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Preferential Inhibition of I_h in Rat Trigeminal Ganglion Neurons by an Organic Blocker

D. Janigro*, M.E. Martenson, T.K. Baumann

Division of Neurosurgery and Department of Physiology and Pharmacology, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, OR 97201-3098, USA

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Abstract. The potency and specificity of a novel organic I_h current blocker DK-AH 268 (DK, Boehringer) was studied in cultured rat trigeminal ganglion neurons using whole-cell patch-clamp recording techniques. In neurons current-clamped at the resting potential, the application of 10 μM DK caused a slight hyperpolarization of the membrane potential and a small increase in the threshold for action potential discharge without any major change in the shape of the action potential. In voltage-clamped neurons, DK caused a reduction of a hyperpolarization-activated current. Current subtraction protocols revealed that the time-dependent, hyperpolarization-activated currents blocked by 10 μM DK or external Cs^+ (3 mM) had virtually identical activation properties, suggesting that DK and Cs^+ caused blockade of the same current, namely I_h. The block of I_h by DK was dose-dependent. At the intermediate and higher concentrations of DK (10 and 100 μM) a decrease in specificity was observed so that time-independent, inwardly rectifying and noninactivating, voltage-gated outward potassium currents were also reduced by DK but to a much lesser extent than the time-dependent, hyperpolarization-activated currents. Blockade of the time-dependent, hyperpolarization-activated currents by DK appeared to be use-dependent since it required hyperpolarization for the effect to take place. Relief of DK block was also aided by membrane hyperpolarization. Since both the time-dependent current blocked by DK and the Cs^+-sensitive time-dependent current behaved as I_h, we conclude that 10 μM DK can preferentially reduce I_h without a major effect on other potassium currents. Thus, DK may be a useful agent in the investigation of the function of I_h in neurons.

Key words: Whole-cell patch-clamp — Time-dependent hyperpolarization-activated current — I_h — Block — K^+ channels

Introduction

I_h is a time-dependent, hyperpolarization-activated current which is found in both excitable and nonexcitable cells [e.g., 14, 21, 25–27, 30, 39]. In primary afferent somatosensory neurons, I_h is found in the cell bodies of trigeminal ganglion (TG) [34] and dorsal root ganglion (DRG) neurons [30] and in dorsal root fibers [3]. The level of expression of I_h among DRG neurons is not uniform and appears to be related to soma diameter [37] and sensory modality [15, 23]. The functional implications are presently not clear.

In CNS neurons, I_h modulates the pattern of rhythmic firing and cell resting potential. The current is involved in the sleep-waking cycle and rhythmic oscillations of thalamic neurons [31], in epileptic discharges of cortical neurons [15, 13], and possibly in the induction of long-term depression of synaptic transmission in the hippocampus [19, 24]. In cardiac cells, where it is labeled i_f, this current plays a pacemaking role [8, 9, 12].

Under physiological conditions, I_h is carried by both sodium and potassium ions [30, 39]. The current is subject to modulation by hormones and neurotransmitters [32] acting through the cyclic AMP second messenger system [1, 18, 21].

It has been known for a long time that I_h is blocked strongly by cesium [30], rubidium [6] and strontium [33], but these blockers are not compatible with electrophysiological studies in vivo. In addition, cesium and rubidium strongly interfere with permeation through a

* *Permanent address:* Department of Neurosurgery, University of Washington, Seattle, WA 98104, USA

Correspondence to: T.K. Baumann

variety of voltage-dependent as well as agonist-induced potassium currents. Thus, specific h-channel blockers would be useful in studies on the physiological role of I_h in primary afferent neurons.

In recent years, there have been reports of organic blockers of i_f [4, 10, 11, 38]. However, at the present time there is still no known substance which blocks the neuronal I_h current exclusively. In this study we have examined the properties of DK-AH 268, a known blocker of i_f in cardiac cells, as a potentially selective blocker of I_h in neurons. Part of this study has been published in abstract form [20].

Materials and Methods

Cell Culture

Adult Sprague-Dawley rats ($n = 3$), were euthanized with an overdose (2–3 ml) of sodium pentobarbital (Abbott). The trigeminal ganglia were dissected and the cells dispersed using a procedure described in detail previously [2]. Briefly, the trigeminal ganglia were minced and allowed to dissociate in a mixture of trypsin/collagenase/DNAse for approximately 1 hr at 37°C. The dissociated cells were then washed free of digestive enzymes and suspended in an L-15/air growth medium containing supplements, nerve growth factor (mouse, 7S, Chemicon), and horse serum (HyClone). The cell suspension was plated onto poly-lysine and laminin-coated glass coverslips and the cultures maintained in an incubator at 37°C. The growth medium was refreshed 3 times per week.

Recording Solutions

During the recordings, the neurons were bathed in a balanced salt solution (BSS) containing (in mmoles/1): NaCl 137.7, KCl 5.0, $CaCl_2$ 1.0, $MgSO_4$ 1.2, H_3PO_4 2.0, D,L-alanine 5.0, glucose 5.5, N-[2-hydroxyethyl] piperazine-N′-[2-ethanesuflonic acid] (HEPES) 32.0 (pH adjusted to 7.35 with 1 N NaOH). The 3 mM cesium solution was made by diluting cesium stock solution (1 M CsCl in BSS). Patch electrodes were filled with a sulfate-containing solution [40] of the following composition (in mM): 75 KCl, 75 KCH_3SO_4, 10 HEPES, 2 Na_2ATP, 0.3 Na_3GTP (pH adjusted to 7.2 with 1 N KOH).

Drugs

DK-AH 268 ((R)(−)-3-[3,4-dimethoxyphenethyl)-3-piperidinyl methyl]-1,3,4,5 tetrahydro-7,8-dimethoxy-2H-3 benzazepin-2-one HCl, Boehringer) was dissolved as a stock solution (10 mM in distilled, deionized water). The stock solution was stored at −20°C. Dilutions (1, 10, and 100 μM DK-AH 268) were made in BSS. (For the remainder of the paper DK-AH 268 is referred to as DK.)

Electrophysiological Recording

After 4 to 11 days in culture, the glass coverslips containing neurons were placed in a bathing chamber and continuously superfused with BSS (0.4 ml/min) via a glass capillary located approximately 100 μm from the neuron under study. A patch-clamp amplifier (Dagan 3900/3911A) was used to obtain tight-seal, whole-cell recordings [17, 28, 29]. Patch electrodes were made from borosilicate glass and had an average resistance of 5 MΩ when filled with the internal solution.

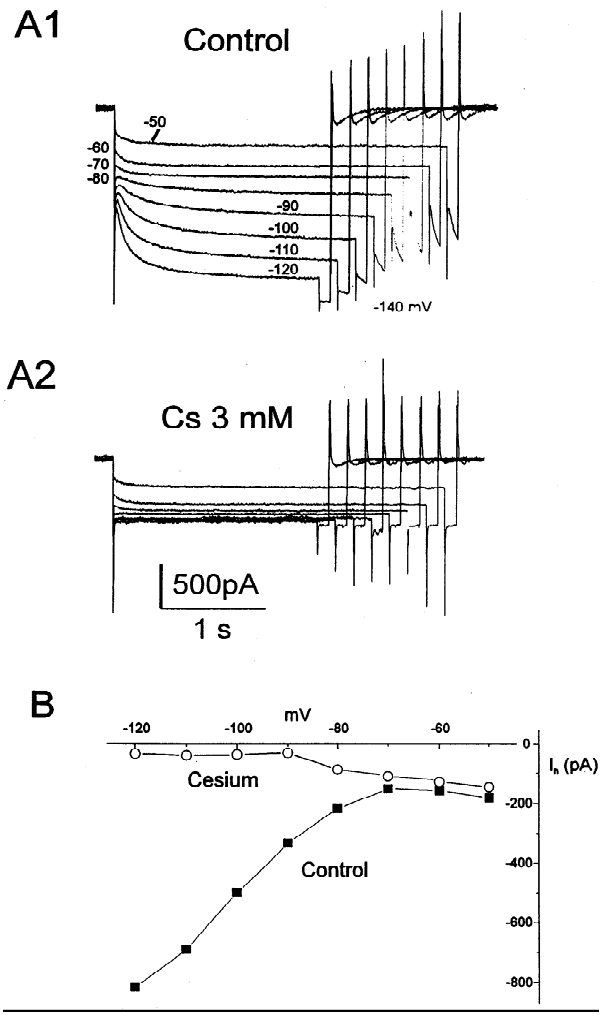

Fig. 1. Hyperpolarization-activated currents in a TG neuron and their block by cesium. (*A*1 Control; (*A*2) blocked by 3 mM Cs; (*B*) steady-state current-voltage relationship of another TG neuron in the absence (Control) and the presence of cesium (instantaneous currents were subtracted from the steady-state currents when constructing the *I-V* relationship in *B*). Hyperpolarization-activated currents elicited by stepping the membrane potential to −125 mV (in steps of −5 mV) from a holding potential (HP) of −40 mV. Each hyperpolarizing pulse was terminated by a brief step to −140 mV to activate the residual current prior to return to HP. Only data for every other trace is shown.

Upon breaking into the cell, the series resistance and capacitance were neutralized. The recording mode was switched to current clamp and the resting membrane potential measured. Threshold for action potential discharge was measured under standardized conditions (i.e., from a membrane potential of −60 mV, $n = 26$). (Small positive or negative holding currents were applied if needed to bring the neuron to −60 mV.) However, action potentials for those neurons used to study the effects of DK under current-clamp conditions ($n = 4$) were evoked from the cell's resting potential. To evoke action potentials, brief constant current pulses (39.5 msec duration, 100 pA increments), were applied at a repetition rate of 1 per 5 sec until threshold for action potential discharge was reached.

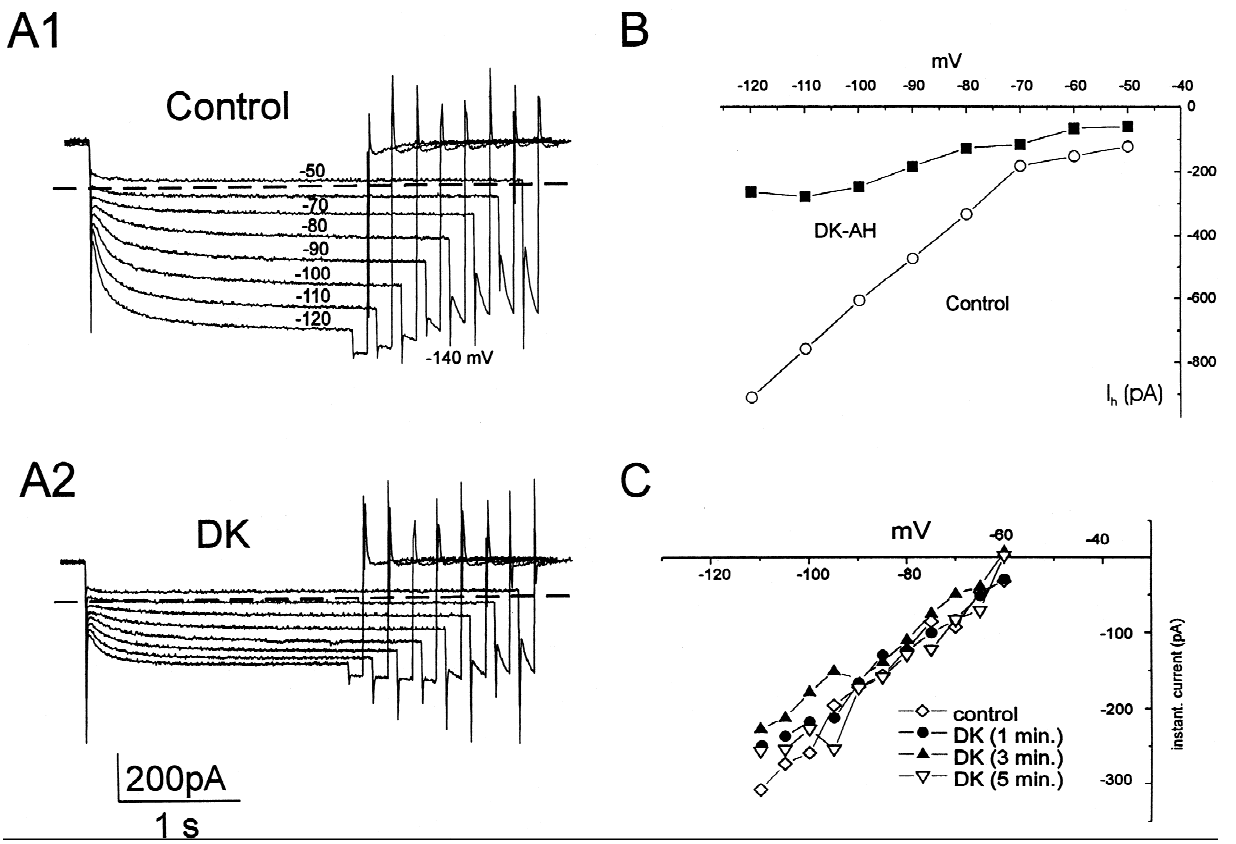

Fig. 2. Block of hyperpolarization-activated currents by DK. (*A*1) control; (*A*2) block by 10 μM DK. (*B*) steady-state current-voltage relationship for I_h in the absence (control) and the presence of DK (instantaneous currents were subtracted). (*C*) Lack of effect of DK on instantaneous hyperpolarization-activated current, even following a prolonged (5 min) DK exposure. Same neuron and drug application in *A, B,* and *C.* (*A*1 was in fact wash following previous DK application under current clamp conditions, *data not shown*).

For most cells, after recording action potentials, the recording mode was switched and responses to Cs or DK were made under voltage-clamp conditions (see figure legends for details). All signals were low-pass filtered at 2 kHz (Frequency Devices, Model 902) and acquired by pClamp 6.0 software (TL-1 interface, Axon Instruments). The DK or cesium-containing solutions were applied by manually operating a 6-way valve. All recordings were performed at room temperature (approximately 21°C), and only one neuron per coverslip was studied to avoid possible drug carryover effects.

Unless otherwise stated, descriptive statistics are reported as means and standard deviations.

Results

Recordings were made from a total of 34 cultured trigeminal ganglion (TG) neurons. The mean resting membrane potential was −62 ± 4 mV (n = 34). None of the neurons displayed spontaneous action potential discharge. All current-clamped cells which were tested with depolarizing current pulses responded with one or two action potentials (mean threshold = 857 ± 556 pA, n = 30). Soma diameters were measured in 31/34 neurons (range 27 to 49 μm).

Hyperpolarization-Activated Currents and Their Block by Cesium

All 30 TG neurons tested under voltage-clamp conditions displayed a time-dependent, hyperpolarization-activated current upon membrane hyperpolarization from a holding potential (HP) of either −40 or −70 mV. The current was variable in magnitude (ranging from −182 to −4306 pA, mean = −1344 pA at −120 mV, n = 30) but displayed reproducible time- and voltage-dependent activation patterns analogous to those described for I_h in dorsal root ganglion neurons [30].

Figure 1 illustrates I_h in a TG neuron held at −40 mV. I_h grew larger in size with larger hyperpolarizing commands, and current increases were associated with faster activation at more negative potentials (Fig. 1*A*1). Extracellular application of a solution containing 3 mM cesium abolished the current (Fig. 1*A*2). Figure 1*B* depicts the *I-V* plot of the I_h current recorded from another TG neuron under identical experimental conditions (Control and 3 mM Cs). Cs-blockade of I_h occurred in a voltage-dependent manner since fully activated currents

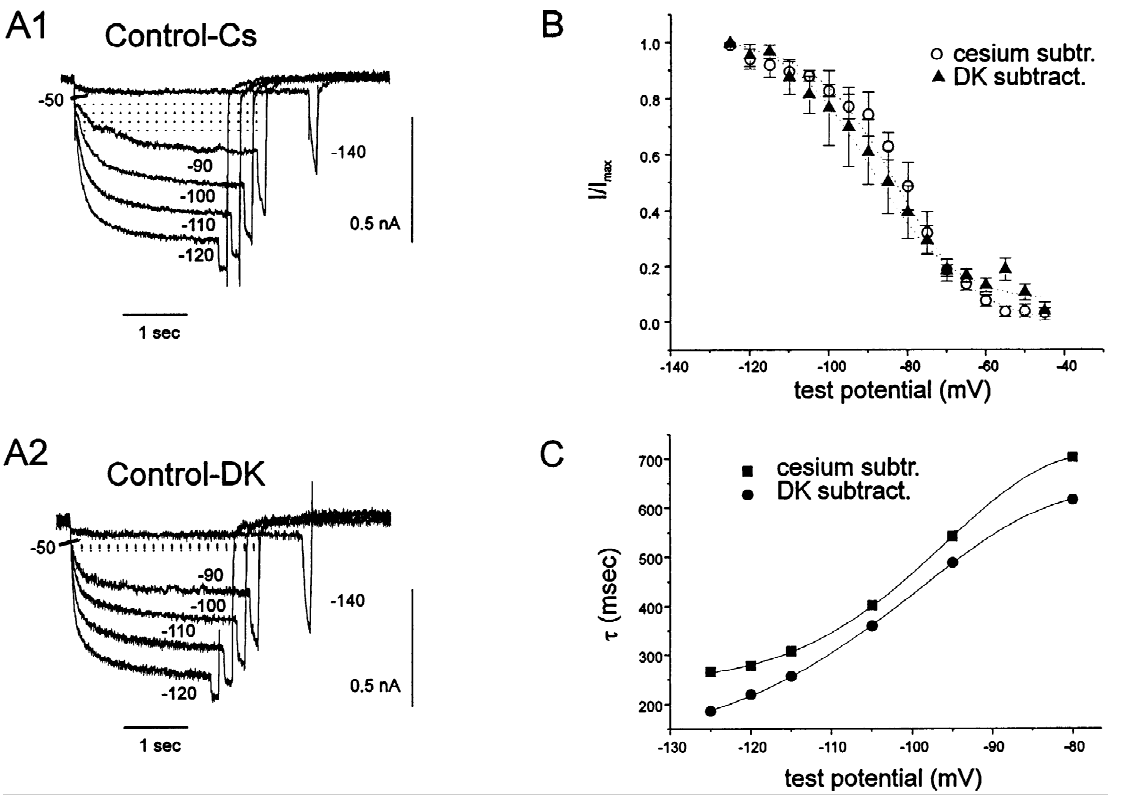

Fig. 3. Net effects of cesium and DK on hyperpolarization-activated currents. (*A*1) Arithmetic difference between currents observed under control conditions and during the exposure of the neuron to 3 mM Cs. (*A*2) arithmetic difference between currents observed under control conditions and during the exposure of the neuron to 10 μM DK. Voltage-clamp protocols as in Figs. 1 and 2. The dashed lines in *A*1 and *A*2 show the relative contribution of the time-independent, instantaneous currents (as measured at the beginning of the voltage-step) to the Cs- or DK-subtracted currents. (*B*) Current activation curves for ''cesium subtracted'' currents (*A*1) and ''DK subtracted'' currents (*A*2) (3 neurons in each group, data expressed as mean ± SEM). To construct the activation curves, the ''tail'' currents obtained by activating the residual current available at each test potential were measured and their values were plotted against the test potential (as described previously [21, 25]). (*C*) Voltage-dependence of the time constants of activation obtained by fitting single exponential functions to the net (''cesium subtracted'' and ''DK subtracted'') currents at each test potential. Data from 2 different neurons.

were more sensitive to Cs blockade than currents evoked by weaker hyperpolarization [39].

To further investigate the properties of the current blocked by Cs, we digitally subtracted currents recorded after Cs application from control currents (Fig. 3*A*1). This procedure revealed that Cs acted primarily on the time-dependent (I_h) current component, albeit a small effect on the time-independent instantaneous current was also observed (as indicated by the dashed lines). All the effects of 3 mM Cs were fully reversible upon removal of the blocking agent from the extracellular solution.

Block of I_h by DK-AH 268

In an analogous set of experiments, I_h was challenged with extracellular application of 10 μM DK. Figure 2 shows the profile of I_h activation before (*A*1), and after perfusion with a low (10 μM) concentration of DK (A2). The *I-V* plots of control and DK-blocked currents (Fig. 2*B*) show that hyperpolarization-activated time-dependent currents were blocked substantially. DK was without effect on the instantaneous currents in the same neuron (Fig. 2*C*). The current subtraction procedure used for the study of the Cs-sensitive current (Fig. 3*A*1) was also applied to the neuron exposed to DK (Fig. 3*A*2). The subtraction confirmed that DK had negligible effect on instantaneous currents (note the nearly superimposed dashed lines) and most of the current affected by 10 μM DK could be accounted for by the blockade of I_h.

To compare the current sensitive to blockade by Cs to that inhibited by DK, we examined the activation profiles (Fig. 3*B*) of the currents obtained after performing the subtraction procedure. Both Cs- and DK-sensitive currents activated at potentials negative to −60 mV and peak activation was reached at or around −130 mV. The Cs-sensitive current was half-activated at −83 mV, while the current blocked by DK had mid-activation point at

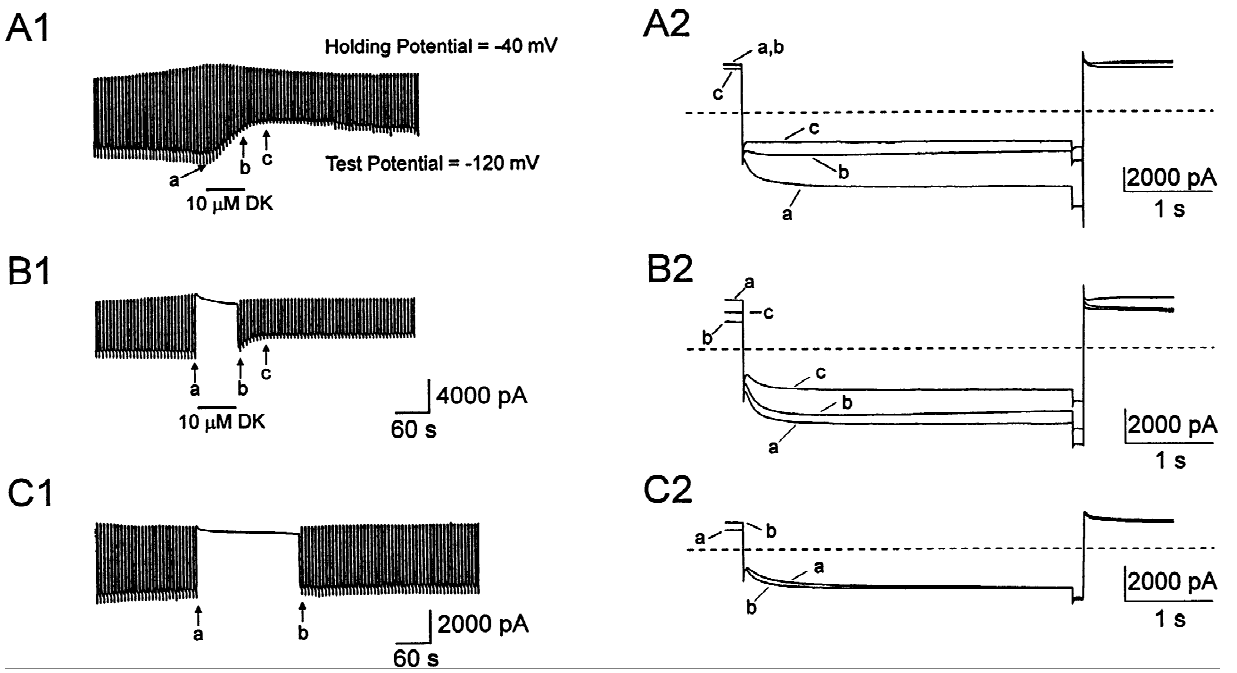

Fig. 4. Use-dependence of the block of I_h by DK. (*A*) block by DK of currents evoked by intermittent hyperpolarizing stimuli. (*B*) Maximal DK effect is shown to be hyperpolarization dependent. Note the relative lack of effect when the neuron was held at −40 mV during the entire application of the drug and the development of a block when hyperpolarizing stimulation resumed during the washout period. (*C*) Control to demonstrate lack of effect of pausing hyperpolarizing stimulation in the absence of DK. Continuous traces in the left panels (*A*1, *B*1, and *C*1) show membrane currents on a slow time-scale; right panels (*A*2, *B*2, and *C*2) are overlaid responses to individual hyperpolarizing stimuli (*a*, *b*, *c*, marked by arrows in left panels). Currents evoked by two-level hyperpolarizing stimuli (−120 mV for 3750 msec, stepped to −140 mV for 125 msec) from a holding potential of −40 mV. Three different neurons in *A*, *B*, and *C*. Same vertical scale in *A*1 and *B*1.

−85 mV. These results suggest that the Cs- and DK-sensitive current components share the same activation properties and that both currents are activated near the resting potential.

We further investigated the identity of Cs- and DK-sensitive currents by comparing the voltage-dependence of the time constants of their activation (Fig. 3*C*). Time-dependent currents obtained after the subtraction procedure were fitted individually by an exponential function according to DiFrancesco [7] and the exponents plotted against the test potentials. Both Cs- and DK-sensitive components showed a marked voltage-dependence of the speed of current activation. The profiles of the fit of the time-constant *vs.* membrane potential were virtually identical. These findings are compatible with the hypothesis that the time-dependent, hyperpolarization-activated currents blocked by Cs and DK are identical.

Use-Dependence

The effect of DK was use-dependent. Membrane hyperpolarization was necessary for DK to exert its blocking effect on I_h (Fig. 4). Intermittent hyperpolarization of the neuron during drug application (Fig. 4*A*) resulted in a gradual reduction of I_h by DK (compare traces *a* and *b*). In the absence of membrane hyperpolarization during the drug application the action of DK was greatly attenuated (Fig. 4*B*, again compare traces *a* and *b*). However, when the intermittent hyperpolarization resumed at the beginning of the washout period, the effect of the drug was evident in the same neuron (trace *c*). Figure 4*C* illustrates a control experiment and shows that interruption of the hyperpolarizing stimulus alone exerted no major effect upon I_h (compare Fig. 4*C* with *B*).

Dose-Dependence

Eight neurons were exposed sequentially to 1, 10, and 100 μM DK. In the neuron illustrated in Fig. 5*A*, brief exposure to 1 μM DK was without an overt effect, while the two higher concentrations of DK caused a progressive reduction in the amplitude of I_h. This result was typical of the entire sample of 8 neurons tested in this manner (Fig. 5*B*1). Even though steady state was not reached before application of the next higher concentration of the drug (or wash) (*see* Fig. 5*A*), 10 and 100 μM DK caused a highly significant average reduction in the

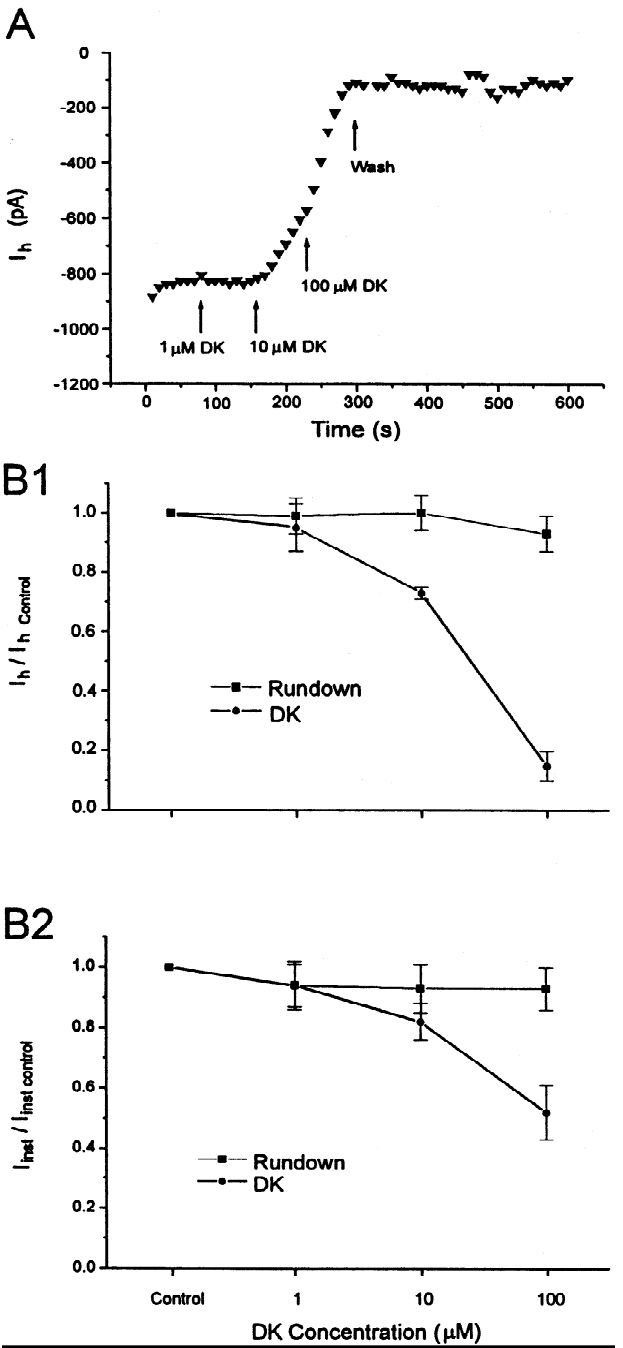

Fig. 5. The dose-response relationship of the block of hyperpolarization-activated currents by sequentially increasing concentrations of DK (1, 10, and 100 μM, each applied for 1 min). (*A*) data for an individual neuron (triangles: peak currents measured for each hyperpolarizing voltage-step from −70 to −120 mV, applied every 10 sec). (*B*1) Normalized data from a total of 8 cells exposed to DK and from 4 neurons serving as matched time-controls for I_h rundown. DK suppressed I_h in a dose-dependent fashion (Repeated Measures ANOVA, 1-tailed, $P < 0.0005$). All pairwise comparisons among DK concentrations were significantly different (Tukey's, $P < 0.001$) with the exception of 1 μM *vs.* control. The decrement in I_h for all DK doses except 1 μM was significantly different from that observed due to rundown alone (*t*-test, one-tailed, $P < 0.0005$ for both 10 and 100 μM DK). (*B*2) Corresponding instantaneous current data for the neurons in *B*1. DK reduced $I_{inst.}$ in a dose-dependent fashion ($P < 0.0005$). Compared with rundown control, $I_{inst.}$ was reduced by 10 and 100 μM DK (*t*-test, one-tailed, $P < 0.025$ and $P < 0.0005$, respectively). Symbols in *B* correspond to amplitudes of currents at the end of either the control or drug-application periods (mean ± SD). The current amplitudes for each neuron were normalized to that neuron's control value. Average control values for I_h and $I_{inst.}$ were −1172 ± 871 and −793 ± 579 pA, respectively.

amplitude of I_h by 27 and 85%, respectively. This decrease far exceeded the expected rundown of I_h during the same period of time (Fig. 5*B*1, see legend for details of analysis).

In a separate set of experiments, 10 μM DK was applied to 3 TG neurons until maximal inhibition of I_h was observed (usually after more than 4 min). In this case, DK caused 68% inhibition of I_h (value corrected for rundown) but had no effect on delayed rectifier or transient outward currents in the same neurons (Fig. 6).

Recovery

While the effects of Cs were promptly reversed upon removal of the blocking agent from the bathing solution (*not illustrated*), DK blockade of I_h was more persistent (Fig. 5*A*). Very limited recovery of I_h was seen even after a 4-min washout period in neurons exposed sequentially to 1, 10, and 100 μM DK. All 8 neurons (same collective as in Fig. 5*B*) showed a lack of recovery similar to the neuron depicted in Fig. 5*A*.

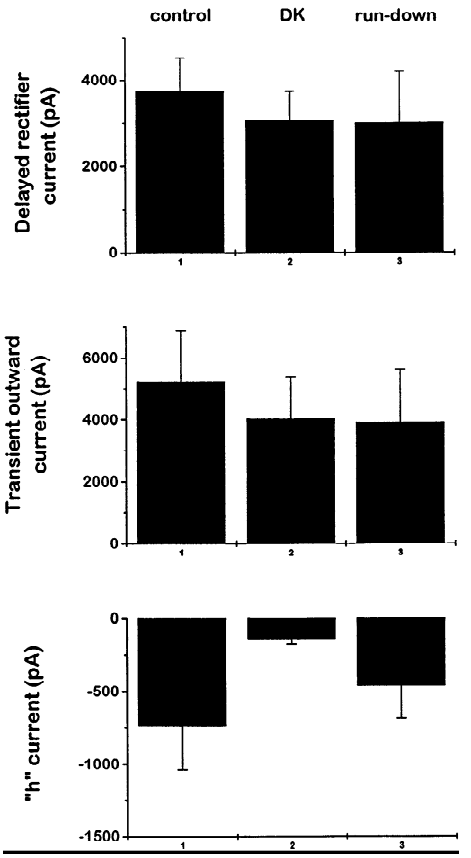

Fig. 6. Reduction of I_h current by 10 μM DK (steady-state values, mean ±SEM). Note the absence of any effect on the transient outward and delayed rectifier currents in the same TG neurons ($n = 3$). I_h was evoked by voltage pulses to −140 mV from a holding potential of −70 mV. Transient outward currents were activated by a depolarization from −140 to +40 mV, delayed rectifier currents were evoked by a depolarization from −70 to +40 mV. Neurons were exposed to DK for 4.3 min.

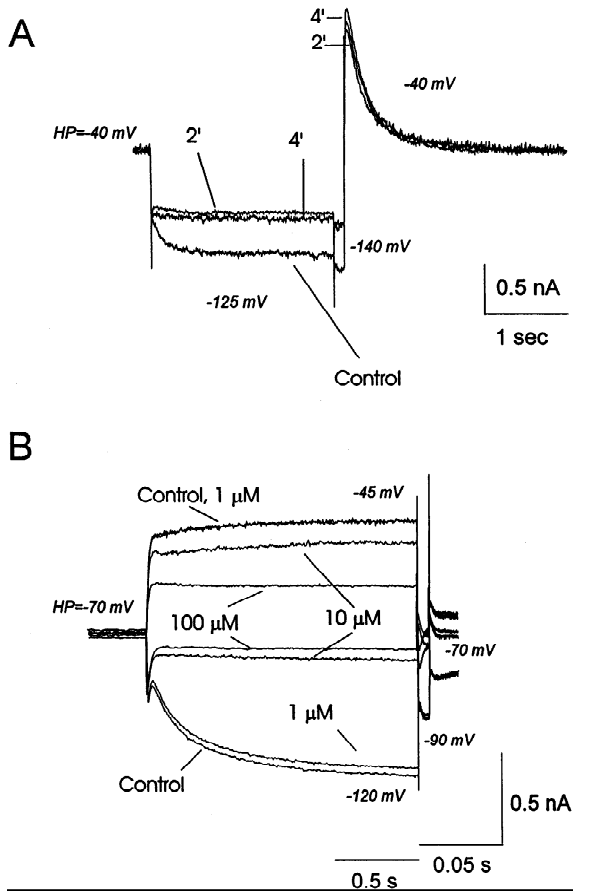

Fig. 7. Specificity of the block of I_h by DK. (*A*) Lack of an effect on a transient outward current (activated by repolarization from −140 mV to the holding potential of −40 mV) even after 4 min of exposure to 10 μM DK which nearly completely blocked I_h in this particular neuron (compare Control to 4′). (*B*) Minimal effect of lower concentrations of DK (1 and 10 μM) on a sustained outward current evoked by a depolarization from −70 to −45 mV. Note full block of I_h by 10 μM in the same neuron and loss of specificity at 100 μM DK. For clarity, holding currents were digitally set to zero in *B*. Two different neurons in *A* and *B*.

Specificity of the Effects of DK-AH 268

Instantaneous currents activated by hyperpolarization were reduced by DK to a far lesser extent than I_h (compare Fig. 5*B*1 with 5*B*2, data from the same 8 neurons).

The relative specificity of the block by DK was further investigated in TG neurons displaying large transient outward currents upon return to the holding potential (HP = −40 mV) following the activation of I_h (Fig. 7*A*). While I_h was almost completely blocked by 10 μM DK, the effect on the transient outward current was negligible even after a 4-min exposure. Likewise, in another population of TG neurons, outward currents evoked by prolonged depolarization to −45 mV (from a holding potential of −70 mV) were only slightly reduced by 10 μM DK which almost fully blocked I_h (Fig. 7). A higher concentration of DK (100 μM), however, blocked the outward current (Fig. 7*B*). Prolonged application of 10 μM DK was without effect on delayed rectifier or transient outward currents in 3 other neurons (Fig. 6).

DK (10 μM) had no overt effect on the shape of the action potential evoked by injection of depolarizing current pulses in 4 current-clamped neurons (Fig. 8, compare *A* with *B*).

Discussion

The use of ions and molecules that alter ion channel permeation properties is a powerful tool in the investigation of the physiological role of ion channels. The results presented in this publication are consistent with the description of a preferential blockade of a neuronal h-type current [30] in rat trigeminal ganglion neurons by a novel organic compound, DK-AH 268 (DK). The actions of DK were compared to those of extracellular

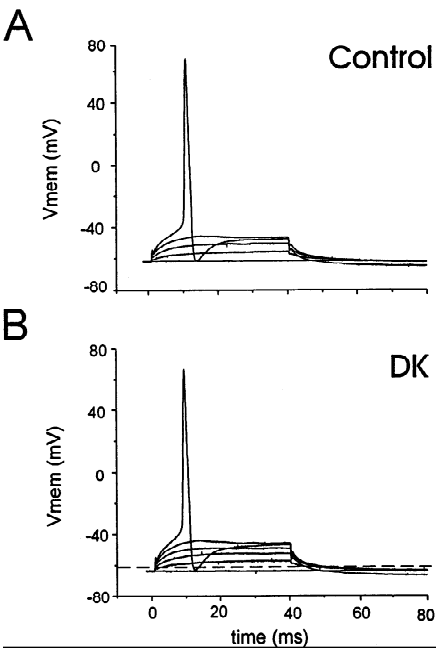

Fig. 8. Effect of DK-AH 268 (DK) on current-clamped trigeminal ganglion (TG) neurons. (*A*) Action potentials evoked by constant current pulses. Note lack of effect of 10 μM DK other than a slight hyperpolarization of the resting membrane potential (from −61 mV in *A* and −64 mV in *B*), coupled with a slight increase in the threshold for action potential discharge (from 400 pA in A to 500 pA in *B*).

cesium ions, which are known to cause a dramatic reduction of h-type as well as other voltage-activated currents.

Our experiments have shown that, in contrast to cesium, DK blockade (at 10 μM) is virtually voltage-independent (*see* Fig. 2*B*) and relatively specific for I_h (compared with the instantaneous inward current). DK-induced blockade was effective at potentials where I_h displayed only minimal activation (−70 to −80 mV). Depolarized cells were minimally affected by the exposure to the drug (Fig. 4). DK required channel activation to exert its blocking action (Fig. 4), suggesting the development of a ''use-dependent'' blockade similar to that described for UL-FS 49, a blocker of the i_f current in the heart [10]. It appears that DK block requires only preactivation of I_h through hyperpolarization, whereas cesium block requires channel activation and electrostatic interaction of the Cs^+ ion with the pore to be effective.

In addition to hyperpolarization-activated currents, we also examined the extent of DK block of outward potassium currents. At concentrations capable of inducing a significant block of h-currents, DK had a minimal effect on outward currents (Figs. 6 and 7). The actions of DK appeared to be more pronounced on a persistent, delayed potassium current (activated by depolarization from −40 mV), since a transient, A-type current activated from more negative holding potentials was virtually unaffected (compare Fig. 7*A* with *B*). Results obtained from current clamp experiments (and presented in Fig. 8) clearly demonstrate that application of 10 μM DK did not result in any appreciable effect on neuronal firing properties, including after-spike repolarization. In fact, the only tangible effect observed after application of DK in current-clamped neurons was a slight cell membrane hyperpolarization, consistent with a specific action of the drug on an inward current activated at rest, i.e., I_h.

Voltage-dependent ion currents play a pivotal role in the regulation of both spontaneous and synaptically evoked electrical signals in neurons [16]. A systematic analysis of ion currents activated at or around resting potential has yielded the characterization of a family of hyperpolarization-activated mixed Na^+/K^+ currents named I_h or I_q [22, 24, 25, 35, 36]. In addition, I_h currents are involved in the generation of repetitive neuronal firing [31], thus resembling the functional role of its cardiac counterpart, i_f [12].

Definitive analysis of the function of h-like currents in neurons has been hindered by the lack of a specific blocker for I_h. Cesium ions have been extensively used as a tool for suppressing mixed Na^+/K^+ currents in neurons [24, 25, 36], but due to their poor specificity and steep voltage-dependence, Cs^+ blocking actions have been difficult to interpret. In fact, cesium blockade is weak around the resting membrane potential [25] and I_h activation profiles overlap considerably with those of another cesium-sensitive current, the inward rectifier [21].

Due to its preferential block of hyperpolarization-activated Na^+/K^+ currents, DK may become a useful tool for improving our understanding of the physiological role of I_h in the regulation of diverse neuronal functions. In addition, DK may be of therapeutic usefulness due to its ''use-dependent'' action.

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