T-Type Ca^{2+} Channel Lowers the Threshold of Spike Generation in the Newt Olfactory Receptor Cell

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ABSTRACT   Mechanisms underlying action potential generation in the newt olfactory receptor cell were investigated by using the whole-cell version of the patch-clamp technique. Isolated olfactory cells had a resting membrane potential of -70 ± 9 mV. Injection of a depolarizing current step triggered action potentials under current clamp condition. The amplitude of the action potential was reduced by lowering external Na$^+$ concentration. After a complete removal of Na$^+$, however, cells still showed action potentials which was abolished either by Ca^{2+} removal or by an application of Ca^{2+} channel blocker (Co^{2+} or Ni^{2+}), indicating an involvement of Ca^{2+} current in spike generation of newt olfactory receptor cells. Under the voltage clamp condition, depolarization of the cell to -40 mV from the holding voltage of -100 mV 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 about one fourth of that of I_{Na}. Depolarization to more positive voltages also induced L-type Ca^{2+} current ($I_{Ca,L}$). $I_{Ca,L}$ was as small as a few pA in normal Ringer solution. The activating voltage of $I_{Ca,T}$ was approximately 10 mV more negative than that of I_{Na}. Under current clamp, action potentials generated by a least effective depolarization was almost completely blocked by 0.1 mM Ni^{2+} (a specific T-type Ca^{2+} channel blocker) even in the presence of Na$^+$. These results suggest that $I_{Ca,T}$ contributes to action potential in the newt olfactory receptor cell and lowers the threshold of spike generation.

KEY WORDS: olfactory receptor cell • calcium channel • action potential • patch clamp • newt

INTRODUCTION

Odorant binding to the receptor protein at the ciliary surface of the olfactory receptor cell leads to the subsequent enzymatic cascades (for review, see Bakalyar and Read, 1991; Breer and Boekhoff, 1992; Reed, 1992; Ronnett and Snyder, 1992) that finally leads to the opening of three types of ionic channels; cAMP-gated cationic channels, Ca^{2+}-gated Cl$^-$ channels (for review, see Gold and Nakamura, 1987; Firestein, 1992; Kurahashi and Yau, 1994), and IP$_3$-gated Ca^{2+} channels (for review, see Reed, 1992; Restrepo et al., 1996; Ronnett and Snyder, 1992). This initial excitation causes a slow and graded voltage change; its amplitude is dependent on stimulus intensities (Trotier and MacLeod, 1983; Kurahashi, 1989a; Firestein et al., 1993). Graded receptor potential is then encoded into spike trains that transmit the olfactory information to the brain. At this step, the information relating to the stimulus intensity is encoded into the spike density. It has long been believed that the action potential of olfactory cells is generated by the influx of Na$^+$; under voltage clamp, the transient inward current has been shown to be mediated by Na$^+$ channel (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).

However, an earlier study by Trotier (1986) has pointed out that the salamander olfactory cell expresses a transient inward current in a Na$^+$-free, tetrodotoxin-containing solution. Furthermore, in preliminary experiments by one of the present authors, Kurahashi (1989a) recorded action potentials using newt olfactory cells bathed in Na$^+$-free medium. These observations raised a possibility that action potential is generated not only by Na$^+$ current, but also by other transient inward current in the amphibian olfactory receptor cells. In the present study, we reexamined the mechanism of spike generation in newt olfactory receptor cells by analyzing membrane currents under the whole-cell clamp condition in further detail. We found that the transient inward current activated by membrane depolarization is a mixture of Na$^+$ and Ca^{2+} currents through two independent ionic channels. The transient Ca^{2+} component was shown to be carried through a T-type Ca^{2+} channel. Activation range for the T-type Ca^{2+} current was approximately 10 mV more negative than that of Na$^+$ current. This observation indicates that T-type Ca^{2+} current determines the threshold of spike generation in the newt olfactory receptor cells.[1]

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[1]After the submission of our original manuscript, Liman and Corey (1996) reported that a T-type Ca^{2+} channel is expressed in the chemosensory neurons from the mouse vomeronasal organ. This observation raises a possibility that T-type Ca^{2+} channels might be expressed not only in newt olfactory receptor cells but also in cells of the other species.

Indeed, at threshold, action potentials were blocked by Ni^{2+}, a selective blocker for T-type Ca^{2+} channel, even in the presence of Na^+.

MATERIALS AND METHODS

Preparation

Solitary receptor cells were enzymatically dissociated from the olfactory epithelium of the newt, *Cynops pyrrhogaster*. Dissociation protocols were similar to those reported previously (Kurahashi, 1989b). In short, the animal was anesthetized by cooling on ice, decapitated, and pithed. The mucosae excised from the olfactory cavity were incubated for 5 min at 30°C in a solution (Table I solution 15) containing 0.1% collagenase (Sigma Chemical Co., St. Louis, MO) with no added Ca^{2+} and Mg^{2+}. The tissue was then rinsed twice with a control solution (solution 1) and triturated. Isolated cells were plated on the concanavalin A–coated glass cover-slip. Cells were maintained at 4°C (up to 10 h) before use. In the present experiment, we selected receptor cells which lost their cilia to study the excitability of the somatic membrane.

Recording Procedures

Membrane currents were recorded by a patch clamp technique in the whole-cell–clamp configuration (Hamill et al., 1981). Pyrex tubing (1.2 mm o.d.) was pulled in two steps on a pipette puller (PP-83; Narishige Scientific Instruments, Tokyo, Japan). To minimize stray capacitance, the external wall of the pipette was coated with an insulating resin (Apiezon wax; Apiezon Products Ltd., London, UK) up to ~100 μm away from the tip. A residual capacitance was compensated electrically. The recording pipette was filled with K^+ solution (in mM): 119 KCl, 1 $CaCl_2$, 5 EGTA, 10 HEPES (pH adjusted to 7.4 with KOH) or Cs^+ solution: 119 CsCl, 1 $CaCl_2$, 5 EGTA, 10 HEPES (pH adjusted to 7.4 with CsOH). Resistance of the pipette was about 20 MΩ.

For recording, the culture dish was mounted on the stage of an inverted microscope with phase contrast optics (Diaphot TMD-2; Nikon, Tokyo Japan). A stainless-steel ring was put into the dish to reduce the dead space of the recording chamber to ~0.15 ml.

Cells were continuously superfused (at a rate of 0.9 ml/min) with one of the solutions listed in Table I. The indifferent electrode was an Ag-AgCl wire connected to the culture dish via an agarose bridge. Current and voltage signals from the amplifier were monitored on an oscilloscope (Tektronix, Beaverton, OR) and a thermal array recorder (WR7900; Graphtec, Fujisawa, Japan). Data were low-pass filtered (4-pole Bessel type) with a cut-off frequency of 5 kHz and then digitized at 10 kHz. All experiments were performed at room temperature (23–25°C).

Application of Drugs

Several pharmacological agents were used for membrane current analyses: tetraethylammonium chloride (TEA) and CsCl for blocking K^+ current, 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS) for blocking Cl^- current, $CoCl_2$, $NiCl_2$, and $CdCl_2$ for blocking Ca^{2+} current. Dihydropyridines (nifedipine and Bay K 8644) and ω-conotoxin GVIA were also used to identify the type of the Ca^{2+} current. Divalent cations and other pharmacological agents were dissolved in the superfusate. These agents were bath applied to the cell from the perfusion system. $CoCl_2$, $NiCl_2$, and $CdCl_2$ were purchased from Nacalai Tesque Inc. (Kyoto, Japan), TEA from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and SITS, nifedipine, Bay K 8644, and ω-conotoxin GVIA from Sigma Chemical Co.

Correction of the Resting Potential

Olfactory receptor cells show a very high input resistance (>2 GΩ; see also Trotier, 1986), so a small leakage current causes an error in the recorded membrane potential. In the present study, therefore, the resting potential was corrected for by using the following equations:

$$1/R_{inp} = 1/R_{mem} + 1/R_{seal},$$

$$(V_{ap} - V_{real})/R_{mem} + V_{ap}/R_{seal} = 0,$$

where R_{inp} is the input resistance, R_{mem} the membrane resistance, and R_{seal} the seal resistance. V_{ap} and V_{real} are the apparent and the real resting potential, respectively. Here, we assumed that the

TABLE I
Superfusates

		NaCl	KCl	$CaCl_2$	$MgCl_2$	CholineCl	$BaCl_2$	TEA-Cl	$CdCl_2$	$NiCl_2$	$CoCl_2$	HEPES	Glucose
1	control	110	3.7	3	1	—	—	—	—	—	—	2	15
2	65 Na^+, 10 Ca^{2+}	65	3.7	10	1	—	—	35	—	—	—	2	15
3	0 Na^+, 10 Ca^{2+}	—	3.7	10	1	65	—	35	—	—	—	2	15
4	65 Na^+, 0 Ca^{2+}	65	3.7	—	1	15	—	35	—	—	—	2	15
5	0 Na^+, 0 Ca^{2+}	—	3.7	—	1	80	—	35	—	—	—	2	15
6	0 Na^+, 10 Ba^{2+}	—	3.7	—	1	65	10	35	—	—	—	2	15
7	0 Na^+, 10 Ba^{2+}, 0.1 Ni^{2+}	—	3.7	—	1	65	10	35	—	0.1	—	2	15
8	65 Na^+, 10 Ca^{2+}, 0.1 Cd^{2+}	65	3.7	10	1	—	—	35	0.1	—	—	2	15
9	0 Na^+, 10 Ca^{2+}, 0.1 Cd^{2+}	—	3.7	10	1	65	—	35	0.1	—	—	2	15
10	65 Na^+, 10 Ca^{2+}, 1 Co^{2+}	65	3.7	10	1	—	—	35	—	—	1	2	15
11	65 Na^+, 0 Ca^{2+}, 1 Co^{2+}	65	3.7	—	1	15	—	35	—	—	1	2	15
12	0 Na^+, 110 $Choline^+$	—	3.7	3	1	110	—	—	—	—	—	2	15
13	0 Na^+, 110 $Choline^+$, 0.1 Ni^{2+}	—	3.7	3	1	110	—	—	—	0.1	—	2	15
14	0.1 Ni^{2+}	110	3.7	3	1	—	—	—	—	0.1	—	2	15
15	0 Ca^{2+}, 0Mg^{2+}	110	3.7	—	—	—	—	—	—	—	—	2	15

pH was adjusted to 7.4 with KOH. All solutions contained phenol red (0.01 mg/ml). Nifedipine, Bay K 8644, ω-conotoxin GVIA, and SITS were tested by adding them to solution 6.

seal resistance at on-cell mode was equal to the resistance of the seal under whole-cell conditions. V_{real} and R_{mem} were estimated from R_{inp}, R_{seal}, and V_{ap} recorded in the experiment. For example, when $R_{inp} = 2$ GΩ, $R_{seal} = 10$ GΩ, and $V_{ap} = -60$ mV, V_{real} and R_{mem} are calculated as:

$$V_{real} = -75 \text{ mV}, R_{mem} = 2.5 \text{ G}\Omega.$$

The resting potentials to be described below are the corrected values.

RESULTS

Action Potentials Recorded under Various Ionic Conditions

The isolated newt olfactory receptor cells had a resting membrane potential of -70 ± 9 mV (mean $\pm$ SD, $n = 18$). Transient and regenerative action potentials were initiated when cells were stimulated by a depolarizing current. Since Na$^+$ was believed to be a dominant charge carrier for spike generation in the olfactory cells (catfish: Miyamoto et al., 1992; xenopus: Schild, 1989; tiger salamander: Firestein and Werblin, 1987; Dubin and Dionne, 1994; rat: Trombley and Westbrook, 1991), we first examined the contribution of external Na$^+$ to spike initiation. In the experiment of Fig. 1, 10-pA current injection induced three repetitive action potentials under the normal condition. When choline$^+$ was substituted for Na$^+$, the rising phase of the action potential became slower and the amplitude of the action potential became smaller. However, the cell still showed a transient action potential that looked similar to that generated in control solution. Similar results were observed in 11 cells out of 12 examined.

Furthermore, as shown with a thick line in Fig. 1, addition of 100 μM Ni^{2+} suppressed the remaining action potential in Na$^+$-free solution completely. Ni^{2+} is known to be a selective blocker for the T-type Ca^{2+} channels in various excitable membranes (Tsien et al., 1988; also see below), and therefore it is highly likely that T-type Ca^{2+} current is involved in spike generation of the newt olfactory receptor cells. Because T-type Ca^{2+} current is known to show a transient time course, it is suitable for generating transient action potentials. Therefore, we reexamined voltage-gated currents of newt olfactory receptor cells in detail under the voltage clamp.

Transient Inward Current and Sustained Outward Current

Under the voltage clamp condition, depolarizing step pulses induced time- and voltage-dependent currents (Fig. 2). Membrane depolarization to voltages between -90 and $+20$ mV from a holding voltage (V_h) of -100 mV induced a rapidly (<15 ms) decaying initial inward current and a late outward current. The transient inward current showed a peak value at about -20 mV (Fig. 2 B).

It has been shown that the outward current consists of two or three types of K$^+$ currents ($I_{K(Ca)}$ and $I_{K(V)}$,
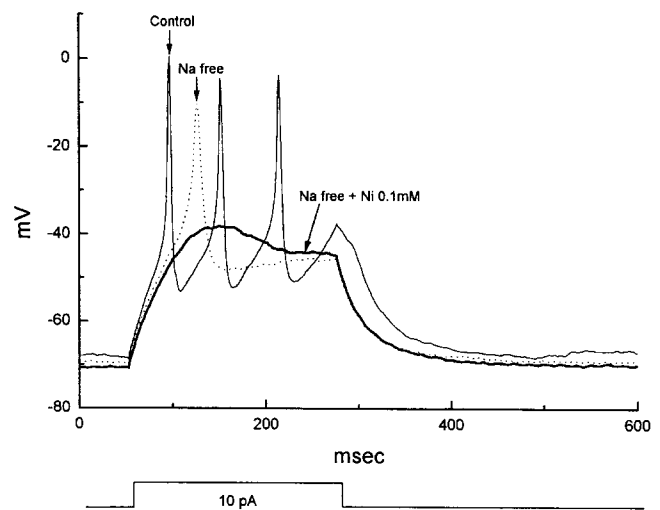

FIGURE 1. Action potentials evoked by injection of 10 pA depolarizing current for 250 ms recorded under current clamp condition. *Continuous line:* recording in control (Table I, solution 1) solution. *Dotted line:* recording in Na$^+$-free solution (solution 12). *Thick line:* recording in Na$^+$-free solution containing 0.1 mM Ni^{2+} (solution 13). Recording pipette was filled with K$^+$ solution.

and possibly $I_{K(A)}$) in the newt (Kurahashi, 1989a) and in other animal species (catfish: Miyamoto et al., 1992; tiger salamander: Firestein and Werblin, 1987; mudpuppy: Dubin and Dionne, 1994; rat: Trombley and Westbrook, 1991; mouse: Maue and Dionne, 1987). These K$^+$ currents are known to be blocked by adding TEA to the bath or by loading cells with Cs$^+$ (see below). In the following experiments, K$^+$ currents were minimized by these treatments.

Inward Currents Consist of Three Components

Fig. 3 A shows a mixture of inward currents observed in a cell loaded with Cs$^+$ and exposed to 35 mM TEA. Depolarizing steps induced a fast transient inward current followed by a small sustained inward current. The peak amplitude of the transient component was $\sim$300 pA. When Na$^+$ was replaced by choline$^+$, the amplitude of the inward current was reduced to $\sim$100 pA. The amplitude of this current component was also dependent on Ca^{2+} concentration; in 3 mM Ca^{2+} the peak amplitude was $\sim$60 pA. The Na$^+$ component could be isolated by subtracting the current under the Na$^+$-free condition from that under the control condition, and was plotted by a dotted line. The Na$^+$ current (I_{Na}) reached a peak $\sim$3 ms after the onset of the stimulus and inactivated in $\sim$4 ms.

The inward current recorded in Na$^+$-free condition was almost completely eliminated by further removing Ca^{2+} from the bathing solution. This result suggests that the current evoked in Na$^+$-free solution was carried solely by Ca^{2+}. The Ca^{2+} currents (I_{Ca}) consisted of
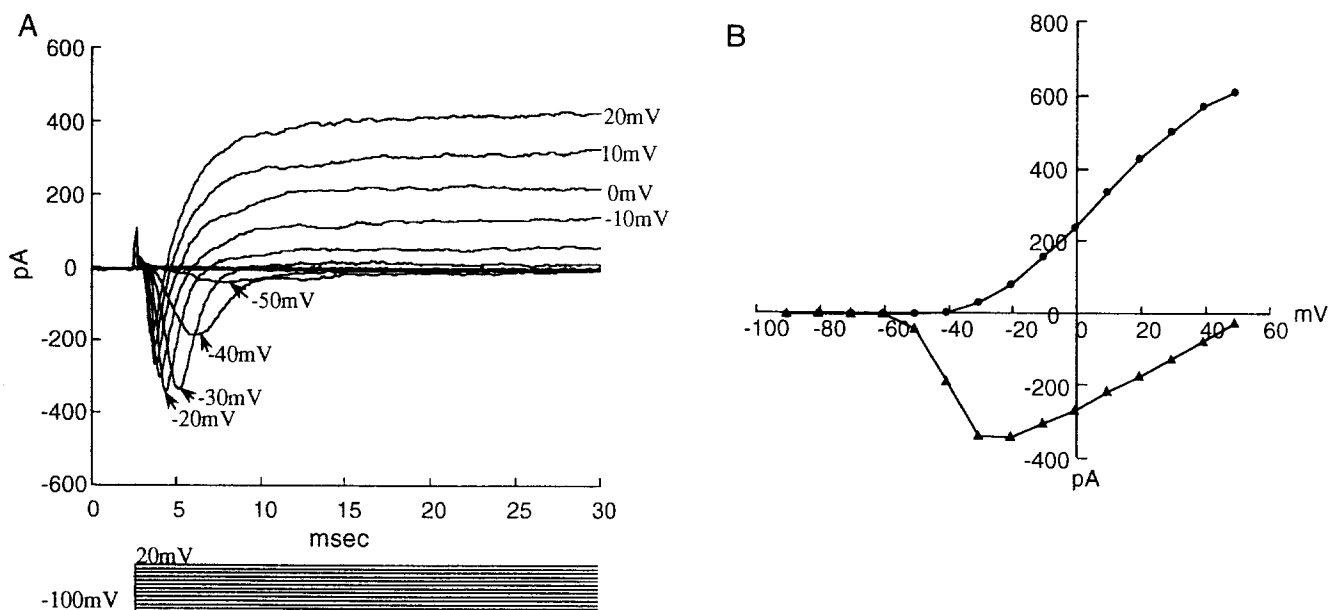
FIGURE 2.   (A) Membrane currents of an isolated newt olfactory receptor cell induced by depolarization from V_h of -100 mV. Command voltages were increased in 10-mV steps from -90 to $+20$ mV. The cell was bathed in control solution (solution 1) and the recording pipette was filled with K^+ solution. (B) I-V relation of the cell shown in A. Peak amplitude of inward ($\blacktriangle$) and outward ($\bullet$) currents was measured and plotted against the voltage.

two components of different kinetics; transient and long-lasting. The time course of the transient current was slower than that of I_{Na} indicating that the transient I_{Ca} is not a residual current through Na^+ channel (see be-

low). The transient I_{Ca} was observed from 42 cells out of 63 examined.

The peak amplitude of the inward current was plotted against the membrane potential in Fig. 3 B. The I-V
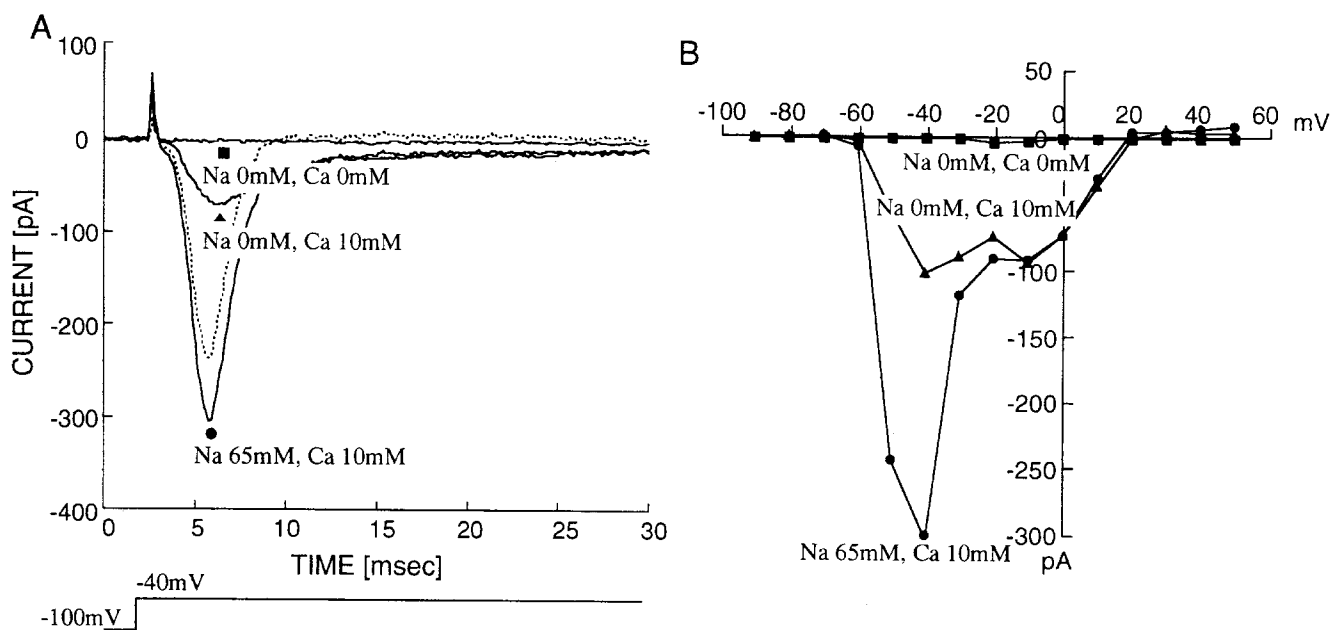

FIGURE 3.   Analysis of inward currents in a newt olfactory receptor cell. (A) Currents induced by depolarization to -40 mV from V_h of -100 mV in a solution containing 65 mM Na^+, 10 mM Ca^{2+} ($\bullet$, solution 2), in Na^+-free solution containing 10 mM Ca^{2+} ($\blacktriangle$, solution 3), and in Na^+, Ca^{2+}-free solution ($\blacksquare$, solution 5). Na^+ or Ca^{2+} was replaced by equimolar choline$^+$. Dotted line represents Na^+ current by computer subtraction ($\bullet$ minus $\blacktriangle$). Pipette was filled with Cs^+ solution. (B) I-V relation of the cell shown in A. The data obtained under the three conditions are plotted with identical symbols as in A.

curve of I_{Ca} was w-shaped showing two remarkable peaks at around -40 and -10 mV, suggesting that Ca^{2+} is carried through two different ionic channels.

Identification of Two Types of Ca^{2+} Currents

To identify the type of Ca^{2+} channels, we studied the pharmacological properties of the transient and long-lasting Ca^{2+} currents. Among several types of Ca^{2+} channels, N-type and L-type Ca^{2+} channels are known to be more permeable to Ba^{2+} than to Ca^{2+}, while T-type shows almost equal permeability to Ba^{2+} and Ca^{2+} (Tsien et al., 1988). Therefore, we first examined Ca^{2+} current by Ca^{2+}/Ba^{2+} replacement.

Fig. 4 A shows membrane currents evoked by voltage pulses in solutions containing 10 mM Ca^{2+} or 10 mM Ba^{2+}. The long-lasting component was markedly increased by replacing extracellular Ca^{2+} to equimolar Ba^{2+} (10 cells tested). I-V curves from a set of experiments show that the current was enhanced at a voltage range from -30 to $+60$ mV. Current amplitude remained the same at the voltage range below -30 mV (Fig. 4 B). Furthermore, the long-lasting component

was suppressed by 100 μM Cd^{2+} (Fig. 4 C, 4 cells). These results are consistent with the previous observation that Ba^{2+} is more permeable through the high-voltage activated (HVA) Ca^{2+} channel and HVA current is sensitive to Cd^{2+} (Tsien et al., 1988).

Among HVA Ca^{2+} channels it is known that the L-type is sensitive to dihydropyridine compounds while the N-type is resistant (Tsien et al., 1988). As shown in Fig. 4 D, the HVA component of the olfactory cell was sensitive to dihydropyridine compounds; nifedipine (1–10 μM) reduced HVA current selectively (6 cells tested). The HVA current was enhanced when the cell was exposed to 0.3 μM Bay K 8644 (not shown, 4 cells). From these results the HVA current of newt olfactory receptor cells was identified as L-type Ca^{2+} current ($I_{Ca,L}$).

In contrast, 100 μM Ni^{2+} suppressed the low-voltage activated (LVA) current component (Fig. 4 E, 6 cells tested). However, 5 μM ω-conotoxin GVIA did not change the current waveform or I-V relation (Fig. 4 F, 4 cells tested). Thus, the LVA Ca^{2+} current in newt olfactory receptor cells was identified as T-type Ca^{2+} current ($I_{Ca,T}$).

In the olfactory receptor cell the influx of Ca^{2+} triggers an activation of Cl^- current ($I_{Cl(Ca)}$) in the ciliary
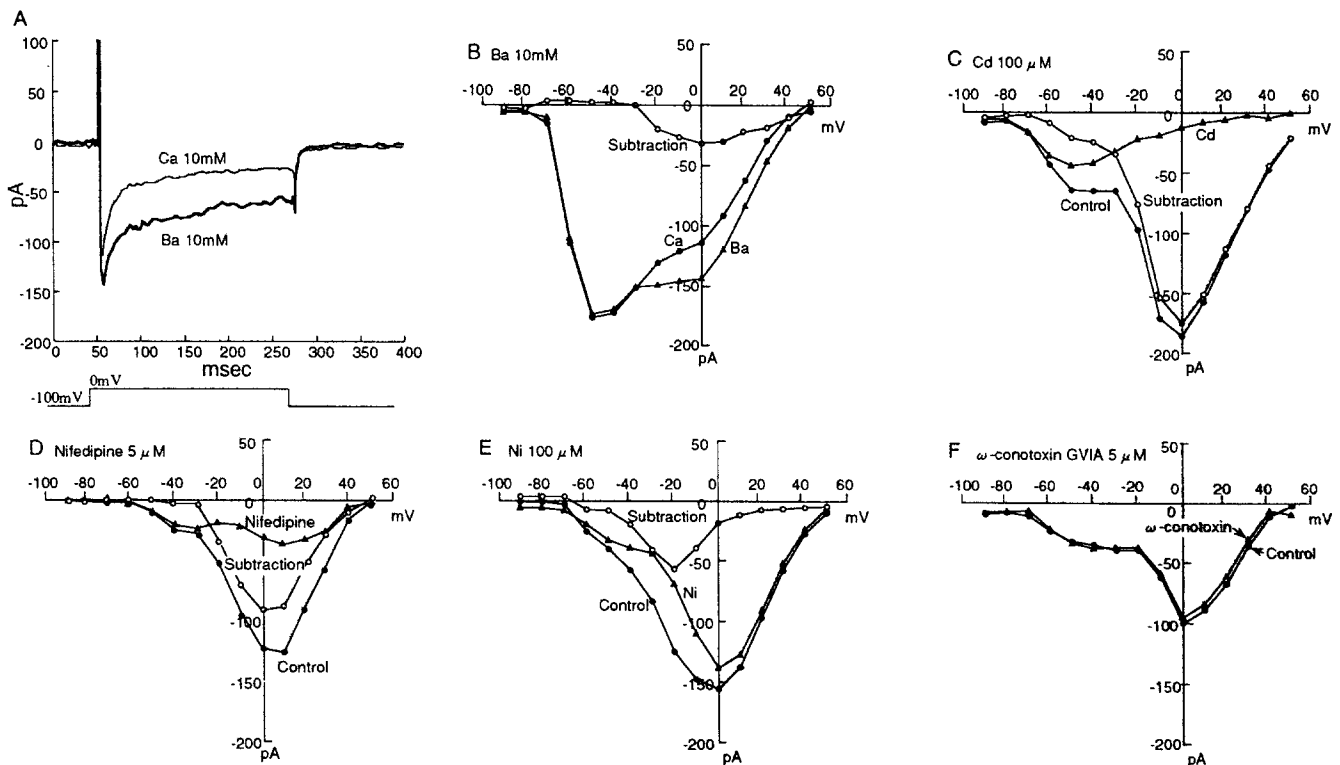

Figure 4. Effects of divalent cations and pharmacological agents on I_{Ca} of a newt olfactory cell. Currents were recorded by using pipettes filled with the Cs^+ solution. (A) Membrane current evoked by depolarization to 0 mV ($V_h = -100$ mV) in a solution containing 10 mM Ca^{2+} (solution 3, thin line) or 10 mM Ba^{2+} (solution 6, thick line). (B) I-V relation of the cell shown in A obtained in 10 mM Ca^{2+} (●) or 10 mM Ba^{2+} (▲). Ba^{2+}-enhanced component (○) was obtained by computer subtraction. (C–F) I-V relations of other olfactory cells to analyze the effects of 100 μM Cd^{2+} (C), 5 μM nifedipine (D), 100 μM Ni^{2+} (E), and 5 μM ω-conotoxin GVIA (F). The effect of these agents was examined in different cells by adding them to Na^+-free, 10 mM Ba^{2+} solution (solution 6). ●: Current recorded under control condition, ▲: current recorded in the presence of test agent, ○: affected fraction obtained by computer subtraction.

membrane (Kleene and Gesteland, 1991; Kleene, 1993; Kurahashi and Yau, 1993; Lowe and Gold, 1993). Before further analyzing the properties of I_{Ca} it is important to examine whether or not the current described above is contaminated by $I_{Cl(Ca)}$. Recording the membrane currents in a solution containing 2 mM SITS eliminated our doubt; the waveform and I-V relation of the current obtained in the solution containing SITS were identical to those obtained in the control solution (not shown). Absence of $I_{Cl(Ca)}$ in our preparation is accounted for by the fact that the cells we used had no cilia.

Voltage Dependence of $I_{Ca,T}$ and $I_{Ca,L}$

An additional difference between $I_{Ca,T}$ and $I_{Ca,L}$ is their voltage dependence. In the experiment of Fig. 5, we activated the two types of currents from several holding voltages (V_h; −100, −80, and −60 mV). When V_h was −100 mV, depolarizing voltage steps elicited both $I_{Ca,T}$ and $I_{Ca,L}$ (Fig. 5 A). When V_h was shifted to −80 mV, the amplitude of $I_{Ca,T}$ was drastically reduced, while the amplitude of $I_{Ca,L}$ was not changed significantly (Fig. 5 B). The amplitude of $I_{Ca,T}$ was further reduced when V_h was shifted to a more positive value (−60 mV, Fig. 5 C).

Kinetic Properties of Two Types of Transient Currents: $I_{Ca,T}$ and I_{Na}

To relate the transient inward currents to the action potential, kinetic properties of $I_{Ca,T}$ and I_{Na} were ana-

lyzed in detail. Fig. 6 A shows $I_{Ca,T}$ evoked by a voltage step in Na^+-free, 10 mM Ca^{2+} solution. $I_{Ca,L}$ was blocked by an application of 0.1 mM Cd^{2+}. The decay phase of the membrane current could be fitted by a single exponential function with a time constant (τ) of 8.3 ms. I_{Na} was also fitted by another single exponential function (τ = 1.4 ms, Fig. 6 B).

Fig. 6C shows the voltage dependence of the decay time constant of $I_{Ca,T}$ and I_{Na}. The time constant of $I_{Ca,T}$ was minimum (9.1 ± 0.9 ms, n = 7) at −40 mV, and increased as the membrane voltage was either hyperpolarized (τ = 16.5 ± 2.4 ms, n = 7, at −70 mV) or depolarized (τ = 20.9 ± 2.8 ms, n = 7, at +20 mV). On the other hand, the time constant of I_{Na} was changed only slightly (τ = 1.6 ± 0.3 ms, at −50 mV; 0.9 ± 0.2 ms at +20 mV, n = 6). Thus, the kinetic behavior of $I_{Ca,T}$ was totally different from that of I_{Na}.

Activation and Inactivation Curves

Fig. 7 A shows the activation curves of $I_{Ca,T}$, I_{Na}, and $I_{Ca,L}$. The cell was depolarized from V_h of −100 mV, and the conductance was normalized to its maximum value. The relation between $g_{Ca,T}$ (*filled triangles*, measured in 10 mM Ca^{2+}) and membrane voltage was fitted by a single Boltzmann function. The half-activation voltage of $g_{Ca,T}$ was −44 mV. Activation curve of I_{Na} (*filled circles*) was also fitted by a single Boltzmann function with a half-activation voltage of −34 mV. This
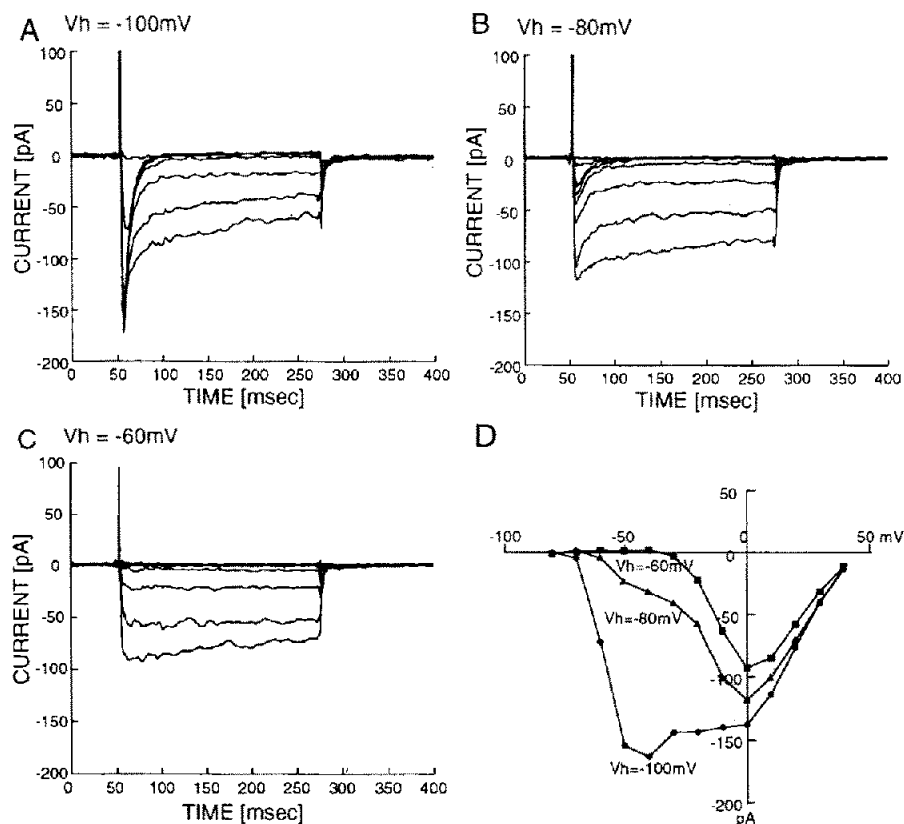

Fig. A: Vh = -100mV, B: Vh = -80mV, C: Vh = -60mV, D: I-V curves

FIGURE 5. Effects of holding voltage on the amplitude of I_{Ca} of a newt olfactory cell. Cells were recorded in Na^+-free, 10 mM Ba^{2+} solution (solution 6) by using pipette filled with CsCl. The cell was depolarized in 10-mV steps from −70 to 0 mV. V_h was −100 mV (A), −80 mV (B), or −60 mV (C). (D) I-V curves of the data shown in A–C. ●: V_h, −100 mV, ▲: −80 mV, ■: −60 mV.
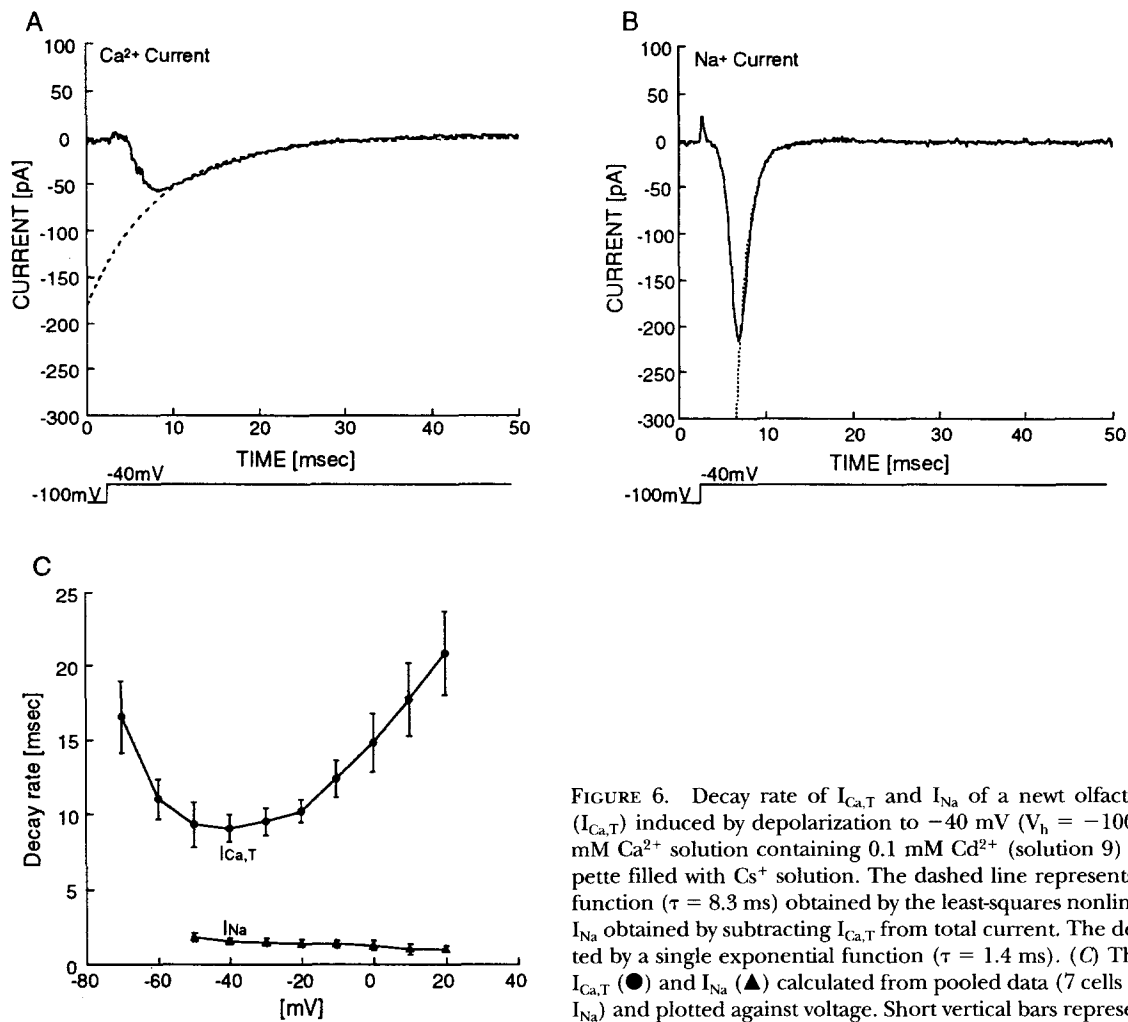
FIGURE 6. Decay rate of $I_{Ca,T}$ and I_{Na} of a newt olfactory cell. (A) Current ($I_{Ca,T}$) induced by depolarization to -40 mV ($V_h = -100$ mV) in Na^+-free, 10 mM Ca^{2+} solution containing 0.1 mM Cd^{2+} (solution 9) recorded by using pipette filled with Cs^+ solution. The dashed line represents a single exponential function ($\tau = 8.3$ ms) obtained by the least-squares nonlinear fit to the data. (B) I_{Na} obtained by subtracting $I_{Ca,T}$ from total current. The decay phase was also fitted by a single exponential function ($\tau = 1.4$ ms). (C) The mean decay rate of $I_{Ca,T}$ (●) and I_{Na} (▲) calculated from pooled data (7 cells for $I_{Ca,T}$ and 6 cells for I_{Na}) and plotted against voltage. Short vertical bars represent SD.

value is 10 mV more positive than that of $g_{Ca,T}$. The half-activation voltage of $g_{Ca,L}$ (*open circles*) was -10 mV.

Inactivation was also voltage dependent (Fig. 7 B). $g_{Ca,T}$ was inactivated by a conditioning polarization (duration $= 1$ s) of more positive than -90 mV and became almost zero by a conditioning polarization of more positive than -30 mV. Half-inactivation voltage was -65 mV. g_{Na} started to be inactivated by a conditioning polarization of more positive than -70 mV. The half-inactivation voltage was -53 mV, 13 mV more positive than that of $I_{Ca,T}$. $g_{Ca,L}$ did not show inactivation within 1 s, and therefore its inactivation curve was not measured in the present experiment.

Ca^{2+} Permeation through Na^+ Channel

In some preparations it has been shown that Ca^{2+} permeates through the Na^+ channel ($P_{Ca}/P_{Na} = 0.01$, squid giant axon, Baker et al., 1971; P_{Ca}/P_{Na}, too small to measure, myelinated nerve fiber, Hille, 1972; tunicate egg, Okamoto et al., 1976). We also examined the possibility of Ca^{2+} permeation through Na^+ channels

in the newt olfactory receptor cell. Currents through Na^+ channels were isolated by the presence of 1 mM Co^{2+} and 35 mM TEA. The response amplitude of the inward current recorded in 65 mM $Na^+/10$ mM Ca^{2+} solution was not changed significantly by removal of external Ca^{2+} (not shown), suggesting that the permeability of Ca^{2+} through Na^+ channels in the newt olfactory receptor cells is extremely low.

Contribution of $I_{Ca,T}$ to the Action Potential

As shown in Fig. 7, the dynamic range of the activation curve of $I_{Ca,T}$ was ~ 10 mV more negative than that of I_{Na}. This observation raises a possibility that $I_{Ca,T}$ contributes to lower the threshold of action potential generation.

To verify this possibility, we recorded action potentials induced by a current injection near threshold. An example is shown in Fig. 8. When the cell was depolarized by injection of 4 pA current in the control medium, a single action potential was generated (Fig. 8 A). Addition of 0.1 mM Ni^{2+} to the solution blocked
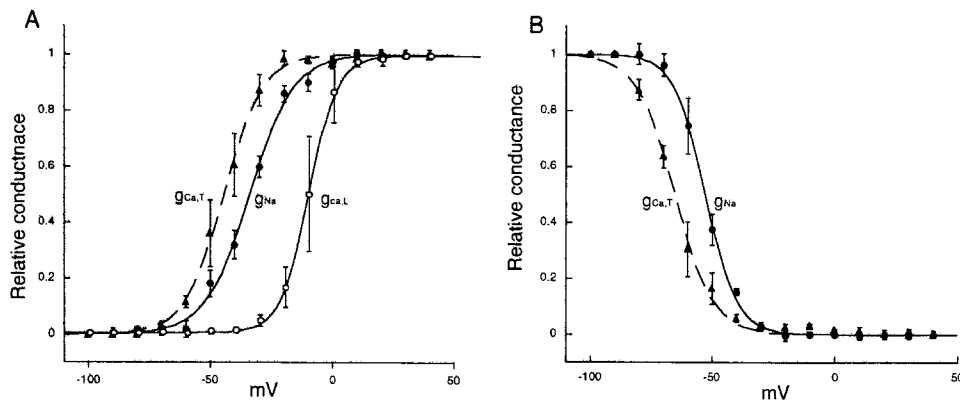
FIGURE 7. Activation and inactivation curves of $I_{Ca,T}$, $I_{Ca,L}$, and I_{Na} of a newt olfactory cell. $I_{Ca,T}$ was recorded in Na^+-free, 10 mM Ca^{2+}, 0.1 mM Cd^{2+} solution (solution 9), $I_{Ca,L}$ in Na^+-free, 10 mM Ba^{2+}, 0.1 mM Ni^{2+} solution (solution 7), and I_{Na} in 65 mM Na^+, Ca^{2+}-free solution (solution 4) by using pipettes filled with Cs^+ solution. V_h was -100 mV in all recordings. (A) Activation curve of $I_{Ca,T}$ (▲), $I_{Ca,L}$ (○), and I_{Na} (●). Relative conductance at a specific membrane voltage was estimated as a ratio of the recorded current amplitude to that expected from the maximum conductance (the linear part of individual I-V curves near the reversal potential). Symbols represent mean of 7 (for $I_{Ca,T}$ and I_{Na}) or 6 (for $I_{Ca,L}$) cells, and vertical bars represent SD. Lines represent a single Boltzmann function obtained by the least-squares nonlinear fit to the data. (B) Inactivation curves of $I_{Ca,T}$ and I_{Na}. Relative conductance was estimated as a ratio of the current amplitude induced by depolarization to -40 mV after a 1-s conditioning pulse of various voltages (-100 to $+40$ mV) to that induced by the same depolarization without conditioning pulses. The relative conductance is plotted against the conditioning voltage. Each symbol represents mean of 7 cells, and vertical bars represent SD. Lines represent a single Boltzmann function obtained by the least-squares nonlinear fit to the data.

the action potential (Fig. 8 A). If more current was injected under this condition, action potential reappeared (Fig. 8 B). However, the rising phase of the action potential was slower than that of the action potential evoked under the control condition. Similar results were obtained from four more cells. These results indicate that $I_{Ca,T}$ is more important for spike generation than I_{Na} near threshold in the newt olfactory receptor cells.

In other words, the action potential was more readily evoked by the presence of the T-type Ca^{2+} channel.

DISCUSSION

In the present study we identified that a transient Ca^{2+} current of the newt olfactory receptor cells is T-type, and showed that both I_{Na} and $I_{Ca,T}$ are involved in spike
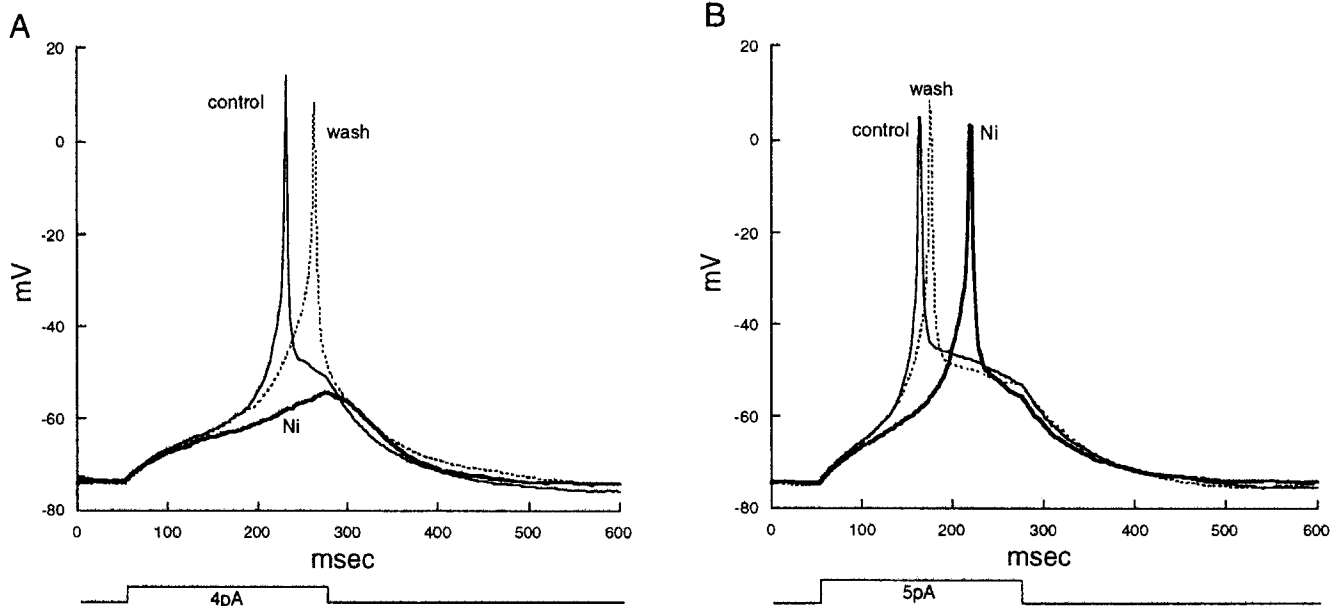

FIGURE 8. Contribution of $I_{Ca,T}$ to action potentials of a newt olfactory cell evoked by a low intensity current injection recorded under the current clamp condition by using pipette filled with K^+ solution. (A) response to near threshold depolarization by injecting $+4$ pA current recorded in control (thin continuous line, solution 1), after addition of 0.1 mM Ni^{2+} (thick line, solution 14), and after washout (dotted line) of Ni^{2+}. (B) Response to a stronger depolarization by injecting $+5$ pA current.

generation. Activation range for the $I_{Ca,T}$ was $\sim$10 mV more negative than that of I_{Na}, indicating that $I_{Ca,T}$ was activated near threshold of spike generation. Indeed, at threshold, action potentials were blocked by addition of Ni^{2+}, a selective blocker for T-type Ca^{2+} channel, even in the presence of Na^+.

In the past, it has been believed that the transient inward current of the olfactory receptor cells 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: Rajendra et al., 1992; Trombley and Westbrook, 1991). Only Trotier (1986) reported a work on salamander olfactory cells suggesting that the transient inward current was also carried by Ca^{2+}. Perhaps, the transient current Trotier reported is identical to $I_{Ca,T}$ of the present study, but at that time classification and characterization of Ca^{2+} channels had not been established. The reason why $I_{Ca,T}$ is not identified in olfactory cells of other animal species is still obscure, but there are several possibilities. (a) The presence of T-type Ca^{2+} currents may depend on the development or regeneration of olfactory cells. It is known that the olfactory epithelia undergo regeneration continuously. The different observations concerning T-type Ca^{2+} channels may be due to different populations of cells under the experimental conditions. It is also reported that in cultured olfactory receptor cells Ca^{2+}-activated K^+ channel is absent (Trombley and Westbrook, 1991), while it has shown ubiquitous in adult olfactory cells (Kurahashi, 1989a; Maue and Dionne, 1987; Miyamoto et al., 1992; Schild, 1989). Expression of Ca^{2+}-activated K^+ channel has been shown to be developmentally related in cultured spinal neurons of Xenopus (Blair and Dionne, 1985). (b) Another possibility is just a simple species difference. It is interesting to note that, very recently, Liman and Corey (1996, see footnote 1) have reported that a T-type Ca^{2+} channel is expressed in the chemosensory neurons from the mouse vomeronasal organ. Since there are so many similarities between principal and accessory olfactory cells in terms of the expression of ionic channels, this observation raises a possibility that T-type Ca^{2+} channels might be expressed not only in newt olfactory receptor cells but also in cells of the other species. Further study would be required to reexamine the presence of T-type Ca^{2+} currents in olfactory receptor cells from other species.

Subtype of Ca^{2+} Channels

The $I_{Ca,T}$ we identified in the newt olfactory cells had properties common to $I_{Ca,T}$ of other sensory neurons (Carbone and Lux, 1984; Nowycky et al., 1985; Fox et al., 1987; Kaneko et al., 1989): activation range, equal permeability to Ca^{2+} and Ba^{2+}, suppression by Ni^{2+},

and insensitivity either to dihydropyridine compounds or to ω-conotoxin GVIA. Also, the $I_{Ca,L}$ we identified in the newt olfactory cells had properties common to $I_{Ca,L}$ of various cells: activation voltages, higher permeability to Ba^{2+} than to Ca^{2+}, inhibition by Cd^{2+}, enhancement by Bay K 8644, and inhibition by nifedipine.

Availability of Na^+ and T-type Ca^{2+} Channels at the Resting Potential

Since activation and inactivation of I_{Na} and $I_{Ca,T}$ are strongly voltage dependent, it is important to know how much Na^+ and T-type Ca^{2+} channels are available at the resting potential to understand their contribution to action potential generation in vivo. In the present study, the resting potential was estimated to be -70 mV. Since the inactivation curves for I_{Na} and $I_{Ca,T}$ were fitted by single Boltzmann functions, fraction of Na^+ and T-type Ca^{2+} channels to be activated by depolarization from the resting potential (-70 mV) can be estimated from the following function:

$$1 / \{ 1 + \exp [(V - V_{half}) / K_h] \} ,$$

where V represents the membrane potential, V_{half} represents the half-inactivation voltage, and K_h represents a coefficient. For $I_{Ca,T}$, V_{half} was -65 mV and K_h was 7.8 mV. Thus, at the resting potential, 64% of T-type Ca^{2+} channels can be activated by depolarization. Similarly, for I_{Na}, V_{half} was -53 mV and K_h was 6.5 mV. Therefore, 93% of Na^+ channels can be activated by depolarization at the resting potential.

As reported by Frankenhaeuser and Hodgkin (1957), the activation voltage of voltage-gated channels is shifted by a change in $[Ca^{2+}]_o$ that affects the surface charge of the plasma membrane. By high $[Ca^{2+}]_o$ the activation voltage will be shifted to more positive voltages, and by low $[Ca^{2+}]_o$ it will be shifted to more negative voltages. Since most of the present recordings were made in 10 mM Ca^{2+} or in 10 mM Ba^{2+}, the activation voltages of $I_{Ca,T}$ and $I_{Ca,L}$ in the physiological $[Ca^{2+}]_o$ are expected to be more negative than the values we obtained; the half-activation voltage was -44 mV for $I_{Ca,T}$ and -10 mV for $I_{Ca,L}$ (Fig. 7 A).

It is also important to evaluate the amplitude of $I_{Ca,T}$ in normal Ca^{2+} concentrations. In 3 mM Ca^{2+} the peak amplitude of $I_{Ca,T}$ was about one-fourth of I_{Na}. Therefore, $I_{Ca,T}$ could carry a significant fraction of inward current.

Comparison with I_{Na} of Other Species

The half-inactivation voltage, V_{half}, of I_{Na} obtained in our studies (-53 mV) is comparable to those obtained for I_{Na} in catfish olfactory receptor cells (-62 mV, Miyamoto et al., 1992), in coho salmon cells (-64 mV, Nevitt and Moody, 1992), in larvel tiger salamander

cells (−50 mV, Firestein and Werblin, 1987), and in cultured rat cells (−63 mV, Trombley and Westbrook, 1991), but is significantly more positive than those obtained from olfactory receptor cells in grass frogs (−82 mV, Pun and Gesteland, 1991) and in adult rats (−108 mV, Rajendra et al., 1992).

Pun and Gesteland (1991) have concluded that I_{Na} located at the cell body of the frog olfactory cell is completely inactivated at the resting potential, based on the relation between the measured resting potential (−52 mV) and the inactivation rate of I_{Na} (V_{half} = −82 mV). However, the resting potential could be even more negative in frog olfactory cells, since the seal resistance under whole-cell mode affects the apparent resting potential, as described in MATERIALS AND METHODS of the present study. They have shown that the mean input resistance of frog olfactory cells is 1–2 GΩ. If the seal resistance was 10 GΩ, the real resting potential of the frog olfactory cells would be in a range between −65 and −58 mV. I_{Na} available in this range would be then estimated to be 12–20% by using the single Boltzmann function with measured parameters (V_{half} = −82 mV, K_h = 12.2 mV, Pun and Gesteland, 1991). Since the peak amplitude of a fully activated Na$^+$ current was larger than 500 pA in frog olfactory receptor cells (Pun and Gesteland, 1991), 12–20% fraction provides 60–100 pA of inward current, which would be sufficient to generate action potentials in such electrically compact cells. Thus, it is highly likely that I_{Na} located at the cell body of the frog olfactory cell provides a significant contribution to the spike generation in frog cells.

Generation of Action Potential

All the data we obtained in the present study strongly suggest that the action potential of newt olfactory cells is generated by activation of both Na$^+$ and T-type Ca^{2+} channels. It has been shown by a current clamp experiment that the action potential is not abolished by complete removal of external Na$^+$. The action potential initiated by the least effective current injection was abolished by Ni^{2+}. The activation voltage of $I_{Ca,T}$ was about 10 mV more negative than that of I_{Na}. From these observations we conclude that the $I_{Ca,T}$ contributes to lower the threshold of spike generation as has been suggested in other neurons (Llinas and Yarom, 1981). In newt olfactory receptor cells this effect helps by elevating the sensitivity to odorants.

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