \text{ mV}$. Instantaneous tail current $I_t$ reflects degree of activation of $I_h$ at the various potentials. Normalized tail currents $I_t/I_{h\text{max}}$ were used to obtain activation curves. Inset: applied voltage protocol. B: activation curves of $I_h$ measured at growth cone (○) and soma (■). Curves: fitted Boltzmann distributions (Eq. 2), yielding $V_{0.5} = -97 \text{ mV}$ at growth cone and $V_{0.5} = -88 \text{ mV}$ at soma. A clear difference between activation curves existed at these 2 sites. C: activation of $I_h$ at soma on membrane hyperpolarization. This type of current was found in 81% of somata tested. Inset: applied voltage protocol. Note time scale difference between C and Fig. 2A. D: growth cone current responses on voltage steps from $-120$ to $-70 \text{ mV}$ after prehyperpolarization to $-140 \text{ mV}$, recorded for measurement of $I_h$. Leakage current was digitally subtracted. Inset: voltage protocol. E: determination of fully activated $I_h$-$V$ relation and $V_{rev}$ of $I_h$ at growth cone and soma. ○: $I_h$ at growth cone. ■: $I_h$ at soma. —: fits according to Eq. 3, yielding $g_{h\text{max}} = 5.1 \text{ nS}$ and $V_{rev} = -27 \text{ mV}$ at soma. Values of $g_{h\text{max}}$ and $V_{rev}$ at these 2 sites were not significantly different from each other. Conductance densities, obtained by dividing $g_{h\text{max}}$ by membrane capacitance, were 0.13 and 0.15 nS/pF, respectively, at growth cone and soma, values that were not significantly different.
**FIG. 4.** Kinetics of $I_h$ in growth cone and soma in perforated-patch configuration. **A:** examples of growth cone $I_h$ responses to 4 potential steps from a holding potential of $-70 \text{ mV}$ (10-mV increments). ●: 1/16 of sample points. A single-exponential function was fitted to the data points and is represented by the solid line. Asymptotic information criterion (AIC) for residual sum of squares (RSS), given by $N \ln \text{RSS} / 2p$, with $N$ being number of data points and $p$ being number of free parameters (Ljung 1987), amounted to $220$ (3 free parameters). **B:** currents from **A** were fitted with a 2-exponential function according to Eq. 4. The 2-exponential function fit is significantly ($F$ test, $P < 0.02$) better than the 1-exponential fit. AIC amounted to $710$ (5 free parameters). Thus the best fit, corresponding to lowest value of AIC, was obtained by Eq. 4, in agreement with the $F$ test. **C:** activation time constants $\tau_f (●, ○)$ and $\tau_s (●, □)$ are voltage dependent at the growth cone (●, ●) and soma (○, □). On membrane hyperpolarization both $\tau_f$ and $\tau_s$ decreased at these 2 sites. At growth cone $\tau_f$ and $\tau_s$ were significantly ($P < 0.02$) larger than those at soma. **D:** amplitudes of 2 components, $I_{h,f} (●, ○)$ and $I_{h,s} (●, □)$ at growth cone (●, ●) and soma (○, □) as a function of membrane potential. Magnitude of $I_{h,f}$ is voltage dependent, whereas that of $I_{h,s}$ is not voltage dependent in voltage range tested.

In conclusion, responses on hyperpolarizing currents were either exponential relaxations (21%) or hyperpolarizations followed by depolarizing sags (79%). Thus a much larger fraction of the somata exhibited a hyperpolarization-activated conductance as compared with that of the growth cones (31%). This conductance clearly influences the soma resting membrane potential and excitability.

$I_h$ properties at the soma in the perforated-patch configuration

In voltage-clamp mode, $I_h$ was present in the voltage range from $-65$ to $-145 \text{ mV}$ (Fig. 3C) in 81% of the somata investigated ($n = 26$). Extracellular application of 2 mM CsCl blocked $I_h$ in a reversible manner. Another hyperpolarization-activated current, similar to $I_{h_0}$ (cf. Arcangeli et al. 1994), was found in 4% of the somata (1 of 26), whereas the remaining 15% of the somata exhibited a leakage current only (cf. Fig. 2, A–C).

The steady-state activation curve of $I_h$ was obtained in a similar way to that of the growth cone. The relationship between the normalized instantaneous component of the tail current ($I_t/I_{t,max}$) and $V_{pp}$ was fitted by Eq. 2 (Fig. 3B), yielding $V_{0.5} = -88 \pm 2 \text{ mV}$ and $k = 11 \pm 1 \text{ mV}$ ($n = 11$). The relationship between the fully activated current $I_t$ and $V$ could be fitted according to Eq. 3, yielding $g_{h,\text{max}} = 5.1 \pm 1 \text{ nS}$ and $V_{rev} = -27 \pm 3 \text{ mV}$ ($n = 7$, Fig. 3E).

The time courses of activation of $I_h$ at the soma could be best fitted by a double-exponential function (Eq. 4), yielding $\tau_f$ and $\tau_s$ as a function of $V$ (Fig. 4C). At a membrane voltage of $-100 \text{ mV}$, $\tau_f$ and $\tau_s$ amounted to $158 \pm 27 \text{ ms}$ and $660 \pm 60 \text{ ms}$, respectively, whereas at $-130 \text{ mV}$, the respective time constants were $62 \pm 16 \text{ ms}$ and $223 \pm 54 \text{ ms}$ ($n = 7$). Both $\tau_f$ and $\tau_s$ were decreased ($P < 0.01$)
on the larger membrane hyperpolarization. In Fig. 4D the relationship between $I_{h,t}$, $I_{h,s}$, and $V$ is depicted. The amplitude of $I_{h,t}$ increased from $453 \pm 52$ pA at $-100$ mV to $614 \pm 47$ pA at $-130$ mV ($P = 0.01, n = 7$). On the contrary, the magnitude of $I_{h,s}$ remained at $\sim 221 \pm 43$ pA ($n = 7$) through the voltage range tested (Fig. 4C).

In summary, as compared with the growth cone, the incidence of $I_h$ at the soma (80%) was higher ($P = 0.001$) and the midpoint potential was more positive ($P = 0.02$). The activation time constants ($\tau_{Ih}$ and $\tau_s$) obtained at the soma were faster ($P = 0.02$) than those at the growth cone. However, the reversal potential and the fully activated current at both regions were not significantly different. We conclude that at growing neurite tips the incidence and gating of $I_h$ were different from those measured at neuron somata.

Modulation of $I_h$ by cAMP at the soma in the conventional whole cell configuration

To study the effect of intracellular cAMP on the properties of $I_h$, we applied the conventional whole cell voltage clamp to the soma perfused with high (100 $\mu$M) and low (0 $\mu$M) cAMP concentrations, a measurement mode impossible to realize for the growth cone because of the very small patch pipette tips. We compared the activation curves of $I_h$, the fully activated current amplitudes, reversal potentials, and kinetics at both concentrations.

Activation curves of $I_h$ in the absence and the presence of 100 $\mu$M cAMP are shown in Fig. 5A. When Eq. 2 was fitted to the normalized tail currents ($I/I_{max}$) the midpoint potential was found to be $-108 \pm 7$ mV ($n = 12$) and $-78 \pm 4$ mV ($n = 8$), respectively, with 0 and 100 $\mu$M cAMP in the pipette. These values were statistically different ($P = 0.001$). Thus 100 $\mu$M cAMP shifted the $I_h$ activation curve $30$ mV toward more depolarized voltages as compared with the absence of cAMP. The slope factors at 100 and 0 $\mu$M cAMP were $11 \pm 2$ mV ($n = 8$) and $11 \pm 1$ mV ($n = 12$), respectively, values that were not significantly different.

The relationship between the fully activated current amplitude $I_h$ and $V$ was fitted by Eq. 3, yielding $g_{h,max} = 3.6 \pm 0.6$ nS and $V_{rev} = -27 \pm 2$ mV ($n = 8$) in the absence and $g_{h,max} = 4.3 \pm 1$ nS and $V_{rev} = -26 \pm 2$ mV ($n = 6$) in the presence of 100 $\mu$M cAMP. These values were not significantly different (Fig. 5B). Thus intracellular cAMP did not change the fully activated conductance and the selectivity of the ion channels, underlying $I_h$.

At both cAMP concentrations, the current recordings in response to hyperpolarizing voltage steps could be best fitted with the use of a double-exponential function according to Eq. 4. In Fig. 5C it is shown that at both cAMP concentrations the activation time constants $\tau_{Ih}$ and $\tau_s$ were dependent on membrane voltage $V$. The magnitudes of $\tau_{Ih}$ and $\tau_s$ at 100 $\mu$M cAMP were significantly smaller ($P = 0.01$) than those at 0 $\mu$M cAMP at all voltages tested. At both cAMP concentrations, the magnitudes of $I_{h,t}$ increased with voltage in the range from $-100$ to $-130$ mV, whereas the magnitudes of $I_{h,s}$ remained approximately constant (Fig. 5D).

Because the steady-state amplitude of $I_h$ varied considerably from cell to cell, the membrane-permeable dibutyryl-cAMP (1 mM) was applied extracellularly to investigate the effect of cAMP on the amplitude of $I_h$ from the same cell in conventional whole cell configuration with 0 $\mu$M cAMP in the pipette. Figure 6A shows that at a voltage close to $V_{rev}$ of $-110$ mV, the amplitude of $I_h$ was significantly increased ($P = 0.005$) from $-176 \pm 12$ pA to $-236 \pm 33$ pA ($n = 4$). However, at the voltage of $-130$ mV, the current amplitudes before and after addition of dibutyryl-cAMP were $-294 \pm 56$ and $-283 \pm 77$ pA ($n = 4$), respectively, not significantly different. This suggested a shift of the activation curve. Indeed, the midpoint potential of $I_h$ activation was significantly ($P = 0.005$) shifted from $-106 \pm 1$ mV to $-97 \pm 1$ mV ($n = 4$, Fig. 6B). Thus 1 mM dibutyryl-cAMP shifted the activation curve $\sim 10$ mV toward depolarizing voltages. Furthermore, the time courses of the current were accelerated by dibutyryl-cAMP (Fig. 6A, $P = 0.03$), in agreement with our preceding conclusions.

From all these results we suggest that cAMP primarily affects the gating kinetics of the channels underlying $I_h$.

DISCUSSION

In the present work we show that under perforated-patch conditions, hyperpolarization-activated currents and depolarizing membrane potential sags were found in $\sim 30\%$ of the isolated growth cones, whereas at the somata the incidences were 80%. On the contrary, 39% of the growth cones possessed an $I_{h,c}$-like current, whereas 4% of the somata exhibited this type of current. Assuming no difference in selection of the cells for growth cones or somata measurements, these findings imply that the density of ion channels carrying $I_h$ and $I_{h,c}$ varied at the two regions of the neuron. A similar difference in $I_h$ and $I_{h,c}$ expression at the soma was also found in rat dorsal motor nucleus of the vagus nerve neurons, where 64% of the cells exhibited $I_h$ and 28% showed $I_{h,c}$ (Travaglini and Gillis 1994). The regional differences in channel occurrence in our preparation suggest differential functions of $I_h$ and $I_{h,c}$-like currents at the soma and the growth cone. It has been proposed that $I_{h,c}$ is essential for neurite outgrowth in neuroblastoma cells (Arcangeli et al. 1994). We do not yet know, however, whether $I_{h,c}$ is important for the growth of rat DRG neurons as well. At present the functions of $I_h$ are not well understood. Extracellular application of Cs+ hyperpolarizes the membrane potentials at the growth cone and soma, which demonstrates that $I_h$ contributes to the setting of resting membrane potential at the growing neurite tip and at the cell body of the rat DRG neuron. Furthermore, we showed that $I_h$ is able to influence the excitability of the growth cone and soma whenever these two sites have been hyperpolarized. Our previous work has demonstrated that an increase of intracellular inositol trisphosphate concentration hyperpolarizes the soma membrane potential up to 30 mV from the resting potential (Wang et al. 1992). Under these conditions and dependent on the concentration of intracellular cAMP (see below), $I_h$ channels could be activated and as a consequence influence cell functions. Because extracellular application of 2 mM CsCl inhibits neurite sprouting (Wang et al. 1995), $I_h$ may be functionally involved in neurite outgrowth as well. Another possibility is that $I_h$ is not fully expressed in the growth cone membrane until the growth cone contacts its target cells from maturation to a sensory nerve terminal requiring $I_h$ for repetitive firing.

At both growth cone and soma, the time course of $I_h$ was
FIG. 5. Effects of adenosine 3',5'-cyclic monophosphate (cAMP) on soma $I_h$ in conventional whole cell configuration. A: activation curves of $I_h$ with 0 (▲) and 100 $\mu$M (■) cAMP in the pipette, respectively. Curves: fitted Boltzmann distributions (Eq. 2), yielding $V_{0.5} = -108$ mV at 0 $\mu$M cAMP and $V_{0.5} = -78$ mV at 100 $\mu$M cAMP. B: fully activated $I-V$ relation in absence (○) and presence (■) of 100 $\mu$M cAMP in the pipette. — : fits according to Eq. 3, yielding $g_{h,\text{max}} = 3.6$ nS and $V_{\text{rev}} = -27$ mV at 0 $\mu$M cAMP and $g_{h,\text{max}} = 4.3$ nS and $V_{\text{rev}} = -26$ mV at 100 $\mu$M cAMP. Results imply that fully activated conductance underlying $I_h$ and value of $V_{\text{rev}}$ are not affected by intracellular cAMP. C: time constants $\tau_f$ (●, ○) and $\tau_s$ (□) with 0 (n = 7, ○, ■) and 100 $\mu$M (n = 6, ○, ■) cAMP in pipette. Both $\tau_f$ and $\tau_s$ are voltage dependent. At 0 $\mu$M cAMP, magnitudes of $\tau_f$ and $\tau_s$ are significantly ($P < 0.01$) larger than those at 100 $\mu$M cAMP. D: amplitudes of $I_h, f$ (●, ○) and $I_h, s$ (□) in absence (○, ■) and presence (●, ■) of 100 $\mu$M cAMP in pipette. Magnitude of $I_h, f$ is voltage dependent, contrary to that of $I_h, s$, in voltage range tested.

Voltage dependent and could be best described by a double-exponential function, consistent with findings in rat auditory brain stem (Banks et al. 1993), mouse DRG neuron somata (Mayer and Westbrook 1983), and rat superior colliculus-projecting neurons (Solomon and Nerbonne 1993). It has been suggested that the multieponential kinetics may originate from channels with multiple open or closed states or from distinct populations of fast and slow channels (Banks et al. 1993; Solomon and Nerbonne 1993). In agreement with results for rat auditory brain stem neurons (Banks et al. 1993), in our preparation the amplitude of the fast component was voltage dependent, whereas the amplitude of the slow component was not. The voltage independence of the slow component amplitude suggests that the conductance underlying the slow component decreases or the current saturates with membrane hyperpolarization (Banks et al. 1993). Furthermore, it has been shown that in some neurons there is a delay following the decay of the capacitive current transient before the time-dependent increase in current (Hille 1992; Solomon and Nerbonne 1993). Various models have been used to account for the delay (Solomon and Nerbonne 1993; and see references therein). We characterized the delay by fitting a power function according to Hodgkin and Huxley (1952) to the measured activation of the current. The value of the exponent of this function varied from 0.98 to 1.4, with a mean value of $1.1 \pm 0.1$ ($n = 16$). Thus, although a delay in the onset of $I_h$ is present in some cells, conventional Hodgkin-Huxley-type kinetics cannot be used to describe the delay. Further kinetic analyses may help to understand the origin of the delay.

The effect of the second messenger cAMP could be well studied at the soma and it affected several properties of $I_h$. First, the $I_h$ activation curve was shifted by intracellular cAMP to more depolarized voltages, in agreement with findings in other neurons and in heart cells (Banks et al. 1993; DiFrancesco and Tortora 1991; Tokimasa and Akasu 1990;
We added 2 mM BaCl₂ extracellularly to block measurements (20 min). Longer observation times probably the small isolated growth cones were healthy during our observations.

Dibutyryl-cAMP increased currents at a voltage of −110 mV, but current amplitude at −130 mV was not significantly changed. Note that activation time courses were accelerated in the presence of dibutyryl-cAMP. B: Activation curve measured in absence and presence of dibutyryl-cAMP. Solid lines: fits according to Eq. 2, yielding $V_{0.5} = −106$ mV and −97 mV (n = 4), before and after addition of the drug, respectively. Slope factors were not changed.

The 30-mV shift in response to cAMP change from 0 to 100 µM in our preparation is close to the 27-mV shift in Purkinje fibers (Tsien 1974), but larger than the 14-mV shift found in the auditory brain stem (Banks et al. 1993). However, it was nearly 3 times larger than the shift of 11 mV in sinoatrial node myocytes (DiFrancesco and Tortora 1991). The activation curve obtained during the perforated-patch mode was halfway between the curves obtained during conventional whole cell mode with 0 and 100 µM cAMP in the pipette. Thus alterations in the intracellular cAMP concentration may cause considerable changes in the activation of $I_h$. Second, both $\tau_f$ and $\tau_i$ were decreased by cAMP. The shift of the activation curve and the acceleration of the activation time course are consistent with an action of cAMP on channel kinetics. Third, the fully activated amplitude of $I_h$ was independent of the intracellular cAMP level. These results are consistent with the findings in heart cells (DiFrancesco and Tortora 1991), but in contrast to those obtained in sympathetic and auditory brain stem neurons (Banks et al. 1993; Tokimasa and Akasu 1990), where extracellular application of 8-bromoadenosine 3′,5′-cyclic monophosphate increased the fully activated amplitude of $I_h$. Finally, the reversal potential of $I_h$ was not altered, implying that the ionic selectivity is independent of cAMP. Thus, in the somata of rat DRG neurons, intracellular cAMP most likely affects solely the gating of channels underlying the hyperpolarization-activated $I_h$. At the isolated growth cone the resting and firing properties were similar to our previous results from growth cones of intact neurons (Wang et al. 1994a). This suggested that the small isolated growth cones were healthy during our measurements (20 min). Longer observation times probably can only be realized with large-diameter (80–100 µm) growth cones from other species, which proved to be viable for several hours after isolation (Haydon et al. 1987; Shaw and Bray 1977; Wessels et al. 1978). Under our experimental conditions, we found a low incidence of $I_h$ at the growth cone as compared with the soma. One possible reason may be the loss of intracellular factors or inflow of Ca$^{2+}$ during the growth cone separation procedure. However, this alternative is unlikely, because we do not expect fast diffusion of factors or of Ca$^{2+}$ through a narrow (1-µm) and long (100-µm) neurite. On the other hand, we cannot exclude a Ca$^{2+}$ increase shortly after the growth cone separation due to stretch of the membrane (Sigurdson et al. 1993), which may cause growth cone degeneration and influence ion channel expression. Furthermore, we used the perforated-patch configuration to avoid the loss of growth cone intracellular substances. Thus the properties of $I_h$ obtained within our measuring period probably represent those of undisturbed growth cones. Under these conditions, we found for the growth cone $I_h$ that the midpoint potential was more negative and that the activation time constants were larger than those measured in the soma. Because we showed that lowering the intracellular cAMP in the soma shifted $V_{0.5}$ to hyperpolarizing values and increased $\tau_f$ and $\tau_i$, our results suggest that the cAMP concentration in the growth cone is lower than that in the soma. We may speculate that a lower intracellular cAMP concentration at the growth cone may be important for its behaviour, because increased intracellular levels at the site inhibited neurite outgrowth (Lankford and Letourneau 1991).

In conclusion, our present work demonstrates for the first time differences in $I_h$ properties and distribution at growing rat DRG neuron growth cones and somata. These differences either reflect developmental stages during neurite outgrowth or are causally involved in neurite sprouting, elongation, or directional growth.

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