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Very Negative Potential for Half-inactivation of, and Effects of Anions on, Voltage-dependent Sodium Currents in Acutely Isolated Rat Olfactory Receptor Neurons

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Abstract. Previous measurements with CsF pipette solutions using whole-cell patch-clamp techniques in dissociated rat olfactory receptor neurons (ORNs) indicated that the sodium currents had very negative inactivation characteristics with the implication that the cell resting potential must also normally have a very negative value. This study supports the conclusions that such an effect was real and not dependent on either the nature of the pipette anions or the recording situation previously used. For all pipette solutions, sodium currents showed a threshold activation ≈ −80 mV and half-maximal activation voltages ≈ −55 with half-inactivation potential ⩽ −100 mV, without being significantly affected by the replacement of F^- by other pipette anions ($H_2PO_4^-$ and $acetate^-$) or the addition of nucleotides and glutathione (which did cause a very slight positive shift). F^-, followed by $H_2PO_4^-$ and to a much lesser extent by $acetate^-$, was the most favorable pipette anion for obtaining good seals and whole-cell sodium currents in these extremely small ORNs. These results implied that resting potentials, for viable responsive cells, should be more negative than about −90 mV, as supported by the observation that action potentials could only be evoked from holding potentials more negative than −90 mV.

Key words: Olfactory receptor neuron — Sodium currents — Fluoride — Phosphate — Acetate — Patch clamp

Introduction

Olfaction is the sensation caused by various volatile odoriferous substances that are identified by ORNs in the olfactory epithelium (Dionne & Dubin, 1994). Although it is still uncertain how different odorants are discriminated and coded, it is generally accepted that the binding of these receptors to airborne odorants activates cyclic nucleotide-gated channels and calcium-dependent Cl^- channels via various intracellular second messenger pathways, which in turn produce depolarizing 'generator' or receptor potentials (Lancet, 1986). The soma of ORNs integrates these generator potentials to trigger action potentials in their axons, and these electrical signals are then delivered to the higher areas of the central nervous system via the olfactory bulb to transduce the sensation of smell (e.g., Shipley & Ennis, 1996).

Both voltage-dependent sodium channels (I_{Na}) and T-type calcium channels ($I_{Ca,T}$) are responsible for the production of action potentials (Hodgkin & Huxley, 1952; Noda, 1993; Schild & Restrepo, 1998). The estimated ratio $I_{Na}/I_{Ca,T}$ during the action potential induced by ramp current injection in newt ORNs was about 1.67 (Kawai, Kurahashi & Kaneko, 1997). Action potentials evoked in rat ORNs must result primarily from a sodium influx since such action potentials were extremely sensitive to low concentrations of TTX (100 nM; Trombley & Westbrook, 1991).

In most sensory systems, information about the intensity of the sensory stimulus is coded in the frequency of action potentials. Extracellular recording from the olfactory nerve clearly demonstrates that repetitive firing increases with odorant concentration (Getchell, 1986). Repetitive spikes have been recorded with prolonged depolarizing current pulses in the ORNs of many animals (Masukawa, Hedlund & Shepherd, 1985; Dubin & Harris, 1997). However, in rat ORNs, only a single action potential was fired in response to long depolarizing pulses, and repetitive firing was only seen if trains of brief current pulses were used to stimulate action potentials (Trombley & Westbrook, 1991).

In the previous studies on dissociated rat ORNs, an unusual property of voltage-dependent sodium channels

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was reported in that its midpoint for voltage-dependent inactivation was very negative, around −108 mV (Rajendra, Lynch & Barry, 1992). This value contrasted somewhat with the value obtained in many other animals, including cultured rat ORNs (Trombley & Westbrook, 1991) and *Necturus* (Dionne, 1992), but it is close to the value obtained in human heart muscle (Deschenes et al., 1998). Because rat ORNs are extremely small cells with a very high input resistance and very few channels contribute to the resting potential (V_m), it is quite reasonable that V_m in these cells may be much closer to the potassium equilibrium potential (E_K). In addition, in vivo, the internal potassium levels may be much higher than usual or else an electrogenic membrane pump may further hyperpolarize the membrane potential so that V_m is even more negative than E_K.

These studies predicted that at a typical resting potential, most of the sodium channels would be inactivated, so that only a few sodium channels may be able to be activated by depolarization and generate action potentials, since they would not have had time to recover from inactivation to fire repetitively. It may be considered that this measured negative inactivation was not typical of the in vivo situation but had been dependent on the experimental recording system, and the particular recording conditions. These might have included the effect of particular ions in the pipette solution or else the absence of some endogenous component within the dialyzed cell or the enzyme used for dissociation. The aim of this study has been to explore these possibilities. In particular, F^- ions have been shown to affect voltage-gated ion channels, including neuronal sodium channels, by altering the properties of a number of membrane and intracellular proteins, such as cAMP-dependent protein kinase (Vargas et al., 1999). Until now, it has not been clear whether F^- could affect the electrophysiological properties of activation and inactivation of these macroscopic sodium currents. Until recently the only pipette anion that we have been able to use to get viable whole cell measurements on these small mammalian ORNs has been F^-. However, recent reports have indicated good odorant whole cell responses in these ORNs using acetate$^-$ (R. Kaur and P.H. Barry, *manuscript in preparation*) and $H_2PO_4^-$ (Vargas et al., 1999). We have therefore used these anions and some others to try and obtain whole cell recording and to compare their effectiveness at producing viable well-clamped Na^+ current responses.

It has been well known in giant axons of the squid, that some anions can affect membrane excitability by decreasing the rising phase of the action potential (Adelman, Dyro & Senft, 1966). The following sequence is the rank-order of a number of anions according to their ability to support action potentials: $SCN^- < I^- < Br^- < NO_3^- < Cl^-$ < acetate$^-$ < HSO_4^- < aspartate$^-$ < glutamate$^-$ < $H_2PO_4^- < F^-$ (Adelman et al., 1966). It is of interest to note that F^- was found to be the best anion to restore sodium conductance to normal values and to eliminate the inhibitory effects of other anions (Tasaki, Singer & Takenaka, 1965; Adelman et al., 1966).

One of the other possibilities was whether the absence of some natural endogenous compound has resulted in a change in voltage sensitivity of the sodium currents in these ORNs. In support of such a possibility, Pun, Kleene & Gesteland (1994) found in frog ORNs that the voltage for half-inactivation ($V_{1/2}$) of sodium currents under whole-cell voltage clamp shifted to more negative potentials with time in the absence of nucleotides. Vargas and Lucero (1999) recently reported a culture system that supported short-term survival of odorant-responsive adult rat ORNs, which exhibited typical voltage-gated Na^+ currents. Their pipette solutions included glutathione and ATP, together with F^-.

The aim of this study was to investigate the role of different pipette anions on these Na^+ channels and to determine whether any of the above factors, or the absence of any critical components, had affected the voltage dependence of these Na^+ channels.

Materials and Methods

Preparation of Acutely Isolated ORNs

Rat ORNs were enzymatically isolated from the epithelia of adult female Wistar rats (<150 grams) according to the method of Maue and Dionne (1987). The rats had first been killed by the inhalation of carbon dioxide. Then, the olfactory epithelium was removed and washed in divalent cation-free DPBS-Dulbecco's Phosphate Buffered Saline (Flow Laboratories, UK). The ORNs were dissociated with 0.022% trypsin solution at 37°C for 29 min. The enzyme dissociation was stopped by the addition of about 0.1 mg trypsin inhibitor (Calbiochem, La Jolla, CA). Finally, the ORNs together with other cells were dispersed by careful and gentle trituration of the tissue.

The cell supernatant was then transferred to the experimental perspex chamber and left for up to half an hour to allow the cells to settle to the bottom. Typical ORNs were identified by their ovoid soma (5–8 μm in diameter) and a single long dendrite (10–30 μm) with a well-defined border. The olfactory cilia and axon were seldom seen, perhaps due either to their loss during the dissociation procedure or to the limited resolving power of the microscope. Cells could generally maintain their viability for up to 8 h. Only cells with typical morphological features, low access resistance and good space clamp control were selected.

Electrophysiological Recording

All tight-seal whole-cell patch recordings were performed at about 21 ± 1°C using standard techniques (Hamill et al., 1981). The recording chamber was perfused at 0.5–2.0 ml/min with General Mammalian Ringers (GMR) solution containing (mM): NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, adjusted to pH 7.4 with 1 M NaOH.

The composition of the traditional pipette solutions (PS) for studying the whole-cell recordings of acutely isolated rat ORNs was (mM): CsF 135, NaCl 10, Ethylene glycol-bis (β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) 1.1 and N-[2-hydroxyethyl]-

Table 1. Composition of some pipette solutions (in mM)

Designation	$[Cs^+]$	$[K^+]$	$[Na^+]$	$[F^-]$	$[Acetate^-]$	$[Cl^-]$	EGTA	[a]HEPES
F^- 1 (PS1)	—	135	10	135	—	10	1.1	10
F^- 2 (PS2)	135	—	10	135	—	10	1.1	10
F^-/Cl^-	65	70	10	70	—	75	1.1	10
$acetate^-$	—	135	10	—	135	10	1.1	10
$acetate^-/Cl^-$	65	70	10	—	70	75	1.1	10
Cl^-	—	135	10	—	—	145	1.1	10

[a] The above solutions were titrated with about 5 mM CsOH to neutralise the HEPES.

piperazine-N′-[2-ethanesulfonic acid] (HEPES) 10, buffered to pH 7.4 with 1 M CsOH. The other test pipette solutions had the same composition except that the 135 mM CsF was replaced by 135 mM test solutions (Table 1), including KF, KCl, KH_2PO_4, Kacetate, KMethanesulphonate, KGlutamate. In some experiments, 1 mM glutathione, 1 mM GTP or 5 mM NaATP was included in PS1. GMR was only used for up to 10 days and 10 mM glucose was also added to it just before use. Pipette solutions were made every 15 days and filtered immediately before use. In the experiments studying the effects of Cs^+ and K^+ on the fast inward currents, the potassium was replaced by a mixture of Cs^+ (110 mM) and tetraethylammonium (TEA, 25 mM) to block outward K^+ currents. Recording electrodes were produced from borosilicate microhaematocrit glass capillaries (#1601, Vitrex, Herlev, Denmark) to a final resistance of between 3 and 5 MΩ.

An Axopatch-1D amplifier (Axon Instruments, Foster City, CA) was used to make current and voltage clamp recordings of sodium currents. Voltage protocols were generated using pCLAMP software (Version 6.0.4, Axon Instruments); membrane currents were digitized and stored for later analysis on a Pentium computer. Current records were filtered at 5 kHz (−3 dB, 4-pole low-pass Bessel filter) and sampled every 50 μsec. An online leak subtraction protocol (P/P4) was employed to automatically subtract leakage current traces from the voltage-dependent Na^+ current traces. The pipette with the cell attached to its tip was usually lifted up as close as possible to the surface of the bath solution but still in the flowing GMR in order to further decrease noise. Capacity transients associated with stray capacity to ground were eliminated and 80% series resistance compensation was applied. Liquid junction potentials between different pipette and bath solutions were carefully calculated and corrected from the recordings, using the Windows version of the JPCalc program (Barry, 1994; contact P.H.B. for program availability). The liquid junction potential correction, $V_{L.J}$, being the potential of the bath solution with respect to the pipette, had to be subtracted from the pipette potential to give the true membrane potential (*see* Table 3 later, for values of $V_{L.J}$).

Data Analysis

Curve fitting was performed using various offline software, such as the commercially available analysis programs, Clampex and Clampfit (Axon Instruments, CA). To minimize any residual series resistance, only leakage-subtracted data from cells with smaller currents were used for analysis. There was no correlation between access resistance, peak I_{Na} and parameters of activation and inactivation. The peak sodium currents at various conditioning pulse potentials were fitted to the following Boltzmann equation:

$$I(V) = G_{max} \cdot (V - E_{Na})/\{1 + \exp[(V - V_{1/2})/k]\} \quad (1)$$

where $I(V)$ is the peak sodium current, $G(V)$ is the sodium conductance at each voltage step, G_{max} is the maximum sodium conductance, V is the membrane potential, E_{Na} is the sodium equilibrium potential, $V_{1/2}$ is the potential for half-activation where $G(V)$ is half of G_{max} and k is the slope constant indicating the steepness of channel activation at different membrane voltages. The current-voltage (I-V) curves were drawn by plotting the peak sodium current amplitudes against test pulse potentials. Since not all experimental curves could be accurately extrapolated to cross the abscissa close to E_{Na}, particularly if there had not been enough data points at positive potentials, all the activation and inactivation data were curve-fitted to pass through the expected theoretical value of E_{Na} (66.6 mV).

The normalized conductance-voltage curves of voltage-activated steady-state activation and inactivation were obtained by plotting the normalized sodium conductance (G_{Na}/G_{max}) against the test membrane potential (V). The conductance of sodium channels (G_{Na}) was determined from:

$$G_{Na} = I(V)/(V - E_{Na}) \quad (2)$$

where G_{Na} is the sodium conductance at each membrane potential, $I(V)$ is the corresponding peak sodium current and V is the membrane potential.

The descending (activation) and ascending (inactivation) phases of sodium current traces elicited by the activation protocol at the holding potential of −100 mV were fitted with single and double exponential expressions, defined as follows:

$$I_t = A \cdot \exp(-(t - k)/\tau_1) + C \quad (3)$$

$$I_t = A_2 \cdot \exp(-(t - k)/\tau_f) + A_1 \cdot \exp(-(t - k)/\tau_s) + C \quad (4)$$

where t is the time from the commencement of the activation process, I_t is the magnitude of the current at time t, A and C are constants, A_2 and A_1 reflect the magnitudes of the fast and slow components of the current, τ_1 is the time constant of sodium currents for a single exponential fit, and τ_f and τ_s are the fast and slow time constants of sodium currents for double exponential fits.

All the data, corrected for liquid junction potentials, were expressed as mean ± SEM Statistical analyses were made using either ANOVA (analysis of variance) followed by Tukey's test for comparing multiple columns, or Student's paired t-test to determine the statistical significance of differences in the results (being significant for $P < 0.05$).

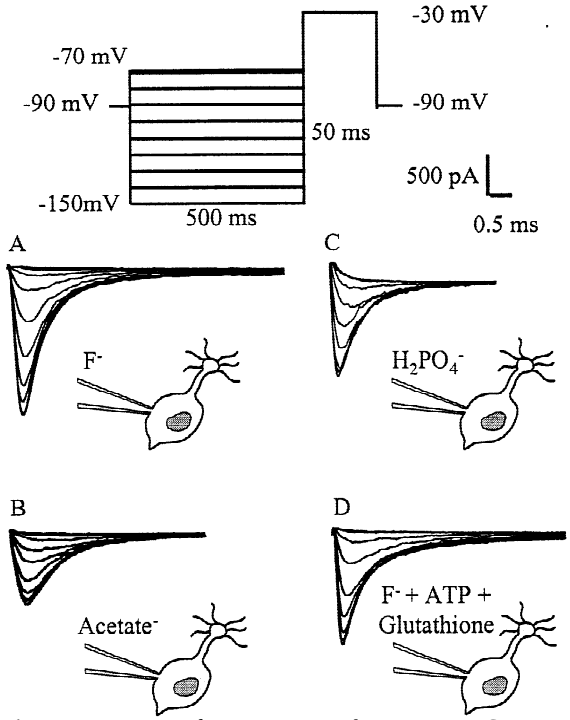

Fig. 1. Inactivation of macroscopic sodium currents. Currents were evoked by test pulses to −30 mV following 500 msec conditioning pulses from −70 to −150 mV with an increment of 10 mV. Cells were held at −90 mV between trials. *A–D* represent sodium currents evoked by the protocol, measured in the F^-, $acetate^-$, $H_2PO_4^-$ and F^- + ATP + glutathione solutions in individual cells. Calibration bars: 500 pA (vertical) and 0.5 msec (horizontal).

Results

Effects of Different Anions on the Kinetics of Sodium Currents

Voltage-dependence of Steady-State Inactivation of Sodium Currents

Steady-state inactivation of sodium currents was studied using the protocol of test pulses to −30 mV following 500 msec conditioning pulses from −70 to −150 mV with an increment of 10 mV. Cells were held at −90 mV between trials. Figure 1 shows representative families of sodium currents measured in the F^-, $acetate^-$, $H_2PO_4^-$ and F^- with ATP plus glutathione solutions. The data were obtained fifteen minutes after the formation of whole-cell configuration. The steady-state inactivation was strongly voltage-dependent and most of the sodium channels became inactivated at membrane potentials more positive than approximately −70 mV in all the recording solutions.

The sodium conductances (G_{Na}) at different test potentials, obtained using Eq. (2), were then normalized by dividing them by the maximal sodium conductance (G_{max}). The normalized values of sodium conductance were plotted against conditioning pulse potential to obtain conductance-voltage (h) curves of steady-state inactivation (Fig. 2). There was no significant difference in the conductance-voltage curves obtained in the F^-, $acetate^-$, $H_2PO_4^-$, F^-/Cl^- and $acetate^-/Cl^-$ solutions (Table 2).

It has been reported in acutely dissociated rat ORNs that the potential for half-inactivation was around −108 mV in the presence of F^- in the pipette solutions (Rajendra et al., 1992). Our studies further supported such a negative value, even at lower concentrations of F^- or with their replacement by other anions. In the absence of nucleotides in the recording pipette solution, the voltages for half-inactivation ($V_{1/2}$) for steady-state sodium currents in F^- and F^-/Cl^- solutions were −106.8 ± 1.5 mV (n = 4) and −101.1 ± 2.4 mV (n = 4), respectively, corresponding well with the previous results. We also found that the application of $acetate^-$, $H_2PO_4^-$ or $acetate^-/Cl^-$ in the pipette solutions did not significantly shift $V_{1/2}$, which for the above pipette solutions were −104.2 ± 1.2, −107.0 ± 2.5, and −105.5 ± 1.4, respectively. In addition, the replacement of potassium with cesium in the pipette solutions did not significantly influence the activation and inactivation of sodium currents (*results not shown*).

Therefore, it may be concluded that the presence of F^- as a major pipette anion doesn't seem to be the reason for the negative value of the half-maximal inactivation in rat ORNs, since the results obtained in all of the recording solutions gave very similar results.

The rate of recovery from inactivation was also studied but only in the F^- and $acetate^-$ solutions. Currents were evoked by a two-pulse protocol with a variable interval t between pulses, ranging from 10 to 1000 msec. Each pulse had a duration of 20 msec. The pulse protocol used steps from various holding potentials (−90, −100, −110, −120, −130 and −140 mV) to −30 mV. If the interval between the conditioning pulse and the test pulse had been too short, the second pulse failed to evoke sodium current since the channels did not have enough time to recover from the inactivation induced by the first pulse. As the interval was increased, the amplitude of the current induced by the second pulse increased as the channels recovered from inactivation. Examples of sodium currents recorded during various stages of recovery at a holding potential of −120 mV are shown in the inset of Fig. 3.

The degree of recovery was determined from the ratio of the peak amplitude of the sodium current evoked by the test pulse (I_p) to that evoked by the conditioning pulse in the absence of inactivation ($I_{p,max}$). The values of $I_p/I_{p,max}$ were plotted against the corresponding time intervals to illustrate the rate of recovery from inactivation. The data at a given holding potential were fitted with both single and double exponential functions:

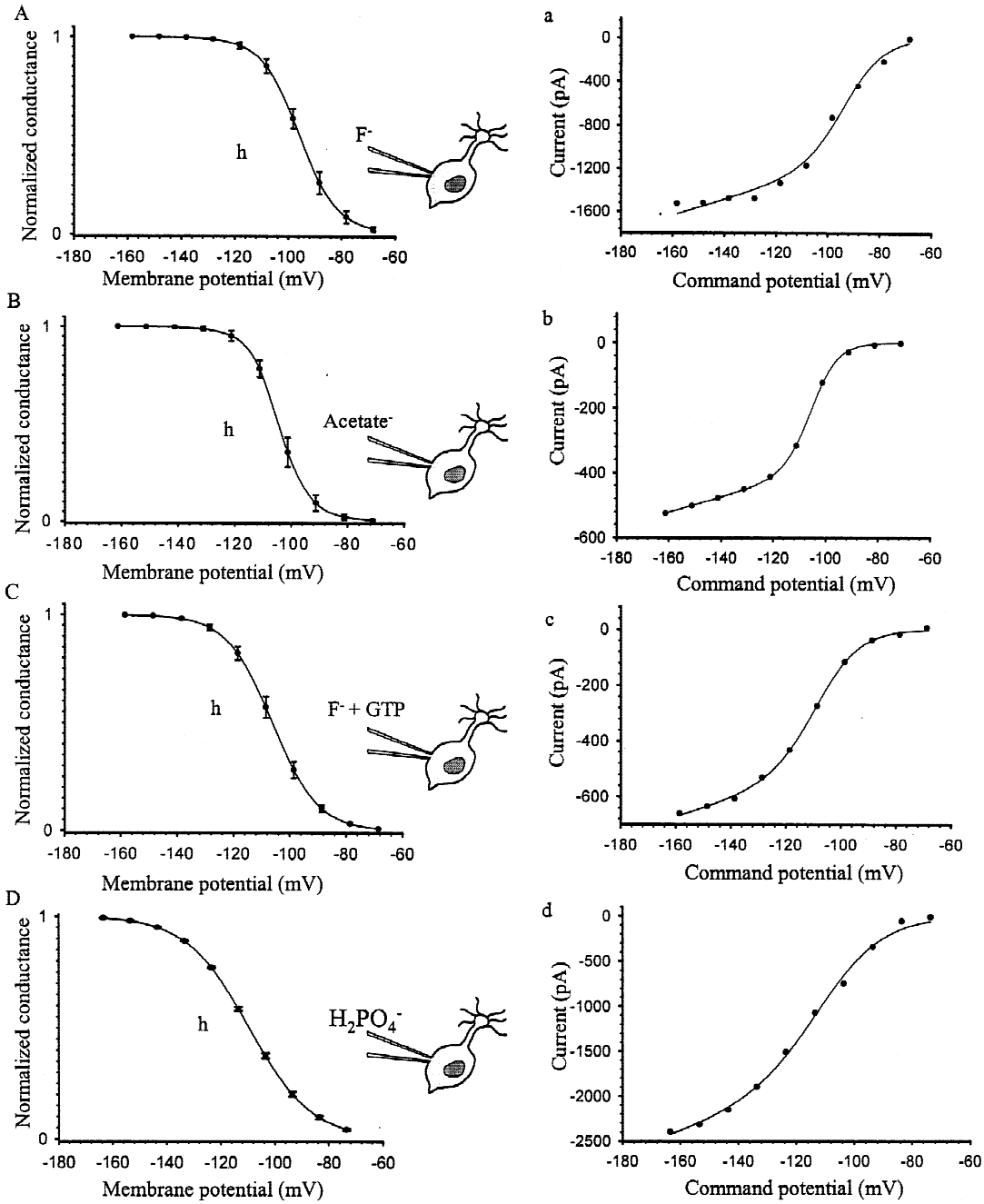

Fig. 2. Normalized conductance-voltage curves (h curves) of voltage-activated steady-state inactivation in different pipette solutions in rat ORNs (*A:* fluoride$^-$; *B:* acetate$^-$; *C:* F^- + GTP; *D:* $H_2PO_4^-$; $n \geqslant 3$). Graphs *a–d* are the representative results in a single olfactory neuron when the obtained currents were fitted with the Boltzman equation (Eq. 1; *a:* fluoride$^-$; *b:* acetate$^-$; *c:* F^- + GTP; *d:* $H_2PO_4^-$). Sodium currents were elicited with an inactivation protocol (Fig. 1) in rat ORNs at 21°C.

$$I_p/I_{p,max} = 1 - \exp(-t/\tau) \quad (5)$$

$$I_p/I_{p,max} = f_1[1 - \exp(-t/\tau_1)] + (1 - f_1)\,[1 - \exp(-t/\tau_2)] \quad (6)$$

where $I_p/I_{p,max}$ is the experimental value of the ratio at time delay t, f_1 is the fractional contribution of the first exponential to the total fit, t is the time delay between conditioning pulse and test pulse and τ is the recovery time constant for the single exponential, and τ_1 and τ_2 are, respectively, the fast and slow recovery time constants for the double exponential fit. A comparison of single and double exponential fits of representative cur-

Table 2. Electrophysiological characteristics of acutely isolated rat ORNs in different pipette solutions

Solutions	Activation			Inactivation		
	$V_{1/2}$ (mV)	G_{max} (pS)	k	$V_{1/2}$ (mV)	G_{max} (pS)	k
F^- ($n = 4$)	−52.3 ± 2.0	18.0 ± 6.7	5.6 ± 0.6	−106.8 ± 1.5	7.3 ± 1.5	7.2 ± 0.8
F^-/Cl^- ($n = 4$)	−60.9 ± 7.5	18.0 ± 7.0	6.4 ± 1.4	−101.1 ± 2.4	11.1 ± 2.1	9.7 ± 1.2
$H_2PO_4^-$ ($n = 4$)	−51.3 ± 0.7	17.7 ± 2.8	4.7 ± 0.2	−107.0 ± 2.5	11.8 ± 1.2	10.8 ± 0.8
$Acetate^-$ ($n = 3$)	−50.6 ± 6.1	3.8 ± 1.1	4.2 ± 1.1	−104.2 ± 1.2	2.3 ± 1.7	5.6 ± 1.2
$Acetate^-/Cl^-$ ($n = 3$)	−56.5 ± 2.4	4.7 ± 1.2	5.4 ± 1.1	−105.5 ± 1.4	2.6 ± 1.7	5.0 ± 1.6
F^- + GTP ($n = 3$)	−49.2 ± 3.2	6.4 ± 0.6	5.4 ± 0.5	−106.8 ± 1.1	3.1 ± 1.1	8.1 ± 1.1
F^- + ATP + glutathione ($n = 7$)	−51.9 ± 0.8	16.7 ± 1.1	4.7 ± 0.2	−100.3 ± 1.3	14.5 ± 1.6	7.2 ± 0.3

rent amplitudes recorded in F^- solutions in one ORN is shown in Fig. 3*A*. All the data were better fitted by the double exponential expression. The recovery became progressively more rapid as the holding potentials became more hyperpolarized. The time required for currents to completely recover from inactivation was very slow, up to 1 sec at a voltage of −100 mV. Similar results were obtained in the $acetate^-$ solutions.

Figure 3*B* compares the change of current amplitudes at the holding potential of −100 and −120 mV recorded in the F^- and $acetate^-$ solutions. When the holding potential was more negative, the recovery from inactivation became faster in both recording solutions. The time course of recovery from inactivation seemed slower in the F^- solutions than it did in the $acetate^-$ solutions at the same holding potential. This indicates that when the membrane potential returns to a resting level after firing an action potential, a longer time must pass before a sufficient number of sodium channels would be available to fire another impulse in the presence of F^-. It is possible that these cells fire single spikes in vivo because in a cell-attached patch, the opening of a single channel in a patch could cause the rapid and complete depolarization of the olfactory cell and generate an action potential current waveform (Lynch & Barry, 1989).

Voltage-dependence of Activation of Sodium Currents

Ability to Obtain Good Whole-Cell Recordings. Whole-cell voltage-clamp recordings of sodium currents in acutely isolated rat ORNs were elicited by depolarization from −95 mV to +65 mV in 10 mV steps from a holding potential of −100 mV (*see* Fig. 4). Reasonable inward sodium currents, with good gigaohm seals (1 to 20 GΩ), were obtained with very good space clamp control. This was judged by visual inspection of the current-voltage curve (Fig. 5). To endeavor to make the pipette solutions more physiologically favorable, some other agents such as GTP, ATP and glutathione have been included in some solutions. Representative families of sodium currents recorded with F^-, $acetate^-$, $phosphate^-$ and ATP together with glutathione pipette solutions are illustrated in Fig. 4.

The nature of the currents was identified by completely replacing sodium with choline in the standard external solution, or adding a nanomolar concentration of tetrodotoxin to the bath solution, which resulted in the reversible reduction of the inward currents (*data not shown*). All of these results confirmed that the rapid, voltage-activated inward current was carried by sodium ions through voltage-dependent sodium channels (*see also* Rajendra et al., 1992; Chabbert et al., 1997).

It has proven to be very difficult to get reasonable whole-cell recordings in these small rat ORNs. Previously it had only seemed possible to obtain satisfactory whole-cell configurations with these ORNs with a substantial concentration of F^- ions in the pipette solutions (e.g., Lynch & Barry, 1991; Rajendra et al., 1992). With some perseverance, recently, we have managed to get results with other anions apart from F^-, but whole-cell recordings with good space clamp were only able to be obtained in F^-, $H_2PO_4^-$, $acetate^-/Cl^-$, $acetate^-$ and $acetate^-/Cl^-$ solutions.

From all the test pipette solutions, it was found that F^- was the most successful in establishing the formation of whole-cell configurations whereas other anions such as $H_2PO_4^-$, $acetate^-$, $glutamate^-$ or $aspartate^-$ were found to be useable, but generally less suitable, for accurate recordings in these small ORNs. The probability of

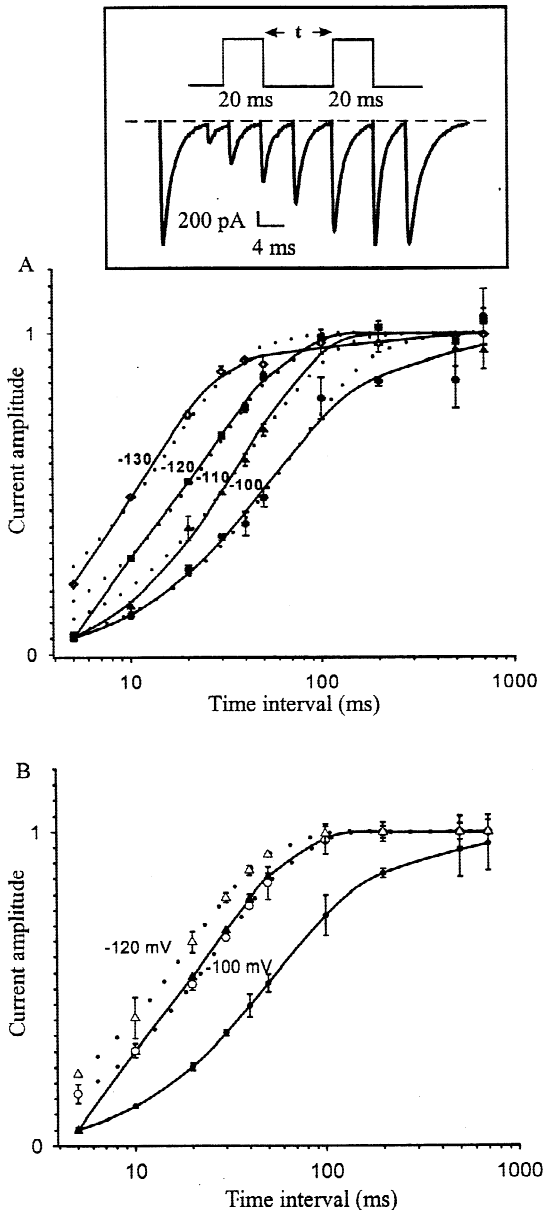

Fig. 3. (*A*) Recovery of sodium currents from steady-state inactivation measured in the fluoride solutions in one rat ORN. Different holding potentials are expressed with various symbols: −100 (circles), −110 (triangles), −120 (squares) and −130 mV (diamonds). Data were fitted with a single exponential expression (Dotted lines) and a double exponential expression (Solid lines). (*B*) Sodium currents evoked in the fluoride solutions (solid lines with filled circles and triangles) recovered more slowly than those in the acetate solutions (dotted lines with open circles and triangles) at the same holding potential.

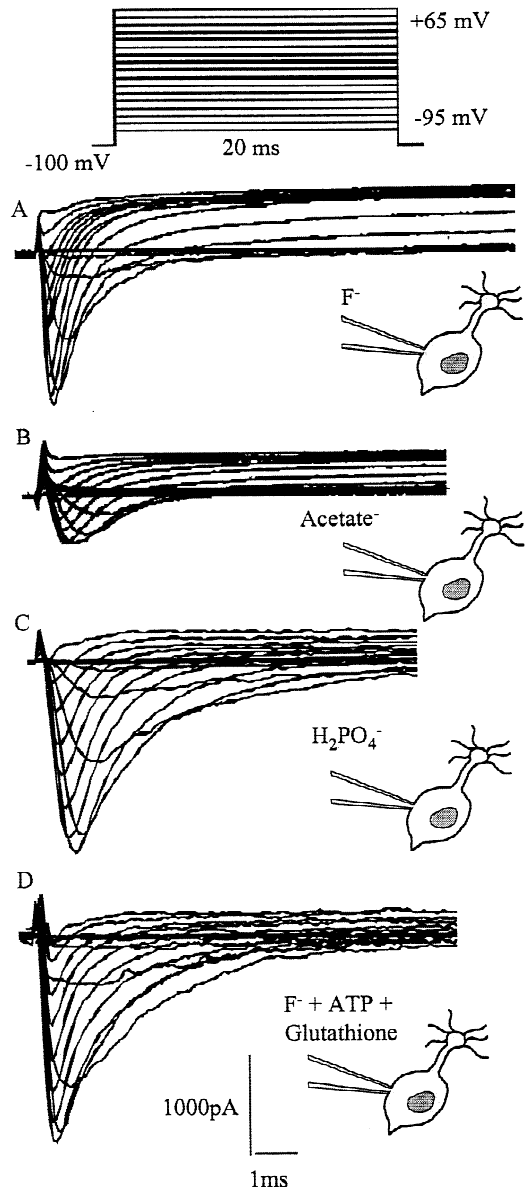

Fig. 4. Representative families of whole-cell recordings of macroscopic sodium currents measured respectively in F^- (*A*), acetate$^-$ (*B*), $H_2PO_4^-$ (*C*) and F^- + ATP + glutathione (*D*) pipette solutions in separate rat ORNs. The inset is the protocol for eliciting fast activating and inactivating sodium currents by depolarizing the membrane potential from a holding potential of −100 mV to a final step of +65 mV in 10 mV increments. Calibration bars refer to all sets of traces. Extracellular and intracellular sodium concentrations were 140 and 10 mM, respectively. The current traces were filtered at 5 kHz.

forming good seals and obtaining reasonable whole-cell recordings with these solutions is summarized in Table 3. It should be noted that the success rate of obtaining satisfactory whole-cell configurations with these ORNs was extraordinarily low when the pipette solutions did not contain a substantial concentration of F^-. Even when a response was eventually obtained with other pipette solutions, the magnitude of the currents tended to be very much smaller and space clamp problems to be greater.

Table 3. The estimation of the probability and the percentage (shown in brackets) of seal formation and current responses measured in different pipette solutions. The liquid junction potentials ($V_{L.J.}$; bath solution-pipette) were calculated for 21°C.

Solutions	$V_{L.J.}$ (mV)	No. of test cells	No. of cells with good seals	No. of cells with Na^+ current recordings	No. of cells with good space clamp recordings
F^-	8.3	100	31 (31%)	16 (16%)	11 (11%)
F^-/Cl^-	6.4	50	16 (32%)	12 (24%)	4 (8%)
$H_2PO_4^-$	13.5	75	28 (37%)	19 (25%)	5 (7%)
$Acetate^-$	11.2	150	20 (14%)	11 (7%)	4 (3%)
$Acetate^-/Cl^-$	7.6	200	40 (20%)	10 (5%)	3 (2%)
$Glutamate^-$	16.8	50	5 (10%)	3 (6%)	—
Cl^-	4.1	50	9 (18%)	2 (4%)	—
$Methanesulfonate^-$	10.5	50	3 (6%)	—	—

Effects of Anions on Peak Sodium Currents. From the data obtained from successful whole-cell recordings, the mean peak magnitude of the inward sodium currents recorded in the presence of F^-, was −1742 ± 680 pA. This was comparable to the values in the presence of $H_2PO_4^-$ (−1487 ± 620) and both were very significantly larger than that in the $acetate^-$ solutions (−453 ± 260; $P < 0.001$; Table 4). The current densities calculated from the peak sodium current and whole-cell capacitance were −471 ± 150 and −129 ± 60 pA/pF in the F^- and $acetate^-$ solutions, respectively, implying a much higher current density in F^- than in $acetate^-$. A similar conclusion was obtained when comparing the results obtained in the F^-/Cl^- and $acetate^-/Cl^-$ solutions (Table 4).

Since the whole-cell capacitance measured from all the recording solutions was reasonably uniform without significant cell-to-cell differences, the size of the dissociated olfactory cells must have been relatively constant. Hence, the variation in maximal inward current recorded in the different pipette solutions must either result from different numbers of functional sodium channels in each cell or from differing amounts of current traversing the same number of channels. In the latter case, this must, in turn, have been due to either differences in single channel conductance or channel open probability.

As already stated, F^- has been shown to be the most "favorable anion" for increasing cell excitability in squid axons. It can restore the sodium conductance toward normal values and remove the inhibitory effect of other "unfavorable anions" (Adelman et al., 1966). Although sodium channels may have been affected by the process of dissociation, the alteration in room temperature or poor clamping conditions, it seems that for our extremely small mammalian ORNs, F^- and $H_2PO_4^-$ are the most efficacious at enabling sodium channels to function normally. It also seems that Cl^-, $acetate^-$ and other mentioned anions are "unfavorable anions" (Adelman et al., 1966), which might in some way diminish the functionality of sodium channels.

Table 4. Electrophysiological characteristics of voltage-dependent sodium currents elicited by the activation protocol in rat ORNs.

Solution	Maximum peak current (pA)	I_{mean} (pA)	Current density (pA/pF)
F^- ($n = 4$)	−3080	−1742 ± 680	−471 ± 150
$H_2PO_4^-$ ($n = 4$)	−2545	−1487 ± 621	−425 ± 117
$Acetate^-$ ($n = 3$)	−440	−453 ± 260	−129 ± 60
F^-/Cl^- ($n = 4$)	−2735	−1518 ± 556	−422 ± 150
$Acetate^-/Cl^-$ ($n = 3$)	−680	−496 ± 130	−191 ± 40

Activation of Sodium Currents. Figure 5 shows the current-voltage curves in F^-, $acetate^-$, $H_2PO_4^-$, and F^- + ATP + glutathione pipette solutions. The activation of sodium currents recorded in all the pipette solutions was typically voltage-dependent with a threshold of activation of approximately −80 mV, and with the voltage for the peak inward currents of around −40 mV. As indicated in Materials and Methods, although the extrapolated linear portions of the sample curves, shown in Fig. 5, did indicate values of reversal potential that corresponded reasonably well with the theoretical predicted value for E_{Na}, because of difficulties in extrapolating accurate values in all experiments, all the activation and inactivation data were curve-fitted to pass through the expected theoretical value of E_{Na} (66.6 mV).

The maximum sodium conductances, recorded in F^-, F^-/Cl^- or $H_2PO_4^-$ solutions, were considerably larger than those in either $acetate^-$ or $acetate^-/Cl^-$ solutions (Table 2). However, there was no significant difference in the slope constants and in the voltage for the half-maximal activation between the F^-, or F^-/Cl^-, solutions and the $acetate^-$, or $acetate^-/Cl^-$, solutions. Since the slope constant should reflect the voltage sensitivity of the channels, it would seem that for the ions tested, the nature of the ion is not affecting the voltage sensitivity of sodium channel activation.

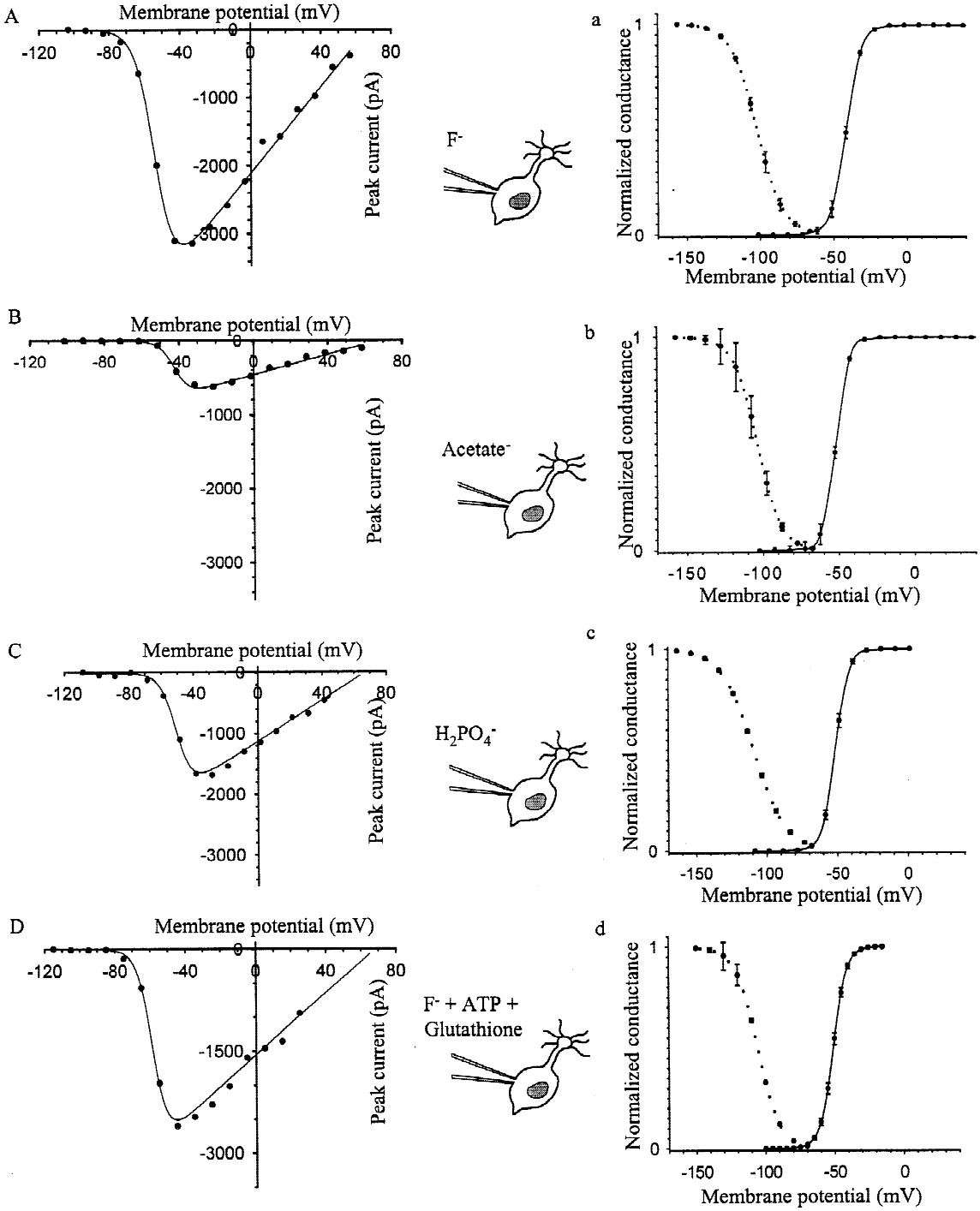

Fig. 5. Representative current-voltage relationship (*I-V* curves) of sodium currents evoked by the activation protocol obtained in F^- (*A*), $acetate^-$ (*B*), $H_2PO_4^-$ (*C*) and F^- + ATP + glutathione (*D*) solutions in individual rat ORNs. *a–d* are whole-cell normalized conductance-voltage curves of sodium currents recorded in different pipette solutions (*a:* F^-, *b:* $acetate^-$; *c:* $H_2PO_4^-$; *d:* F^- + ATP + glutathione) in rat ORNs ($n \geqslant 3$). The data points were obtained by dividing the fitted conductance by the maximum sodium conductance. The small window currents occurred at approximately −75 mV.

At room temperature, the kinetics of activation of sodium currents was extremely rapid. The averaged time-to-peak of sodium currents, less than 1 msec in all the recording solutions, could be well fitted with a single exponential expression, with, for example, time constants of 0.50 msec for F^- and 0.85 msec for $acetate^-$. The data indicate a faster activation of sodium channels in F^- solutions than in $acetate^-$ solutions. Similar results

were obtained for times-to-peak for the F^-/Cl^- and the $acetate^-/Cl^-$ solutions, with values of 0.45 and 0.95 msec, respectively.

The activation phases of the sodium currents measured in the F^- and $acetate^-$ solutions were well fitted with a single exponential. When the holding potential was more negative than −30 mV, the time constants for activation were slower in $acetate^-$ than in F^- solutions (*results not shown*). The inactivating phase of sodium currents is a spontaneous process, unaffected by amplitude of the peak sodium current (Hodgkin & Huxley, 1952). The time courses of inactivation were well fitted with double exponential functions, for steps to −55 mV or more depolarized values, different from the kinetics classically described in the squid axon (Hodgkin & Huxley, 1952). Similar results have been found in frog ORNs (Pun et al., 1994) and human hippocampal dentate granule cells (Reckziegel et al., 1998). It seems that the inactivation of sodium currents in rat ORNs is a second order process. The time constants for fast inactivation in F^- solutions were significantly larger than those in the $acetate^-$ solutions, indicating that inactivation was slower in F^- than in $acetate^-$, at least, when the potential was more negative than −30 mV. According to the Hodgkin and Huxley model, the amplitude of the peak sodium currents and the maximal sodium conductance are determined by the process of both activation and inactivation. Currents activated more quickly and inactivated more slowly would be expected to be larger and this may explain the larger mean peak current amplitude and larger maximal sodium conductance in the F^- and F^-/Cl^- solutions.

Effects of Nucleotides and Glutathione on Macroscopic Sodium Currents

To determine whether nucleotides affect the properties of sodium currents, we applied GTP (100 μM) or ATP (5 mM), together with F^- in the pipette solution. Figure 4*D* shows the representative sodium currents elicited by the activation protocol in F^- together with ATP plus glutathione solutions. The addition of nucleotides (*not shown*), or nucleotides plus glutathione (Fig. 5*D*), to F^- in the recording pipette did not significantly change the *I-V* curves and the normalized conductance-voltage curves measured in both ATP and glutathione pipette solutions (Fig. 5*d*) were similar to those obtained in $acetate^-$ (Fig. 5*b*), which were slightly shifted towards a more negative value.

The inactivation of sodium currents was also studied using pipette solutions which together with F^- contained nucleotides (*not shown*), or nucleotides and glutathione (Fig. 1*D*). The $V_{1/2}$ measured was not significantly different between their values and those obtained in the F^- solutions. The mean values were −106.8 ± 1.1 mV in GTP + F^- ($n = 3$) and −108.9 ± 2.2 in ATP + F^- pipette solutions ($n = 3$; not included in Table 2) and were not significantly different from each other ($P > 0.5$). Therefore, the effects of nucleotides on the macroscopic sodium currents were not very obvious in the extremely small rat ORNs, although a guanine nucleotide-mediated and a G-protein-mediated event has been suggested to be responsible for regulating the steady-state inactivation of sodium channels in frog ORNs (Pun et al., 1994). When glutathione was added to the F^- pipette solution, the value of $V_{1/2}$ became slightly more positive than the results recorded in the other test solutions ($0.001 < P < 0.05$). However, it was still more negative than about −100 mV and there was no significant difference in the slope factor ($P > 0.05$). Hence, the omission of nucleotides and glutathione do not seem to be the reason for the very negative inactivation midpoint (about −100 mV) observed in these rat ORNs.

Membrane Excitability

The membrane excitability of rat ORNs was examined using current clamp techniques after fast activating and inactivating inward currents had been obtained under voltage-clamp recordings. Hyperpolarizing current pulses failed to elicit any spikes from any holding potentials. Overshooting action potentials usually could be recorded following short (2 msec) depolarizing current pulses (Fig. 6).

The resting membrane potential of acutely isolated ORNs, was expected to be approximately −83 mV, similar to the potassium equilibrium potential. Attempts to measure this value in the whole-cell configuration, seemed to indicate a value close to zero millivolts ($n = 7$) although the concentrations of internal potassium and external sodium were similar to those in a natural physiological state. However, this was not unexpected, given that the magnitudes of the seal resistance with these large pipettes were quite low (about 1–3 GΩ; *see below*) and that the values of the cell input resistance were high (e.g., 26–40 GΩ; Lynch & Barry, 1989, 1991 in CsF solutions), as expected, with only a few leakage potassium channels open at rest in Cs^+/TEA^+ solutions. It can readily be shown that the zero current pipette potential (V_p) will be given by:

$$V_p = (R_s V_c + R_c V_s)/(R_s + R_c) \quad (7)$$

where R_s and R_c represent the resistances of the seal and cell respectively and V_s and V_c the potentials across the seal and the cell potential. For example, if $V_s \approx 0$ mV, then

$$V_p = V_c R_s/(R_s + R_c).$$

For example, if $V_c \approx -90$ mV, $R_s \approx 2$ GΩ and $R_c \approx 30$

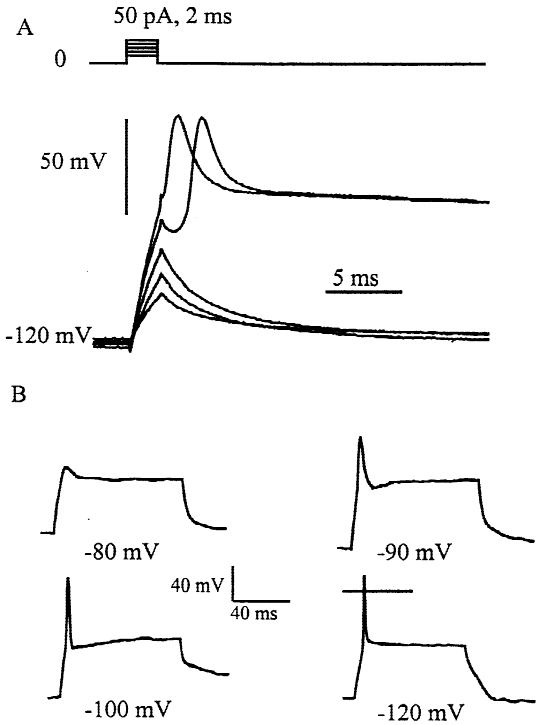

Fig. 6. Excitability of acutely isolated rat ORNs. (*A*) Example of action potential elicited in one cell by depolarizing current steps in the current clamp mode (2 msec, 50 pA). The cell was maintained at about −120 mV by a holding current of −10 pA. (*B*) The same neuron was clamped at different potentials, from −80 to −120 mV. Action potentials could only be recorded when the holding potential was more negative than −90 mV, indicating that the resting membrane potential was more negative than about −90 mV.

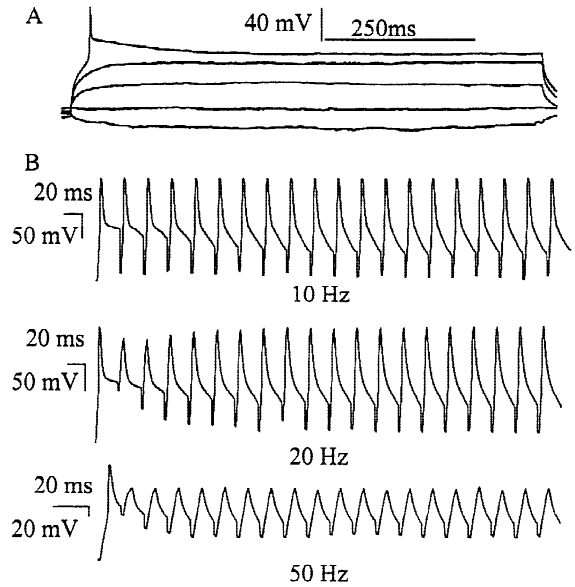

Fig. 7. Properties of action potentials in acutely isolated ORNs. (*A*) Only a single spike was elicited by the 500-msec duration depolarizing 50-pA current pulses from the resting potential of −100 mV. No action potential was activated by hyperpolarizing current pulses. (*B*) Repetitive action potentials obtained by trains of brief 30-pA current pulses at a frequency of 10, 20, 50 Hz. It is still difficult to tell what physiological advantage such a negative voltage dependence of the steady-state inactivation of sodium currents may have. This value suggests that the resting potential is also very negative, perhaps more negative than about −90 mV in acutely isolated rat ORNs.

GΩ, then $V_p \approx -3$ mV. The input resistance near the resting membrane potential was measured from the current generated to a 10-mV pulse under voltage-clamp conditions. The values measured in both high-potassium solution and Cs^+/TEA^+ solution were about 1–3 GΩ ($n = 7$), and probably primarily reflect the relatively low ratio of seal resistance R_s to cell resistance (R_c). The capacitance of the cells in all the test solutions, which was measured by integrating the area under the current capacitive transient to the 10-mV pulse, was between 3 and 5 pF. An ORN of 5–10 μm diameter with a 2 μm diameter 30 μm long dendrite, and six cilia of diameter 0.2 μm and length 30 μm, should have an estimated surface area of about 600 μm^2 (6×10^{-6} cm^2). Based on this membrane surface area, the specific capacitance should be between 0.5 and 0.83 μF cm^{-2}. From estimates of specific membrane resistance of 10^5 Ωcm^2 (Lynch & Barry, 1989), R_c should be about 16 GΩ. However, assuming a typical membrane capacitance of 1 μF cm^{-2}, the area would actually be between 3×10^{-6} cm^2 and 5×10^{-6} cm^2 and hence R_c should be between 20–33 GΩ.

Since it is very difficult to record the resting membrane directly in these small neurons due to their high input resistance, different holding potentials were applied to estimate the voltage limit for the generation of action potentials (Fig. 6*B*). When the holding potential was increased above −90 mV, no spike could be evoked, in agreement with our prediction that the resting membrane potential should be about −90 mV (or maybe even more negative).

Only a single action potential could be generated with prolonged suprathreshold depolarizing current injections (e.g, 500 msec, 30 pA; $n = 7$; Fig. 7*A*), as had been previously reported in cultured rat ORNs (Trombley & Westbrook, 1991), although repetitive firing has been reported in the ORNs of other animals (Dubin & Dionne, 1994). Repetitive action potentials could only be elicited in response to a series of brief depolarizing current pulses (Fig. 7*B*), the maximum firing rate being approximately 20 Hz.

Discussion

The present study has investigated the effects of various pipette solutions on the unusually negative midpoint of inactivation of Na^+ channels in dissociated rat ORNs and the functional significance of such negative inactivation.

We have clearly shown that none of the manipulations of pipette solution tested resulted in any significant change in this property. We did find, however, that F^- supported good stable whole-cell recordings and large sodium currents. The sequence of anions that were able to support good whole-cell recordings were: $F^- > F^-/Cl^- > H_2PO_4^- > acetate^- > acetate^-/Cl^- > glutamate^- > Cl^- > methanesulphonate^-$, while the sequence that elicited the largest peak sodium currents was:

$$F^- > F^-/Cl^- > H_2PO_4^- > acetate^-/Cl^- > acetate^-$$

Possible Effects of Anions on Membrane Excitability and the Properties of Sodium Channels

A number of previous studies in isolated neurons and axons have demonstrated that different anions used in the intracellular perfusate can greatly affect voltage-dependent inward currents and leakage currents. In the perfused squid axon, F^- has previously been found to be the most favorable anion for maintaining axonal excitability and for reducing leakage currents (Adelman et al., 1966). It could restore the sodium conductance toward normal values and remove the inhibitive effect of other "unfavorable" anions. These early studies also suggested that the inward currents in response to a constant voltage pulse were also larger in the presence of F^-. The reason why larger inward currents were recorded in the F^- solutions is still obscure. Similar results had also been reported by Kostyuk, Krishtal and Pidoplichko (1975), when they perfused F^- internally in a single neuron isolated from molluscan ganglia. They supposed that F^- suppresses the calcium conductance by destroying an associated asymmetry current and calcium inward currents could be made to transport sodium by the introduction of F^-.

We have confirmed a preferential role for the presence of F^- ions in the pipette solution in being able to obtain satisfactory whole-cell measurements, and especially reliable recordings of well-clamped voltage-activated Na^+ currents, in olfactory receptor neurons. In particular, we have shown that the voltage dependence of activation and inactivation of the channels was essentially unaffected by the nature of intracellular anions. In contrast, over most of the voltage range (between −90 and −30 mV), the time course of activation was significantly more rapid when F^-, rather than other anions, was present in the pipette solution. In addition, in the presence of F^-, inactivation was also slower at voltage steps ranging from −90 to −30 mV. If the peak current reflects a balance between the extent of activation and inactivation (i.e., they overlap a bit at the peak) then this could, at least partially, explain the increase in amplitude of the peak current.

Interestingly, in isolated neurons from *Helix aspersa*, it was found that F^- was the least favorable anion for maintaining excitability and eliciting large sodium currents (Lee, Akaike & Brown, 1977). In addition, Vargas et al. (1999) reported in cultured rat ORNs that the internal potassium F^- can inhibit the activity of cAMP-dependent protein kinase and lead to a 16-mV hyperpolarizing shift in $V_{1/2}$ of hyperpolarization-activated channels. Their measured value of $V_{1/2}$ obtained in the absence of F^- was still about 30 mV more negative than that obtained in identified cultured rat ORNs (Trombley & Westbrook, 1991). In rat brain cells, phosphorylation of voltage-dependent sodium channels by cAMP-dependent protein kinase did not cause any significant change in the kinetics or voltage dependence of activation or inactivation of the channel but only reduced peak sodium currents (Li et al., 1992). Therefore, there may not be any definite relationship between the shift in $V_{1/2}$ and the phosphorylation of sodium channels. It is possible that the function or even the molecular identity of the sodium channels we recorded from is different, as a result of different recording conditions or the particular procedure required for the isolation of these ORNs.

The reasons for specific effects of anions like F^- are not really known, although there are certain possibilities. Internal perfusion of different anions in giant axons of squid indicated that an unfavorable anion can affect the resting potential, action-potential magnitude and excitability by affecting the macromolecular structure of the membrane macromolecular conformations and changing membrane permeability (Tasaki et al., 1965). It should also be appreciated that in simulating in vivo conditions, the replacing anions have to mimic the effect of the large endogenous organic anions. Possible effects of different anions on sodium channels may include screening effects, ion binding effects and salting-out effects. When ions get close to the membrane surface, they tend to gather around the charged groups of sodium channels and could shift voltage dependence. These effects are called screening effects, which are determined by the different concentration and valence of the ions in the medium (Adelman et al., 1966). In this study, all the test anions are monovalent ions at the same concentration, thus ruling out simple screening effects as a mechanism for explaining the observed electrophysiological differences between F^- and $acetate^-$.

Ion binding effects in sodium channels could arise from the specific binding of different anions to various positively charged groups on the glycoprotein. These effects may influence permeability of sodium channels by reducing the effective surface charge concentration and surface potentials. The normal channel excitability could also be affected by disrupting the intermolecular and intramolecular linkages. It has been shown that F^- ions have the least affinity to positively charged groups

of various proteins and the least ion binding effects (citations in Adelman et al., 1966). Therefore, F^- could be the most "favorable anion" for keeping the normal excitability of sodium channels in rat ORNs. However, these different binding affinities can have their effects at low ion concentrations. In this study, the replacement of a substantial proportion of the pipette anions had to be F^-, $H_2PO_4^-$ or acetate$^-$ in order to produce a significant effect, suggesting that ion-binding effects did not play a major role in affecting sodium conductance and the voltage-sensitivity of the channels.

The final possibility, salting-out effects, which have nothing to do with the charged groups in sodium channels, are caused by an alteration of the water structure. Different anions can react with water molecules and form a layer around each anion. They produce hydrophobic bonds within channels (Hamaguchi & Geiduschek, 1962), which are important in stabilizing the structure of sodium channels. F^- ions are considered to have the greatest salting-out effects (Von Hippel & Wong, 1964). It is possible that these effects, maybe together with some ion binding effects, might have made F^- the most "favorable anion" for keeping sodium channels in a good electrophysiological state and eliminating the inhibitory effects of other "unfavorable anions." This may be the mechanism for the larger maximal sodium conductance and peak amplitude of sodium currents in the presence of F^-. A better understanding of these ion interactions awaits further investigation, probably in a more isolated and better-defined system.

It is a significant finding that one of the least favored anions was Cl^-, especially in light of the fact that this anion is commonly used in whole-cell recordings. These results suggest that some caution should be paid to the nature of the solution bathing the internal membrane surface and suggest, at least for investigation of whole-cell sodium currents, that F^- should be included in the pipette solution. Even half F^- and half Cl^- were sufficient to support good recordings.

Effects of Trypsin on Activation and Inactivation of Sodium Currents

Trypsin is an enzyme used in these experiments for the dissociation of rat ORNs. Although it has been pointed out that long exposure to protease could decrease the channel density and reduce the maximal conductance in rat ventricular myocytes (Saint, 1998), it is still not clear whether the application of external trypsin can affect the physiological function of sodium channels and alter their structures. It has been reported that the pretreatment with trypsin did not affect the anionic sequence in isolated neurons located in the subesophageal ganglion of *Helix aspersa* (Lee et al., 1977). They found that trypsin destroyed the TTX-sensitivity of neuronal membrances without affecting the magnitude and time course of the inward currents. This may explain why sodium channels were less sensitive to TTX in acutely isolated rat ORNs than in cultured ones (Rajendra et al., 1992; Trombley & Westbrook, 1991). We tried to get recordings of macroscopic sodium currents without using trypsin for dissociation of olfactory receptor neurons but failed to achieve any good viable results.

Possible Effects of Nucleotides and Glutathione on Sodium Currents

Under odorant stimulation, various hormones and neurotransmitters in vivo regulate membrane electrical properties and behavior of ORNs by affecting the threshold for action potential generation and the frequency of action potential firing. This is achieved by regulating sodium channel number and their function and open probability via adenylate cyclase and PKA (Kalman et al., 1990; D'Arcangelo et al., 1993).

The G-protein related cAMP second messenger pathway is a well-established mechanism for regulation of electrical excitability in mammalian cells (Li et al., 1992). In unstimulated excitable cells, most of sodium channels are substantially phosphorylated (Rossie, Gordon & Catterall, 1987). The increase of cytosolic cAMP can enhance the level of phosphorylation of sodium channels, resulting in the decrease of peak sodium current and slowed inactivation (Numann, Catterall & Scheuer, 1991). One example of modulation of the gating of voltage-dependent sodium channels involves the β-subunit which is supported by the S5-S6 loops of DI and DIV of the α-subunit (Schreibmayer, Wallner & Lotan, 1994; Makita, Benett & George, 1996). The residues located in the cytoplasmic linker connecting domains I and II can be phosphorylated by PKA (Murphy & Catterall, 1993).

The results obtained in frog ORNs showed that in the absence of nucleotides, especially GTP, in the recording pipette, there was a 10 to 20-mV negative shift in the voltage for half-inactivation due to the indirect modulation of G proteins and phosphorylation processes (Pun et al., 1994). KF has also been shown to inhibit the activity of PKA, so it is possible that the presence of internal F^- may decrease the phosphorylation of voltage-sensitive ion channels and cause the negative shift of a very negative $V_{1/2}$ of inactivation (Vargas et al., 1999). However, in acutely dissociated rat ORNs, the effect of internal F^- was not obvious and the addition of GTP or ATP alone did not significantly alter the activation and inactivation of sodium currents. It is possible that the application of both cAMP and ATP may cause the positive shift of voltage-dependence of sodium currents.

Redox modulation has been shown to contribute to the gating of rat axonal sodium channels (Strupp et al.,

1992). Glutathione (GSH) is a tripeptide composed of glutamine, cystine and glycine (γ-L-glutamyl-L-cysteinyl-glycine) and best known as a free radical scavenger. GSH itself can induce sodium currents through its own receptor-mediated channels in the neocortex (Shaw, Pasqualotto & Curry, 1996). The cytoplasmic application of the reducing agent GSH affected the inactivation kinetics of sodium currents in rat motor and sensory nerve fibres (Mitrovic, Quasthoff & Grafe, 1993). In a recent report on short-term cultured adult rat ORNs, GSH was applied in the recording pipette and the voltage-dependence of inactivation of sodium currents was found to be more positive than in the present study (Vargas & Lucero, 1999). Inclusion of 1 mM glutathione in our pipette solution, however, had no effect on the activation, inactivation and the peak sodium currents of our ORNs.

Perforated-Patch Recording

An alternative approach to whole-cell recording that would leave the internal composition of the cell reasonably intact, especially as far as large organic components are concerned, would be to use the perforated-patch technique (e.g., Marty & Neher, 1995). However, we did not attempt such measurements because we considered that the problems in precisely determining the cell potential (because of difficulties in accurately determining the contribution of the Donnan potential across the patch) and a slowing of the voltage clamp (because of an enhanced series resistance component across the patch) would have made the interpretation of the results too uncertain. Nevertheless, if these difficulties could be overcome, this would be a good approach to investigate whether the presence of any other endogenous cell constituents do affect the negative voltage dependence of these Na^+ channels.

Electrophysiological Implications of Such Negative Voltage-Dependence of Sodium Currents

The results that we have presented suggest that the very negative midpoint of inactivation is a real property of sodium channels in this preparation. This value seems to make sodium channels unavailable for the generation of action potentials. It has been reported in ORNs of catfish that mixtures of amino acids can shift activation and inactivation of sodium currents without generation of detectable currents (Ivanova & Caprio, 1992). Modulation of sodium channels can lead to an alteration in the activities of these channels and subsequently, a change in the membrane electrical properties of the cell (Zhainazarov & Ache, 1997). Therefore, the properties of voltage-dependent sodium channels in vivo may be different from what we have obtained under the present experimental situation. Transient potassium currents can also affect cell excitability and the rate of repetitive firing. In rat ORNs, transient K^+ currents have a significantly higher threshold for activation than the sodium currents, indicating their critical role in spike repolarization (Lynch & Barry, 1991). These currents recover from inactivation very slowly and the potential for half-inactivation was only −45 mV. This difference may relate to a unique population of sodium channels in these cells.

Expression studies have suggested that α subunits of sodium channels alone can act as functional channels and accurately express normal channel gating in mammalian cells (Scheuer et al., 1990). They contain four homologous internal repeats, each of which has six putative transmembrane segments (Stuhmer et al., 1989; their Fig. 8). The positive charges in segment S4 are postulated to reside within the membrane and are important in the voltage-sensing mechanism for activation of the channel (Noda et al., 1986; Stuhmer et al., 1989). Studies of cytoplasmic application of antibodies suggested that the repeats III and IV were involved in the process of inactivation (Vassilev, Scheuer & Catterall, 1988; reviewed by Jan & Jan, 1989).

Molecular biological studies have revealed that a large diversity of α subunits, and to a lesser extent, β subunits are present in the mammalian CNS (Catterall, 1995). Three distinct types of α subunit, types I, II and III, were cloned in the rat brain (Noda et al., 1986; Auld et al., 1988), which were also widely distributed in other different tissues (Kayano et al., 1988). Recombinant cardiac (hH1) and skeletal (hSkM1) muscle sodium channel α subunits expressed in tsA-201 cells indicated that the midpoint of the steady-state inactivation curve was approximately 25 mV more negative for hH1 compared with hSkM1 (Wang, George & Bennett, 1996). Although the alteration in structure of α subunits did not always result in the change of biophysical properteis of sodium channels (Barchi, 1984), the difference in the properties of activation and inactivation of sodium currents in various animals and tissues is definitely due to the change in their subtypes. Therefore, one possible mechanism for the difference in voltage-dependence of sodium currents in acutely isolated and cultured rat ORNs may be a variation in the particular sodium channel subtype.

This present study supports a very negative $V_{1/2}$ of inactivation of sodium currents and suggests that it has functional implications for the ORNs, particularly in response to a prolonged depolarizing current injection. In contrast to the responses observed for cultured ORNs, acutely isolated rat ORNs could only fire single action potentials in response to a long duration of depolarizing current injection. Even when repetitive short current pulses were applied, a condition likely to be less physi-

ologically relevant compared to a more prolonged current injection, as presumably occurs in response to odorant binding, these neurons failed to fire full spikes at frequencies as low as 20 Hz, when held at membrane potentials more negative than about −90 mV. This behavior differs from other cells, such as mature ORNs of salamander and *Xenopus laevis* (Masukawa et al., 1985; Iida & Kashiwayanagi, 1999), where larger long-duration current injection resulted in increased firing frequency. Lynch and Barry (1989) reported that the activation of a single channel in a cell-attached patch, could trigger an action potential in an ORN. This suggested that action potentials could be elicited by only one or two odorant molecules. This action potential could, in turn, be transmitted to the olfactory bulb glomeruli, which can also integrate the convergence of 50 million ORNs to the higher olfactory sensory system (Allison, 1953).

Conclusion

In conclusion, we have shown that a number of different anions and anion combinations could support the achievement of good stable whole-cell recording and the achievement of large sodium currents to differing extents. By far the best response was obtained with F^-, F^-/Cl^-, $H_2PO_4^-$ and, to a much lesser extent, $acetate^-$ and $acetate^-/Cl^-$.

Our results also supported the previous suggestion that the potential for half-inactivation of sodium currents in acutely isolated ORNs is more negative than −100 mV. This, in turn, implies that the resting potential is also very negative, maybe even more negative than −90 mV with further implications for membrane excitability in these mammalian ORNs.

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