Enhancement by T-Type Ca^{2+} Currents of Odor Sensitivity in Olfactory Receptor Cells

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Mechanisms underlying action potential initiation in olfactory receptor cells (ORCs) during odor stimulation were investigated using conventional and dynamic patch-clamp recording techniques. Under current-clamp conditions, action potentials generated by a least effective odor-induced depolarization were almost completely blocked by 0.1 mM Ni^{2+}, a T-type Ca^{2+} channel blocker, but not by 0.1 mM Cd^{2+}, a high voltage-activated Ca^{2+} channel blocker. Under voltage-clamp conditions, depolarizing voltage steps induced a fast transient inward current, which consisted of Na^+ (I_{Na}) and T-type Ca^{2+} ($I_{Ca,T}$) currents. The amplitude of $I_{Ca,T}$ was approximately one-fourth of that of I_{Na} (0.23 ± 0.03, mean ± SEM). Because both I_{Na} and $I_{Ca,T}$ are known to show rapid inactivation, we examined how much I_{Na} and $I_{Ca,T}$ are activated during the gradually depolarizing initial phase of receptor potentials. The ratio of $I_{Ca,T}/I_{Na}$ during a ramp depolarization at the slope of 0.5 mV/msec was 0.56 ± 0.03. Using the dynamic patch-clamp recording technique, we also recorded $I_{Ca,T}$ and I_{Na} during the generation of odor-induced action potentials. This ratio of $I_{Ca,T}/I_{Na}$ was 0.54 ± 0.04. These ratios were more than twice as large as that (0.23) obtained from the experiment using voltage steps, suggesting that $I_{Ca,T}$ carries significant amount of current to generate the action potentials. We conclude that $I_{Ca,T}$ contributes to enhance odor sensitivity by lowering the threshold of spike generation in ORCs.

Key words: odorant; amyl acetate; odor response; T-type Ca^{2+} channel; newt; action potential; patch clamp

The initial step in olfactory sensation involves the binding of odorant molecules to specific receptor proteins on the ciliary surface of olfactory receptor cells (ORCs). Odorant receptors coupled to G-proteins activate adenylyl cyclase leading to the generation of cAMP, which directly gates a cyclic nucleotide-gated cationic channel in the ciliary membrane (Bakalyar and Reed, 1991; Breer and Boekhoff, 1992; Firestein, 1992; Reed, 1992; Ronnett and Snyder, 1992; Kurahashi and Yau, 1994; Restrepo et al., 1996). This initial excitation causes a slow and graded depolarizing voltage change, which is encoded into a train of action potentials that travels to the higher olfactory center (Trotier and MacLeod, 1983; Trotier, 1986; Kurahashi, 1989; Pun and Gesteland, 1991).

Action potentials of ORCs are known to be generated by voltage-gated Na^+ currents (catfish, Miyamoto et al., 1992; coho salmon, Nevitt and Moody, 1992; *Xenopus,* Schild, 1989; tiger salamander, Firestein and Werblin, 1987; Dubin and Dionne, 1994; newt, Kawai et al., 1996; rat, Trombley and Westbrook, 1991) and T-type Ca^{2+} currents (newt, Kawai et al., 1996). In this last study, we showed that T-type Ca^{2+} currents ($I_{Ca,T}$) lowered the threshold of action potentials induced by injection of current steps into newt ORCs, which have lost their cilia during the dissociation procedure (Kawai et al., 1996). It is unclear, however, whether $I_{Ca,T}$ lowers the threshold of action potentials induced by odor stimuli in intact ORCs and how $I_{Ca,T}$ regulates odor sensitivity of ORCs. To test these, we examined the effects of voltage-gated Ca^{2+} channel blockers on action potentials of ORCs induced by puffer application of the odorant amyl acetate using the whole-cell patch-clamp recordings. We found that $I_{Ca,T}$ lowers the threshold of the odor-induced action potentials in intact ORCs.

Furthermore, to investigate mechanisms underlying action potentials evoked by odor stimuli, first we recorded voltage-gated Na^+ currents (I_{Na}) and $I_{Ca,T}$ activated by depolarizing voltage steps under voltage-clamp conditions. Because it is known that both I_{Na} and $I_{Ca,T}$ show inactivation during graded depolarizations (Hodgkin and Huxley, 1952; Nowycky et al., 1985; Tsien et al., 1988; Kaneko et al., 1989), we examined how much I_{Na} and $I_{Ca,T}$ are activated during a ramp depolarization, of which the slope is similar to that of the odor-induced depolarization. In addition, using the dynamic patch-clamp recording techniques, we also recorded $I_{Ca,T}$ and I_{Na} during the generation of odor-induced action potentials and compared their ratio. We found that $I_{Ca,T}$ carries a significant amount of current to generate action potentials in ORCs during odor stimuli.

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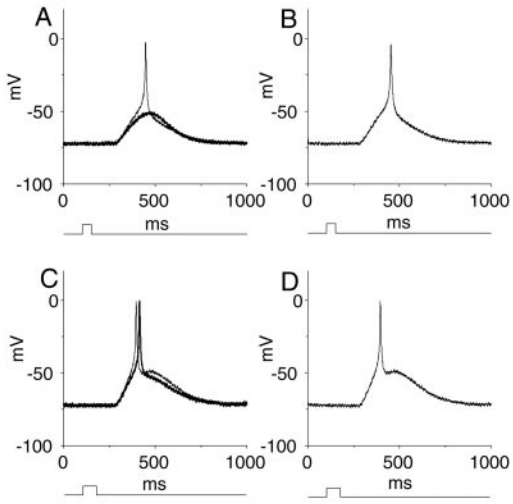

Figure 1. Contribution of $I_{Ca,T}$ to action potentials of an isolated ORC evoked by the odorant amyl acetate. *A*, Response to near threshold depolarization induced by puffer application of 0.1 mM amyl acetate for 50 msec in control (*thin line*) and after addition of 0.1 mM Ni^{2+}, a T-type Ca^{2+} channel blocker (*thick line*). Recording pipette was filled with K^+ solution. Timing of odorant stimulation is indicated by the upward deflection shown below the voltage traces. *B*, Response to near threshold depolarization induced by 50 msec amyl acetate puff after washout of Ni^{2+}. *C*, Response of the same ORC as in *A* to depolarization induced by puffer application of 0.1 mM amyl acetate for 75 msec in control (*thin line*) and after addition of 0.1 mM Ni^{2+} (*thick line*). *D*, Response to 75 msec amyl acetate puff after washout of Ni^{2+}.

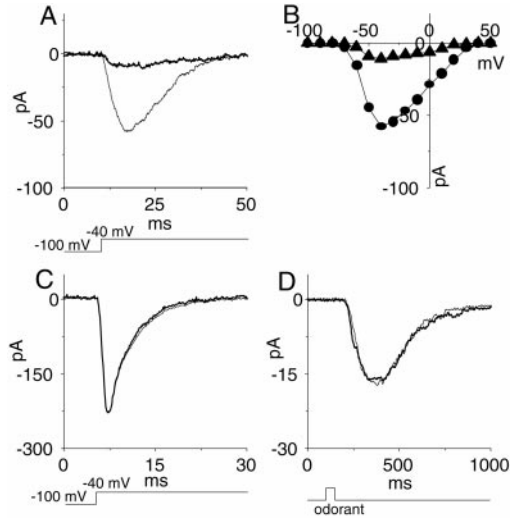

Figure 2. Blockage by Ni^{2+} of $I_{Ca,T}$ in ORCs. *A*, $I_{Ca,T}$ induced by a depolarizing voltage step to −40 mV from a V_h of −100 mV in a control solution (*thin line*) and the solution containing 0.1 mM Ni^{2+}, a T-type Ca^{2+} channel blocker (*thick line*). Currents were recorded using pipettes filled with the Cs^+ solution. The bath solution contained (in mM): 110 choline-Cl, 3.7 KCl, 3 $CaCl_2$, 0.1 $CdCl_2$, 2 HEPES, and 15 glucose. $CdCl_2$ (0.1 mM) was added to the bath to block L-type Ca^{2+} currents selectively. *B*, *I–V* relation of the cell shown in *A* in the control solution (*filled circles*) and the solution containing 0.1 mM Ni^{2+} (*filled triangles*). *C*, I_{Na} induced by depolarization to −40 mV from a V_h of −100 mV in a control solution (*thin line*) and the solution containing 0.1 mM Ni^{2+} (*thick line*). Recording pipette was filled with Cs^+ solution. The bath solution contained (in mM): 110 NaCl, 3.7 KCl, 3 $CoCl_2$, 2 HEPES, and 15 glucose. $CoCl_2$ (3 mM) was added to the bath to block voltage-gated Ca^{2+} currents. *D*, Odorant-induced currents recorded from an ORC at a V_h of −70 mV in a control Ringer's solution (*thin line*) and the solution containing 0.1 mM Ni^{2+} (*thick line*). Timing of 0.1 M amyl acetate puff for 50 msec is indicated by the upward deflection shown below the current traces.

MATERIALS AND METHODS

Preparation and recording procedures. ORCs were dissociated enzymatically from the olfactory epithelium of the newt *Cynops pyrrhogaster,* as reported (Kawai et al., 1996). Isolated cells were viewed on an Olympus upright microscope with differential interference contrast optics (40× water-immersion objective). Membrane voltages and currents were recorded in the whole-cell configuration (Hamill et al., 1981) using a patch-clamp amplifier (Axopatch 200B; Axon Instruments, Foster City, CA) linked to a computer. Recording procedures were controlled by pClamp software (Axon Instruments). Data were low-pass-filtered (four-pole Bessel type) with a cutoff frequency of 5 kHz and then digitized at 10 kHz by an analog-to-digital interface. All experiments were done at room temperature (23–25°C).

Solutions and odorant stimuli. The recording pipette was filled with pseudointracellular (K^+) solution (in mM): 119 KCl, 1 $CaCl_2$, 5 EGTA, and 10 HEPES (pH adjusted to 7.4 with KOH) or Cs^+ solution: 119 CsCl, 1 $CaCl_2$, 5 EGTA, and 10 HEPES (pH adjusted to 7.4 with CsOH). The resistance of the pipette was ~6 MΩ. The control bath solution used to record voltage responses and the odorant-induced currents contained (in mM): 110 NaCl, 3.7 KCl, 3 $CaCl_2$, 2 HEPES, and 15 glucose, the solution used to record I_{Na} contained 110 NaCl, 3.7 KCl, 3 $CoCl_2$, 2 HEPES, and 15 glucose, and the solution for $I_{Ca,T}$ contained 110 choline-Cl, 3.7 KCl, 3 $CaCl_2$, 0.1 $CdCl_2$, 2 HEPES, and 15 glucose. $CdCl_2$ (0.1 mM) was added to the bath to block L-type Ca^{2+} currents selectively. For odorant stimuli, amyl acetate (100 μ mM) was dissolved in the control bath solution, included into a puffer pipette, and applied from a pressure ejection system.

RESULTS

$I_{Ca,T}$ enhances odor sensitivity by lowering the threshold of spike generation in ORCs

We examined whether $I_{Ca,T}$ lowers the threshold of action potentials induced by odor stimuli in intact ORCs with cilia attached. To test this, we recorded action potentials generated by a least effective odor-induced depolarization. An example is shown in Figure 1. Isolated newt ORCs had a resting potential of −73 ± 3 mV (mean ± SEM; n = 34). When 0.1 mM amyl acetate was applied to an ORC for 50 msec from a puffer pipette in control Ringer's solution, a single action potential was generated (Fig. 1*A*, *thin line*). Addition of 0.1 mM Ni^{2+}, a T-type Ca^{2+} channel blocker, to the bath blocked the action potential (Fig. 1*A, thick line*), and this effect was reversible (Fig. 1*B*), suggesting that $I_{Ca,T}$ may be involved in spike generation. If 0.1 mM amyl acetate was applied for longer (75 msec) under this condition, an action potential reappeared (Fig. 1*C,D*). Similar results were obtained in nine cells. These suggest that $I_{Ca,T}$ may enhance odor sensitivity by lowering the threshold of spike generation in ORCs. In contrast, addition of 0.1 mM Cd^{2+}, a high voltage-activated (HVA) Ca^{2+} channel blocker, failed to block the action potential (data not shown), indicating that HVA Ca^{2+} currents are not involved in spike generation.

To elucidate mechanisms underlying spike generation in ORCs, we examined the effects of 0.1 mM Ni^{2+} on $I_{Ca,T}$ and I_{Na} under voltage-clamp conditions. In control conditions, membrane depolarization to −40 mV from a holding potential (V_h) of −100 mV induced $I_{Ca,T}$ of approximately −60 pA (Fig. 2*A*). Addition of 0.1 mM Ni^{2+} to the bath decreased the peak amplitude of $I_{Ca,T}$ by 71 ± 6% (n = 7; Fig. 2*A, thick line*). The reduction of the $I_{Ca,T}$ amplitude was observed over the entire voltage range tested (Fig. 2*B*). In contrast, 0.1 mM Ni^{2+} did not change I_{Na} significantly (Fig. 2*C*). To exclude the possibility that Ni^{2+} may affect directly cyclic nucleotide-gated (CNG) cationic channels in olfactory cilia, we also tested the effects of Ni^{2+} on the odorant-induced cur-

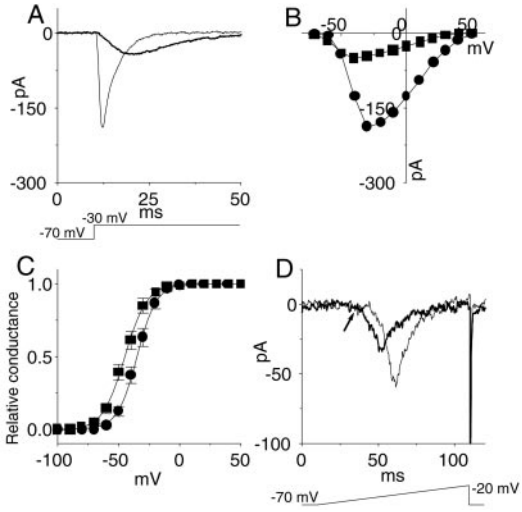

Figure 3. Comparison of $I_{Ca,T}$ and I_{Na}. *A*, $I_{Ca,T}$ (*thick line*) and I_{Na} (*thin line*) induced by depolarization to −30 mV from a V_h of −70 mV. Each current–response was recorded from the same ORC. Recording pipette was filled with Cs^+ solution. The bath solution used to record I_{Na} contained (in mM): 110 NaCl, 3.7 KCl, 3 $CoCl_2$, 2 HEPES, and 15 glucose, and the solution for $I_{Ca,T}$ contained 110 choline-Cl, 3.7 KCl, 3 $CaCl_2$, 0.1 $CdCl_2$, 2 HEPES, and 15 glucose. *B*, *I–V* relation of the cell shown in *A*. Peak amplitude of $I_{Ca,T}$ (*filled squares*) and I_{Na} (*filled circles*) was plotted against the voltage. *C*, Activation curves of $I_{Ca,T}$ (*filled squares*) and I_{Na} (*filled circles*) recorded at a V_h of −100 mV. *Symbols* represent mean of eight cells, and vertical bars represent SEM. *Lines* represent a single Boltzmann function obtained by the least-squares nonlinear fit to the data. *D*, $I_{Ca,T}$ (*thick line*) and I_{Na} (*thin line*) induced by ramp depolarization to −20 mV from a V_h of −70 mV at the rate of 0.5 mV/msec. Each current–response was recorded from the same ORC.

rents. However, 0.1 mM Ni^{2+} did not change significantly the current induced by puffer application of 0.1 mM amyl acetate (Fig. 2*D*). These results suggest that the inhibition by Ni^{2+} of spike generation shown in Figure 1*A* is caused by blockage of $I_{Ca,T}$ rather than blockage of I_{Na} or the CNG currents.

A ratio of $I_{Ca,T}$ amplitude to I_{Na} amplitude during the generation of action potentials

Under voltage-clamp conditions, the peak amplitude of $I_{Ca,T}$ induced by a depolarizing voltage step to −30 mV from a V_h of −70 mV, which is near the resting potential of ORCs, was approximately one-fourth of I_{Na} (0.23 ± 0.03; $n = 8$; Fig. 3*A*). $I_{Ca,T}$ began to be activated at −60 mV and was maximal at −40 mV, whereas I_{Na} began at −50 mV and was maximal at −30 mV (Fig. 3*B*). Figure 3*C* shows activation curves of $I_{Ca,T}$ and I_{Na}. The relation between $I_{Ca,T}$ (*filled squares*) and membrane voltage was fitted by a single Boltzmann function. The half-activation voltage of $I_{Ca,T}$ was −45 mV. The activation curve of I_{Na} (*filled circles*) was also fitted by a single Boltzmann function with a half-activation voltage of −35 mV. This value is 10 mV more positive than that of $I_{Ca,T}$.

As shown in Figure 1, puffer application of amyl acetate to ORCs induces a gradually depolarizing receptor potential. Because it is known that both I_{Na} and $I_{Ca,T}$ show inactivation during graded depolarizations (Hodgkin and Huxley, 1952; Nowycky et al., 1985; Tsien et al., 1988; Kaneko et al., 1989), next we examined how much I_{Na} and $I_{Ca,T}$ are activated during a ramp depolarization, of which the slope is similar to that of the odor-induced graded depolarization. Depolarizing voltage ramp at the slope of 0.5 mV/msec activated $I_{Ca,T}$ (Fig. 3*D, thick line*) earlier

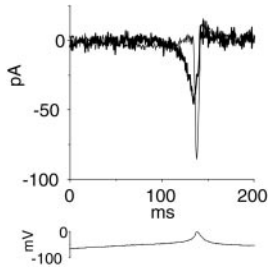

Figure 4. $I_{Ca,T}$ and I_{Na} induced by depolarization using a waveform of the odorant-induced action potentials under voltage-clamp conditions. The waveform of the action potential shown in Figure 1*A* (*thin line*) was used as a command voltage. $I_{Ca,T}$ (*thick line*) and I_{Na} (*thin line*) were recorded from the same ORC at a V_h of −70 mV and are shown at the faster time scale. The waveform of the command voltage was shown below the current traces.

than I_{Na} (*thin line*); $I_{Ca,T}$ started at ~35 msec (*arrow* in the figure), whereas I_{Na} started at ~45 msec. This result suggests that $I_{Ca,T}$ contributes to membrane depolarization initially. The peak amplitude of $I_{Ca,T}$ was 33 pA and that of I_{Na} was 58 pA ($I_{Ca,T}/I_{Na}$ ratio, 0.57). A similar ratio (0.56 ± 0.03, mean ± SEM) was obtained in six cells. This ratio was more than twice as large as the ratio (0.23) obtained by depolarizing voltage steps. The difference was caused by significantly smaller I_{Na} during the ramp depolarization than the step depolarization (Fig. 3*A,D*).

Furthermore, using the dynamic patch-clamp recording techniques, we also recorded $I_{Ca,T}$ and I_{Na} during the action potentials induced by puffer application of amyl acetate and compared the $I_{Ca,T}/I_{Na}$ ratio. The waveform of the action potential, which was shown in Figure 1*A* (*thin line*), was used as a command voltage for the voltage-clamp recordings. This command voltage also activated $I_{Ca,T}$ (Fig. 4, *thick line*) earlier than I_{Na} (*thin line*). The peak amplitude of $I_{Ca,T}$ was 46 pA, and that of I_{Na} was 84 pA ($I_{Ca,T}/I_{Na}$ ratio, 0.55). A similar ratio (0.54 ± 0.04, mean ± SEM) was obtained in six cells. This ratio was also significantly larger than the ratio (0.23 ± 0.03) obtained by depolarizing voltage steps. These results suggest that $I_{Ca,T}$ carries a significant amount of charge to depolarize the membrane potential during odor stimuli.

DISCUSSION

Effects of $I_{Ca,T}$ on action potentials induced by odor stimuli

In various preparations, $I_{Ca,T}$ is known to be involved in the generation of action potentials. Hagiwara et al. (1988) reported that Ni^{2+} prolonged the pacemaker depolarization of rabbit sinoatrial node cells by blocking $I_{Ca,T}$. A similar contribution of $I_{Ca,T}$ to spike generation is also reported in inferior olivary neurons (Llinas and Yarom, 1981). In this last study, we showed that $I_{Ca,T}$ lowered the threshold of action potentials induced by injection of current steps into ORCs (Kawai et al., 1996). It was not clear, however, whether $I_{Ca,T}$ lowers the threshold of action potentials induced by odor stimuli.

All the data we obtained in the present study suggest that $I_{Ca,T}$ has a role of enhancing the sensitivity to odorants by lowering the threshold of action potentials. (1) Under voltage-clamp conditions, addition of 0.1 mM Ni^{2+} to the bath decreased the peak amplitude of $I_{Ca,T}$ by 71 ± 6% but did not change significantly I_{Na} or the odorant-induced current. These suggest that the inhibition by Ni^{2+} of action potentials evoked by a least effective odor stimulation (Fig. 1*A*) is caused by blockage of $I_{Ca,T}$ rather than blockage of I_{Na} or the CNG currents. Divalent cations in the bath

are known to decrease the CNG currents in ORCs (Zufall and Firestein, 1993; Frings et al., 1995; Kleene, 1999; Gavazzo et al., 2000). In the present experiment, however, 0.1 mM Ni^{2+} did not change significantly the CNG currents, probably because 3 mM Ca^{2+} was always added to the bath, when the CNG currents were recorded. (2) The half-activation voltage of $I_{Ca,T}$ is 10 mV more negative than that of I_{Na}, indicating that $I_{Ca,T}$ is more important for spike generation than I_{Na} near threshold. Indeed, an action potential generated by a least effective odor-induced depolarization was blocked by 0.1 mM Ni^{2+}. In contrast, when the odorant was applied for a longer time in the bath containing 0.1 mM Ni^{2+}, an action potential reappeared (Fig. 1*C*). This is probably caused by I_{Na} activated directly by a more depolarizing odorant-induced receptor potential. From these observations we conclude that $I_{Ca,T}$ contributes to enhance odor sensitivity by lowering the threshold of spike generation in ORCs

In addition, $I_{Ca,T}$ in newt ORCs may also contribute to make the olfactory sensation robust. Regardless of changes in the external environment, ionic concentrations of the mammalian extracellular solution are known to be fairly invariable. In contrast, it is known that ionic concentrations of the fish and amphibian extracellular solution change as the external environment changes. A decrease in the extracellular Na^+ concentration would reduce the inward current to generate action potentials in ORCs, if Na^+ was the sole current carrier. When a Ca^{2+} component through T-type Ca^{2+} channels is present, however, such a reduction in I_{Na} would be compensated by $I_{Ca,T}$, thus maintaining the effectiveness of the mechanism to generate action potentials. Another mechanism of compensation for environmental changes is reported in olfactory cilia (Kurahashi and Yau, 1994). They showed that a Ca^{2+}-activated Cl^- current in olfactory cilia would compensate changes in inward currents through CNG cationic channels.

Comparison of $I_{Ca,T}$ and I_{Na} for the role of spike generation

In a voltage-clamp experiment using depolarizing voltage steps, the maximum $I_{Ca,T}$ was 45 pA, whereas that of I_{Na} was 190 pA, giving an $I_{Ca,T}/I_{Na}$ ratio of 0.24. By contrast, the amplitude of $I_{Ca,T}$ during the ramp depolarization was 33 pA, and that of I_{Na} was 58 pA, giving an $I_{Ca,T}/I_{Na}$ ratio of 0.57. A similar ratio (0.54 ± 0.04) was also obtained by the voltage-clamp experiments using the waveform of the odorant-induced action potentials. These results indicate that $I_{Ca,T}$ contributes to spike generation more than we expected based on data obtained by the experiments using voltage steps. One of the reasons for this difference is that I_{Na} is more rapidly inactivated by the membrane depolarization than $I_{Ca,T}$. In fact, the inactivation kinetics of I_{Na} in ORCs (time constant = 1–1.5 msec; Trotier, 1986; Firestein and Werblin, 1987; Schild, 1989; Trombley and Westbrook, 1991; Miyamoto et al., 1992; Kawai et al., 1996) is much faster than that of $I_{Ca,T}$ (time constant = 10–20 msec; Kawai et al., 1996).

$I_{Ca,T}$ started to flow earlier than I_{Na} during the ramp depolarization (Fig. 3*D*), and the odorant-induced depolarization (Fig. 4). These observations are interpreted by a lower activation voltage for $I_{Ca,T}$ than I_{Na}. Lower activation voltage and slower inactivation of $I_{Ca,T}$ than I_{Na} support that $I_{Ca,T}$ carries a significant amount of charge to generate action potentials in ORCs.

$I_{Ca,T}$ in ORCs

In the past, it has been believed that the transient inward current of ORCs contributing to action potential generation is solely carried by Na^+ (catfish, Miyamoto et al., 1992; coho salmon, Nevitt and Moody, 1992; *Xenopus,* Schild, 1989; tiger salamander, Firestein and Werblin, 1987; Dubin and Dionne, 1994; rat, Trombley and Westbrook, 1991; Rajendra et al., 1992). The reason why $I_{Ca,T}$ was not identified in ORCs of other animal species is still obscure, but there are several possibilities. (1) The presence of $I_{Ca,T}$ may depend on the development or regeneration of ORCs. It is known that the olfactory epithelia undergo regeneration continuously (Firestein, 1992; Reed, 1992; Ronnett and Snyder, 1992; Restrepo et al., 1996). The different observations concerning $I_{Ca,T}$ may be attributable to different populations of cells under the experimental conditions. In cultured ORCs Ca^{2+}-activated K^+ channel is absent (Trombley and Westbrook, 1991), whereas it has shown to be ubiquitous in adult ORCs (Maue and Dionne, 1987; Kurahashi, 1989; Schild, 1989; Miyamoto et al., 1992). Expression of Ca^{2+}-activated K^+ channel has been shown to be developmentally related in cultured spinal neurons of *Xenopus* (Blair and Dionne, 1985). (2) Another possibility may be just a simple species difference. However, it is interesting to note that Liman and Corey (1996) have reported that $I_{Ca,T}$ is expressed in the chemosensory neurons from the mouse vomeronasal organ. Because there are so many identities between principal and accessory ORCs in terms of the expression of ionic channels, this observation raises a possibility that $I_{Ca,T}$ might be expressed not only in newt ORCs but also in ORCs of other species. Further study would be required to reexamine the presence of $I_{Ca,T}$ in ORCs from other species.

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