Modulation by PKA of the Hyperpolarization-activated Current (I\textsubscript{h}) in Cultured Rat Olfactory Receptor Neurons

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Abstract. The hyperpolarization-activated I\textsubscript{h} channel is modulated by neurotransmitters acting through the cAMP messenger system. In rat olfactory receptor neurons (ORNs), dopamine, by inhibition of adenylyl cyclase, shifts the voltage of half-maximal activation (V\textsubscript{1/2}) of I\textsubscript{h} to more negative potentials and decreases I\textsubscript{h} maximal relative conductance. Whether these effects result from a phosphorylation-dependent mechanism is unclear. Therefore, we used whole-cell patch-clamp recording techniques to study cAMP-dependent phosphorylation via PKA on I\textsubscript{h} in rat ORNs. General protein kinase inhibition (50 nM K252a) produced a hyperpolarizing shift in I\textsubscript{h} V\textsubscript{1/2} and decreased I\textsubscript{h} maximal conductance. Specific inhibition of PKA with H-89 (500 nM) also shifted the V\textsubscript{1/2} of I\textsubscript{h} to more negative potentials, and, in some cells, decreased I\textsubscript{h} maximal conductance. PKA-mediated phosphorylation (cBIMPS, 50 μM) shifted I\textsubscript{h} V\textsubscript{1/2} more positive, modulated the kinetics of I\textsubscript{h} channel activation and increased I\textsubscript{h} peak current amplitude. Internal perfusion of the catalytic subunit of PKA (84 nM) also shifted I\textsubscript{h} V\textsubscript{1/2} positive and this shift was blocked by co-perfusion with PKI (50 nM). These results show that in rat ORNs, the voltage dependence of I\textsubscript{h} activation can be modulated by PKA-dependent phosphorylation. We also show that PKA and other protein kinases may be involved in the regulation of I\textsubscript{h} maximal conductance. Our findings suggest that changes in the phosphorylation state of ORNs may affect resting properties as well as modulate odor sensitivity.

Key words: Olfaction — Hyperpolarization — Protein kinase A — I\textsubscript{h} — Voltage-gated — Phosphorylation

Introduction

The hyperpolarization-activated current (I\textsubscript{h}) is a voltage-gated conductance that was first described as I\textsubscript{f} in the heart (Brown & DiFrancesco, 1980), and as I\textsubscript{h} in central (Halliwell & Adams, 1982; Spain, Schwindt, & Crill, 1987; Pape & McCormick, 1989; Maccaferri et al., 1993) and peripheral neurons (Mayer & Westbrook, 1983; Hestrin, 1987; Pearce & Duchen, 1994; Scroggs et al., 1994), including vertebrate and invertebrate ORNs; (Lynch & Barry, 1991; Corotto & Michel, 1994). The I\textsubscript{h} channel activates upon hyperpolarization of the cell membrane and produces a slowly activating, non-inactivating, inwardly rectifying current. The current is carried by both Na\textsuperscript{+} and K\textsuperscript{+} ions, and under normal physiological conditions has a reversal potential more positive than the resting membrane potential (Mayer & Westbrook, 1983; Spain et al., 1987; McCormick & Pape, 1990). Pharmacologically, I\textsubscript{h} is identified by its sensitivity to external Cs\textsuperscript{+} and relative insensitivity to Ba\textsuperscript{2+}, tetrodotoxin, tetraethylammonium (TEA) and 4-aminopyridine (Mayer & Westbrook, 1983; McCormick & Pape, 1990; Lynch & Barry, 1991; Ludwig et al., 1998). Functionally, I\textsubscript{h} plays important roles in the electrophysiological properties of many neurons. When active, it contributes to the resting membrane potential of central and peripheral neurons (Trotier & Doving, 1996; Bal & McCormick, 1997; Lamas, 1998; Wellner-Kienitz & Shams, 1998; Doan & Kunze, 1999), shapes the patterns of neuronal rhythmic firing and controls cell excitability (McCormick & Pape, 1990; Akasu, Shoji, & Hasuo, 1993; Maccaferri & McBain, 1996; Tabata & Ishida, 1996; Wang, Van den Berg, & Ypey, 1997; Hughes, Cope, & Crunelli, 1998; Lüthi, Bal, & McCormick, 1998; Magee, 1998; Wellner-Kienitz & Shams, 1998). In the heart, it plays a key role in cardiac pacemaking activity by the generation and control of diastolic depolarization.
and spontaneous firing rate of sinoatrial node cells (Brown & DiFrancesco, 1980; DiFrancesco, Ducouret, & Robinson, 1989).

Another property of the $I_h$ channel is its modulation by neurotransmitters and hormones that regulate basal adenyl cyclase activity and intracellular levels of cAMP ([cAMP]). Activation of adenyl cyclase and subsequent increase in [cAMP], result in an enhancement of $I_h$ due to a depolarizing shift in the voltage dependence of half-activation ($V_{1/2}$) of $I_h$ (DiFrancesco et al., 1986; Bobker & Williams, 1989; Banks, Pearce, & Smith, 1993; Ingram & Williams, 1996; Larkman & Kelly, 1997), while adenyl cyclase inhibition and decrease in [cAMP]ₙ produces a decrease in $I_h$ and a hyperpolarizing shift in $V_{1/2}$ (DiFrancesco & Tromba, 1988; Chang & Cohen, 1992; Pape, 1992; Ingram & Williams, 1994; Vargas & Lucero, 1999b).

In bull-frog sympathetic neurons, canine Purkinje fibers and ventricular myocytes, PKA-dependent phosphorylation of the channel underlies the regulation of the voltage dependence of $I_h$ activation (Tokimasa & Akasu, 1990; Chang et al., 1991; Yu, Chang, & Cohen, 1993, 1995). In addition, cloning of members of the $I_h$ channel superfamily showed that a PKA consensus phosphorylation site is present in some members of this channel superfamily (Santoro et al., 1998) and that the native channel can exist in a phosphorylated state (Gauss, Seifert, & Kaupp, 1998).

In contrast to the effects of PKA-dependent phosphorylation on $V_{1/2}$ of $I_h$, the effects of phosphorylation on $I_h$ maximal conductance ($g_{max}$) seem to be more variable. In sympathetic neurons, stimulation of adenyl cyclase activity enhanced $I_h$ by increasing its $g_{max}$ and shifting its $V_{1/2}$ to more positive potentials; protein kinase inhibition reversed this enhancement of $I_h$ (Tokimasa & Akasu, 1990). However, in Purkinje fibers, $I_h$ channel phosphorylation regulated only the $V_{1/2}$ of $I_h$ activation; it had no effect on $g_{max}$ of $I_h$ (Chang et al., 1991; Yu et al., 1993). In isolated ventricular myocytes, $I_h$ phosphorylation also produced a positive shift in the $I_h$ activation curve, but it was not clear whether the conductance was also regulated (Yu et al., 1995). Interestingly, in sinoatrial node cells, $I_h$ phosphorylation resulted only in an increase in $g_{max}$ of $I_h$ (Accili, Redaelli, & DiFrancesco, 1997). Therefore, the regulatory effects of phosphorylation on $I_h$ seem to vary among different cell types.

In rat ORNs, dopamine modulates $I_h$ through activation of $D_2$ dopamine receptors (Vargas & Lucero, 1999b), which results in an inhibition of adenyl cyclase activity and a decrease in [cAMP]ₙ (Mania-Farnell, Farbman & Bruch, 1993; Coronas et al., 1999). Activation of $D_2$ receptors in rat ORNs produces a hyperpolarizing shift in the $V_{1/2}$ of $I_h$ and a decrease in $g_{max}$ whereas increasing intracellular cAMP produces a depolarizing shift in the $V_{1/2}$ (Vargas & Lucero, 1999b). Whether these modulatory actions of dopamine on $I_h$ are due to decreased cAMP alone or result from a reduction in cAMP-dependent phosphorylation (PKA) is still unclear. Therefore, we used whole-cell, voltage-clamp recording techniques to study the modulatory effects of PKA-mediated phosphorylation on the basal properties of $I_h$ in rat ORNs. We found that PKA regulates the voltage dependence of $I_h$ activation and, in some cells, the $I_h$ $g_{max}$. These results demonstrate that, in rat ORNs, $I_h$ is modulated by phosphorylation. Since $I_h$ can contribute to the modulation of cell excitability and to spike frequency adaptation during the excitatory response to odorants (Lynch & Barry, 1991), these findings suggest a mechanism by which dopaminergic regulation of [cAMP] and phosphorylation state in ORNs may set resting properties as well as modulate odor sensitivity.

**Materials and Methods**

**Cell Preparation and Culture Conditions**

Rat ORNs were dissociated and kept in culture as previously described (Vargas & Lucero, 1999a). Briefly, adult male Simonsen albino rats (~200 g) were handled according to the Policy on Humane Care and Use of Laboratory Animals established by the Public Health Service. Rats were deeply anaesthetized (150 mg/kg ketamine + 15 mg/kg rompun, Mallinekrodt Veterinary, Munde- lein, IL) and sacrificed by decapitation. The olfactory epithelium from the nasal septum and turbinate of one rat was dissected under 100% oxygen vapor saturated with rat Ringer’s, placed in enzyme solution (10 mg/ml bovine serum albumin (BSA), 1 mg/ml collagenase (Gibco BRL; Grand Island, NY), 50 µg/ml deoxyribonuclease II and 44 U/ml dispase (Gibco BRL) in divalent cation-free rat Ringer’s (in mm: 145 NaCl, 5.6 KCl, 10 Hepes, 10 glucose, 4 EGTA) pH 7.4, 300 mOs/ml, and incubated with gentle shaking (80 rpm) at 37°C for 45 minutes. Following this incubation period, the tissue was washed with fresh divalent cation-free rat Ringer’s and gently triturated using a fire-polished Pasteur pipette. The resulting cell suspension was filtered, and 200 µl were plated onto Conca- navalin A (10 mg/ml, Sigma type IV)-coated glass coverslips placed in 35-mm petri dishes. Following a 20-minute settling time, 2 ml of culture medium was added to each dish. The dishes were placed at 37°C in a CO₂ incubator until used (2-4 days). The culture medium [Dulbecco’s Modified Eagle Medium (Gibco BRL) supplemented with 100 µM ascorbic acid, 1:100 Insulin-Transferrin-Selenium100 × (Gibco BRL), 5% fetal bovine serum (Gibco BRL), and 2 mm Glutamine, 100 U/ml Penicillin G, 100 mg/ml Streptomycin (Irvine Scientific; Santa Ana, CA)] was replaced daily. Chemicals were purchased from Sigma (St. Louis, MO) unless stated otherwise.

**Electrophysiological Recordings**

Whole-cell, voltage-clamp recording techniques (Hamill et al., 1981) were performed on rat ORNs kept in culture for up to 4 days. As reported previously (Vargas & Lucero, 1999a, 1999b), no changes in the electrophysiological properties of the cells were observed over time in culture. Electrodes (10–12 MΩ resistance in 25 mm K⁺ (pH Ringer’s) were pulled from thick-walled (0.64 mm) borosilicate filament glass (Sutter Instrument; San Rafael, CA) on a Flaming/Brown P87 puller. Coverslips with adherent cells were placed into the recording chamber and superfused with the external