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Preparation of isolated mouse olfactory receptor neurons

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Abstract. A method is described for producing large numbers of isolated olfactory receptor neurons from adult mouse nasal epithelium. The dissociated neurons and other cell types isolated from nasal epithelium retain their morphology and can be identified visually. The neurons were judged to be intact and viable by trypan blue dye exclusion, the presence of olfactory marker protein (OMP), and a variety of electrophysiological measurements indicating the presence of substantial membrane potentials, low levels of intracellular Ca^{2+}, and the ability to fire action potentials. The receptor neurons and other cell types produced by this method are amenable to the patch-clamp technique and to immunohistochemical studies.

Key words: Olfaction – Epithelium – Dissociation – Receptor neuron – Patch-clamp – Olfactory marker protein

Introduction

The olfactory receptor neurons in the nasal epithelium are key elements of the olfactory pathway, detecting and discriminating among odorants and providing this information directly to the brain. Because of their importance, considerable effort has focused on developing methods to study the membrane properties of these neurons. Intracellular techniques have been used in studies of both intact epithelia and explant cultures (Getchell 1977; Masukawa et al. 1983, 1985; Trotier and MacLeod 1983; Gonzales et al. 1985). However, intracellular recording is technically difficult because the receptor neurons are usually very small. In addition, penetration with a microelectrode often damages the cell, necessitating the use of high resistance electrodes with low current passing capabilities that limit control of the membrane potential. The recently developed patch-clamp technique (Hamill et al. 1981) is well suited to working with small cells and allows transmembrane currents to be measured with a high degree of spatial and temporal resolution. It is often less damaging to the cell, allowing stable recordings for long periods of time. Often prerequisite to its utilization, however, is the development of methods that remove connective tissue and extracellular material from the cell surface, so that high resistance seals can be formed between the cell and the patch electrode.

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Apart from difficulties with the techniques that have been used, there are disadvantages to studying the receptor neurons in situ or in tissue explants. These relate to the unambiguous identification of cell type, control over the ionic milieu of the neurons, limitations to the application of pharmacological agents to parts of the cell not exposed on the epithelial surface, and difficulties restricting the application of odorants to individual cells. The use of isolated receptor neurons circumvents these problems, enabling experiments to be carried out under well defined biochemical and pharmacological conditions and allowing selected regions of the receptor neurons to be examined in detail.

We report here a method for producing large numbers of isolated olfactory receptor neurons from mouse nasal epithelium. The neurons retain their morphology and so can be easily distinguished from the other cell types of the epithelium. They are viable as judged by a variety of criteria and contain olfactory marker protein (OMP). They have proved amenable to study with the patch-clamp technique, and preliminary results of these studies have been reported (Maue and Dionne 1984, 1986).

Materials

Nine to sixteen week old "nude" mouse heterozygotes (balb/c/Nu) were obtained from the Athymic Mouse Facility, University of California, San Diego (supported by the National Cancer Institute, DHEW, CA, USA). Nine week old male balb/c and timed pregnant Swiss mice (for embryonic tissues) were obtained from MTS laboratories (San Diego, CA, USA). The mice were raised in pathogen-free conditions at these facilities, and we received routine health reports. The mice were used within 3 days of arrival, during which time they were housed in sterilized containers equipped with air filters, had free access to food pellets and water, and were maintained on normal day/night schedules at room temperature and humidity. All enzymes were purchased from Sigma Chemical Co (St. Louis, MO, USA). All media referred to as DMEM, unless otherwise noted, was divalent-ion free Dulbeccos Modified Eagles Medium. The "normal saline" consisted of (mM): 140 NaCl, 5.6 KCl, 2.0 $MgCl_2$, 2.0 $CaCl_2$, 9.4 glucose, and 5.0 sodium N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), pH 7.4. The hemocytometer for cell counts was from American Optical, the trypan blue from Grand Island Biological Co, (Grand Island, NY, USA), and all serum and immunocytochemical reagents, with the exception of the goat anti-OMP serum, were purchased from Vector Laboratories (Burlingame, CA, USA). The goat anti-OMP serum was a generous gift of Dr.

Frank L. Margolis, Roche Institute of Molecular Biology (Nutley, NJ, USA). Photographs were taken on Tri-X-Pan film with an Olympus OM-1 camera mounted on a Zeiss WL microscope or with a Zeiss photomicroscope. Both microscopes were equipped with differential interference contrast (Nomarski) optics and the photomicroscope with an exciter/barrier filter combination optimized for fluorescein isothiocyanate (FITC) fluorescence.

Methods

Tissue dissection. The mice were killed by cervical dislocation, and the olfactory tissues quickly removed as follows: The lower jaw was cut away, the skin, tissue, and eye removed from one side of the skull, and the zygomatic bone, mandible, and teeth were cut away. The palate was then lifted off and the bony plates of the skull gently pried away to expose the ethmoid turbinates on one side of the nasal cavity. These turbinates, the posterior half of the tissue covering the septum, and the turbinates on the other side were removed, rinsed in DMEM, and placed in DMEM on a glass slide. The tissue was separated from the supporting cartilage, placed in fresh DMEM, and minced into approximately 1 mm pieces. Removing the tissue from the mouse was accomplished within 5 min and the minced tissue was added to the enzymes within 30 min of the death of the animal. Similar procedures were used to obtain and prepare embryonic and neonatal tissue, except that tissue from 4–6 animals was used and the tissue was minced without removing it from the supporting cartilage.

Tissue dissociation. Single cells were dissociated from the tissue pieces using enzymatic treatment, divalent-free media, and mechanical disruption; the entire procedure was carried out at 37° C. Specifically, the minced tissue was incubated in 2.5 ml of DMEM containing 0.025% trypsin (Type III) for 40 min. After 10 min in trypsin, the tissue pieces were gently triturated once every 5 min using a fire polished Pasteur pipette, a procedure continued until the cells were plated. The trypsin treatment was terminated by adding 1.5 ml of DMEM containing 0.025% trypsin inhibitor (Type I-S) and 1.5 mM $MgCl_2$. Ten minutes later, an additional 1.0 ml of DMEM containing 0.004% DNAase (Type I) and 10 mM $CaCl_2$ was added to dissolve the DNA released from broken cells. After 10 min, 2 ml of the cell-containing supernatant were applied to a concanavalin A (Con A) coated glass coverslip (see below) in a 35 mm tissue culture dish. The dish was left undisturbed for 30–60 min at room temperature to allow the cells to settle from the suspension and adhere to the coverslip before they were used.

Preparation of Con A coated glass coverslip. The procedure used to covalently link Con A to glass coverslips was a modification of the method of Edelman et al. (1971). All glass and containers were sterilized prior to use and solutions were sterilized by filtration. Glass coverslips, each in a 35 mm dish, were immersed in 2 ml of saline containing a water-soluble carbodiimide (WSC), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, 75 mg/ml, for 2–3 min. The solution was removed and 2 ml of saline containing 10–15 mg/ml of Con A (Type V) was added to each dish. After 1 h, the coverslips were rinsed 2–3 times with saline and stored refrigerated under sterile conditions until needed.

Estimates of cell number. The number of cells produced by this dissociation procedure was estimated by counting aliquots of the cell suspension on a hemocytometer at 400 × with phase contrast optics. Counts of both receptor neurons and total cells were made using standard techniques (Merchant et al. 1964). Neurons were identified by their morphology: cells with a small round soma and one long process from which cilia radiated. These criteria explicitly excluded neurons without a dendritic process and detectable cilia.

Trypan blue exclusion. The percentage of cells which excluded trypan blue dye was estimated using standard assay procedures (Merchant et al. 1964). One hour after the cells had been plated on the coverslips, cells were exposed to a trypan blue solution (1 ml of 0.4% trypan blue added to 5 ml of normal saline) for 10 min. They were then rinsed with normal saline and counted at 400 × magnification using phase contrast optics.

OMP labelling. The OMP labelling was done at room temperature and all reagents and sera were diluted in DMEM. One hour after plating, cells were lightly fixed in 0.25% paraformaldehyde for 10 min and then permeabilized with 0.2% saponin for 4 min. Following this, the cells were rinsed with rabbit serum (1:100), exposed to goat anti-OMP (1:200) for 1 h, rinsed again with rabbit serum (1:100), and then exposed to a biotinylated rabbit anti-goat IgG (1:100). One hour later the cells were rinsed with DMEM, exposed to avidin-fluorescein (1:100) for 1 h, and then rinsed again with DMEM. The coverslip containing the cells was then mounted on a glass slide with gylcerol containing 5% n-propyl-gallate and refrigerated until examination. Control experiments omitted the anti-OMP serum from the protocol or substituted nonimmune goat serum.

To evaluate the labelling, cells were first located and identified using Nomarski optics. Labelling of a cell was then judged under fluorescent illumination by comparing the intensity of its fluorescence to the background level of illumination and to the other cells in the vicinity.

Results

Isolated cells

The dissociation procedure described above produced approximately 1 million (972,000 ± 400,000; $n = 6$) isolated cells from the nasal epithelium of an adult mouse; of this total, approximately 17% (167,900 ± 60,000; $n = 6$) were olfactory receptor neurons. The remainder were either one of several nonneuronal cell types or simply unidentifiable. Although not quantitated, the number of cells produced from preparations of embryonic tissue was much smaller. This might be due to many fewer neurons present in the embryonic epithelium, as suggested by anatomical studies of rat embryonic tissue (Chuah et al. 1985). The isolated cells retained many of the morphological characteristics found in situ.

Receptor neurons. Isolated receptor neurons (Figs. 1A, B; 4A, C) comprised the largest fraction of identifiable cells. They remained largely intact, except for the axon, which presumably was severed from the soma during the dissociation procedure. Somata were spherical or ovoid, 5–8 μm

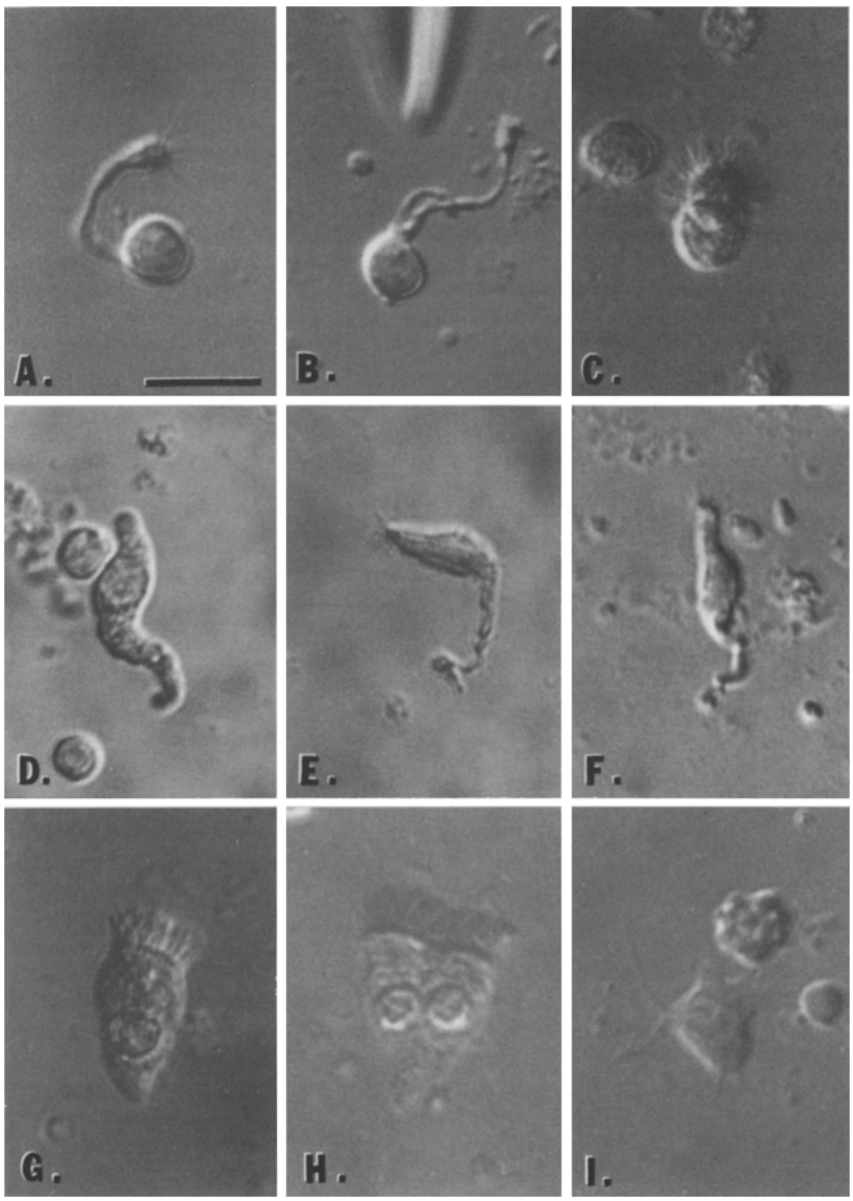

Fig. 1A–I
Cell types produced by the dissociation procedure. **A, B** Isolated olfactory receptor neurons. A patch-clamp electrode, slightly above the cell and out of focus, is visible in the upper portion of **B**. **C** An isolated "Medusa" cell. **D, E, F** Individual support cells. **G** Isolated respiratory epithelial cell. **H** A pair of respiratory epithelial cells. **I** "Fibroblast-like" cell. *Calibration bar* in **A** represents 10 μm and applies to all of the photographs

in diameter. A dendrite approximately 1 μm in diameter extended from the soma and terminated in a swelling or "knob" bearing several fine cilia. Dendrites were usually 5–15 μm long, although dendrites as long as 30–35 μm were observed. Most cells appeared to have 5–10 cilia, with 15–20 visible in some cases. Cilia were generally 10–25 μm long, occasionally extending 40–45 μm. Often the cilia were among the parts of the cell that adhered to the coverslip. However, in many cases only the soma or the soma and a portion of the dendrite were in contact with the coverslip and the cilia projected up into the bath solution. Even in these instances the cilia were immotile, as expected for mammalian olfactory neurons (Lidow and Menco 1984). Similar dendritic dimensions and numbers of cilia have been reported for receptor neurons in intact tissue from mice and other mammals (Allison 1953; Kerjaschki and Horander 1976; Menco 1980).

Nonneuronal cells. Several distinctive nonneuronal cell types were dissociated from the epithelium, some corresponding to the types of cells described in the olfactory epithelium of other species. Respiratory epithelial cells (Figs. 1G, H; 4G) were isolated from both adult and embryonic tissue. Although occasionally rounding up in suspension, these columnar cells were approximately 15–25 μm long and 10–15 μm wide at the broader "apical" end. A brush-like border of synchronously beating, 5 μm long cilia covered the apical surface. The nucleus was prominent.

Cells similar to type I sustentacular cells of the salamander described by Rafols and Getchell (1983) were observed routinely (Figs. 1E, F; 4E). These cylindrically shaped cells were approximately 10–15 μm long, 3–5 μm wide, and at one end tapered to approximately 1 μm diameter before extending another 5–10 μm and expanding into a broader terminal ending or "basal foot". The nucleus was located in the narrower end of the soma. At the apical end of the cell were many short, fine processes, which may have been the microvillus projections described for this type of cell (Getchell et al. 1984).

Cells resembling type II support cells of salamander (Rafols and Getchell 1983) and sustentacular cells dissociated from frog olfactory epithelium (Kleene and

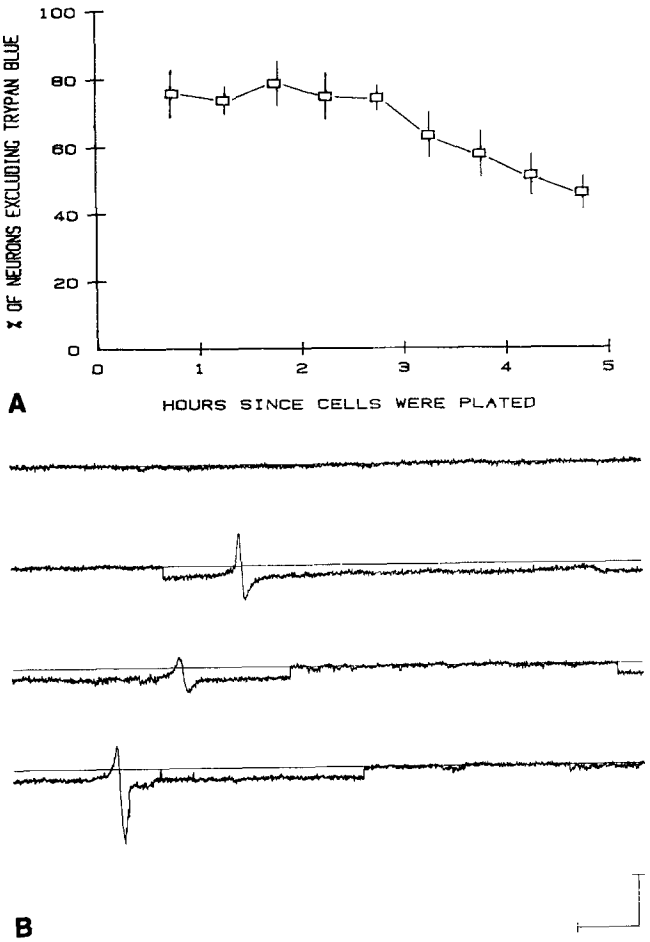

Fig. 2A, B. Viability of the isolated olfactory neurons. **A** Trypan blue exclusion. At the times indicated, aliquots of the cell suspension were exposed to trypan blue, and the number and types of cells excluding the dye were noted, with a minimum of 60 neurons examined at each time point. Plotted are the means and standard deviations that resulted from analysis of 4 preparations. **B** Current recording made in the cell-attached configuration after formation of a "gigohm seal" on a receptor neuron. The 4 current records are contiguous; a baseline has been drawn through the records where all of the single channels observed in the membrane patch were closed. Openings of individual channels in the patch (and in this case, inward currents) are represented by downward deflections from the baseline level of current. In this example, currents associated with action potentials in the cell occur soon after the channel openings; these currents also occurred when the channels in the patch were closed, although at a much lower frequency. The patch pipette contained an elevated K^+ saline; the pipette potential with respect to the bath was 37 mV. *Calibration bar* is 10 pA; 20 ms

Gesteland 1981) were also observed. Approximately 25 μm long, these cells had a prominent nucleus and granular appearance (Figs. 1D; 4E).

Cell preparations from adult and from embryonic tissue also contained a cell type which did not resemble any of the commonly described types of cells in the olfactory epithelium (Fig. 1C). The cells were ovoid, 8–12 μm in diameter, with a very distinctive "cap" of asynchronously beating cilia, which led to the moniker "Medusa cells". The asynchronicity, along with the relative size and appearance of the patch of cilia, smaller cell size, and lack of a large, prominent nucleus, distinguished these cells from respiratory epithelial cells. Although previous reports of "Medusa" cells apparently do not exist, other cell types, such as "brush cells", have been described in mammalian olfactory epithelium (Menco 1980).

Other less distinctive cells were also found in the preparation. These included small, round, relatively featureless cells, and flat fibroblast-like cells that would often extend processes onto the coverslip (Fig. 1I).

Cell viability

Cell viability was evaluated with criteria which depended upon the integrity of the membrane. Although most work focused on the receptor neurons, there were indications that other cell types were also viable: ciliary motility, an energy requiring process, was often observed in the respiratory and "Medusa" cells. Furthermore, on several occasions the application by pressure-ejection from a pipette of 50 μM forskolin or 50 μM acetylcholine to "Medusa" cells resulted in a dramatic increase in the frequency and vigor of the ciliary motility. Motility gradually declined to the initial level within minutes after removal of the compounds. This response was not observed with control applications of saline and could be elicited repeatedly in a given cell.

Trypan blue exclusion. When assayed 1 h after plating, 75–80% of the isolated neurons excluded trypan blue dye. This percentage remained unchanged for 2–3 h and then gradually declined to slightly more than 50% by 5–6 h after plating (Fig. 2A). In neurons which took up the dye, the color change was easily discernible and the cells were noticeably swollen; although not quantitated, these cells generally looked damaged and often lacked cilia or had only the twisted remains of a dendrite. This suggested that visual inspection of the cells during physiological experiments could help confine studies to cells that were likely to be viable.

In contrast to the receptor neurons, nonneuronal cells appeared to survive the preparative procedure less well. Only 10–12% of the respiratory cells and 50–60% of the "Medusa" cells excluded dye. Although not quantitated, less than 50% of the small, round unidentified cells initially excluded dye, and by 5–6 h after plating almost all of them had taken up the trypan blue.

Action potentials. Isolated receptor neurons impaled with microelectrodes containing 1 M K^+-acetate and then stimulated with depolarizing current pulses often displayed overshooting action potentials followed by afterhyperpolarizations. In addition, currents associated with spontaneous action potentials in the isolated receptor neurons were occasionally recorded in the patch pipette from cell-attached membrane patches (Fig. 2B). This has been reported for other cells and can occur when the input resistance of the cell is high (Fenwick et al. 1982; Forda et al. 1982; Ashcroft et al. 1984). The currents were biphasic, usually occurring spontaneously at infrequent and irregular intervals, occasionally occurring in "trains" or "bursts" of 2–3 or more. Less frequently their appearance could be elicited by rapid hyperpolarizations of the membrane patch (which can depolarize the rest of the cell; see Fenwick et al. 1982). We also observed that the opening of single ion channels can trigger action potentials in these cells (Fig. 2B). This has been described in pancreatic beta cells (Ashcroft et al. 1984) and analyzed in more detail in chromaffin cells (Fenwick et

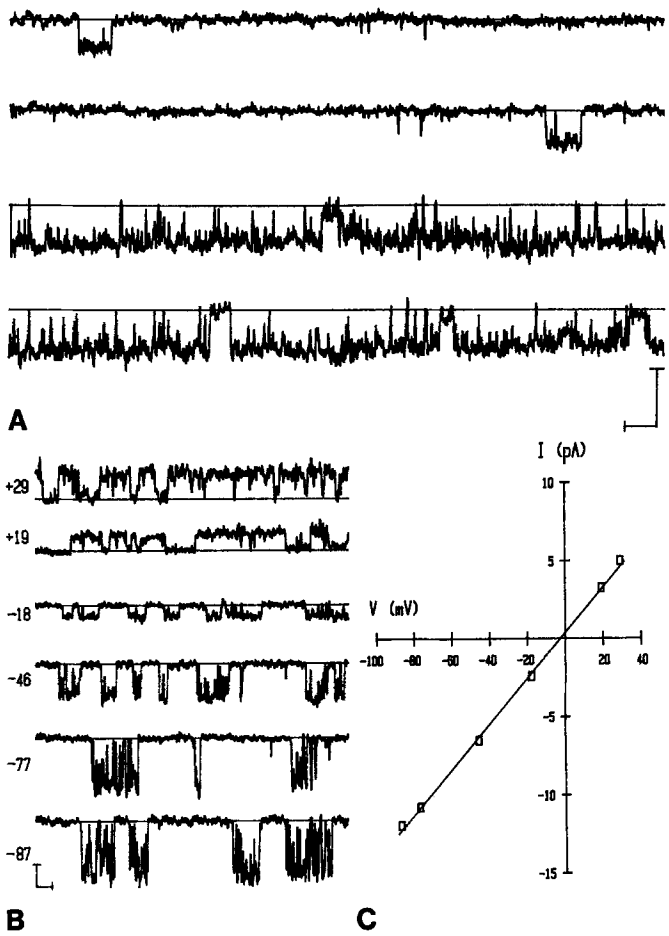

Fig. 3A–C. Single channel recordings from isolated receptor neurons. **A** Ca^{2+}-activated K^+ channel from a neonatal mouse neuron. In all 4 current records downward deflections represent openings of the channel (the patch pipette contained elevated K^+ saline). The top two recordings are contiguous and illustrate the level of activity of the channel in the "on-cell" configuration (low intracellular $[Ca^{2+}]$, estimated membrane potential -20 mV). The lower two records are also contiguous and show the marked increase in activity in the same patch when the $[Ca^{2+}]$ at the intracellular surface was increased to approximately 2 mM by excising the patch into bath saline (membrane potential 0 mV). *Calibration bars* are 5 pA; 5 ms. **B** K^+ channel activity in an excised patch of membrane. In all 6 current records, the baseline indicates where the channels are closed. Displayed to the left of each record is the membrane potential at which the activity was recorded. The current records suggest the kinetic behavior of the channel was voltage dependent and that the percentage of time the channel was open increased at depolarized potentials. Detailed analysis (Maue and Dionne, in press) confirmed this impression. Saline solutions on both sides of the membrane contained elevated K^+, with the bath having a $[Ca^{2+}]$ of 0.1 μM. *Calibration bars* are 5 pA; 5 ms. **C** The current-voltage relationship for the channel activity shown in **B**. The relationship between current and voltage appeared to be linear over the range examined; the reversal potential of the single channel currents was near 0 mV. The single channel conductance, estimated by linear regression, was approximately 140 pS

al. 1982), and also suggests that the isolated receptor neurons had high input resistances.

Evidence of low intracellular Ca^{2+} levels. Among the ion channels we have studied in isolated receptor neurons with the patch-clamp technique are Ca^{2+}-activated K^+ channels (Maue and Dionne 1984). The activity of these channels in the cell-attached configuration was very infrequent (Fig. 3A), and based upon analysis of their Ca^{2+} sensitivity (Maue and Dionne, in press) suggested that the $[Ca^{2+}]$ inside the cell was less than 0.1 μM. This low value could be maintained only if the cells were viable and intact.

Evidence of membrane potentials. Single channel recordings of K^+ channels (Fig. 3A, B), as well as intracellular recordings, provided estimates of the membrane potentials of the isolated neurons. When recordings of the K^+ channels were made in the cell-attached configuration with 145 mM K^+ saline in the recording pipette the reversal potential of the single channel currents could be used as a rough indication of the membrane potential of the cell. Although this method is indirect and has several sources of error, the sum of the errors is likely to cause the membrane potential to be underestimated. The estimates suggested the neurons had substantial membrane potentials, averaging -52 ± 17 mV ($n = 10$), with a range of -30 to -80 mV. Membrane potentials measured in isolated receptor neurons impaled with microelectrodes were consistent with these estimates, averaging -37 ± 6 mV ($n = 11$). These values are similar to those obtained from other olfactory preparations (Getchell 1977; Masukawa et al. 1983, 1985; Trotier and MacLeod 1983; Gonzales et al. 1985).

OMP Labelling

Olfactory marker protein (OMP) is a small, cytosolic protein that is unique to the receptor neurons of the nasal epithelium and is a widely used biochemical marker for these cells. Its developmental appearance and cellular localization in maturing receptor neurons (Farbman and Margolis 1980; Monti Graziadei et al. 1980), as well as its wide distribution phylogenetically (Keller and Margolis 1975), have been well characterized. OMP labelling of individual neurons was observed in the preparation of cells described here (Fig. 4B, D). Over 50% ($52 \pm 7\%$, $n = 3$) of the neurons were labelled as defined by the criteria described above. No specific labelling was observed in any of the other identified cell types (Fig. 4F, H). However, a small fraction of the round, unidentified cells appeared to label, suggesting that some of these cells might have been receptor neurons which lost their axons and dendrites during the dissociation procedure. In the control experiments, no labelled receptor neurons or other cell types were observed.

Discussion

Other methods to isolate olfactory receptor neurons have been published (Ash et al. 1966; Hirsch and Margolis 1979; Kleene and Gesteland 1981, 1983; Noble et al. 1984); however most were plagued by one or more problems including relatively low yields, altered receptor cell morphology, and questionable viability. In addition, there was no indication that neurons isolated by these methods were amenable to electrophysiological study. Although some viable and identifiable cells were isolated, methods to dissociate receptor neurons from rabbit olfactory tissue (Ash et al. 1966) and from rat nasal epithelium (Hirsch and Margolis 1979) were developed primarily for biochemical characterizations of the tissue. Kleene and Gesteland (1983) reported a method

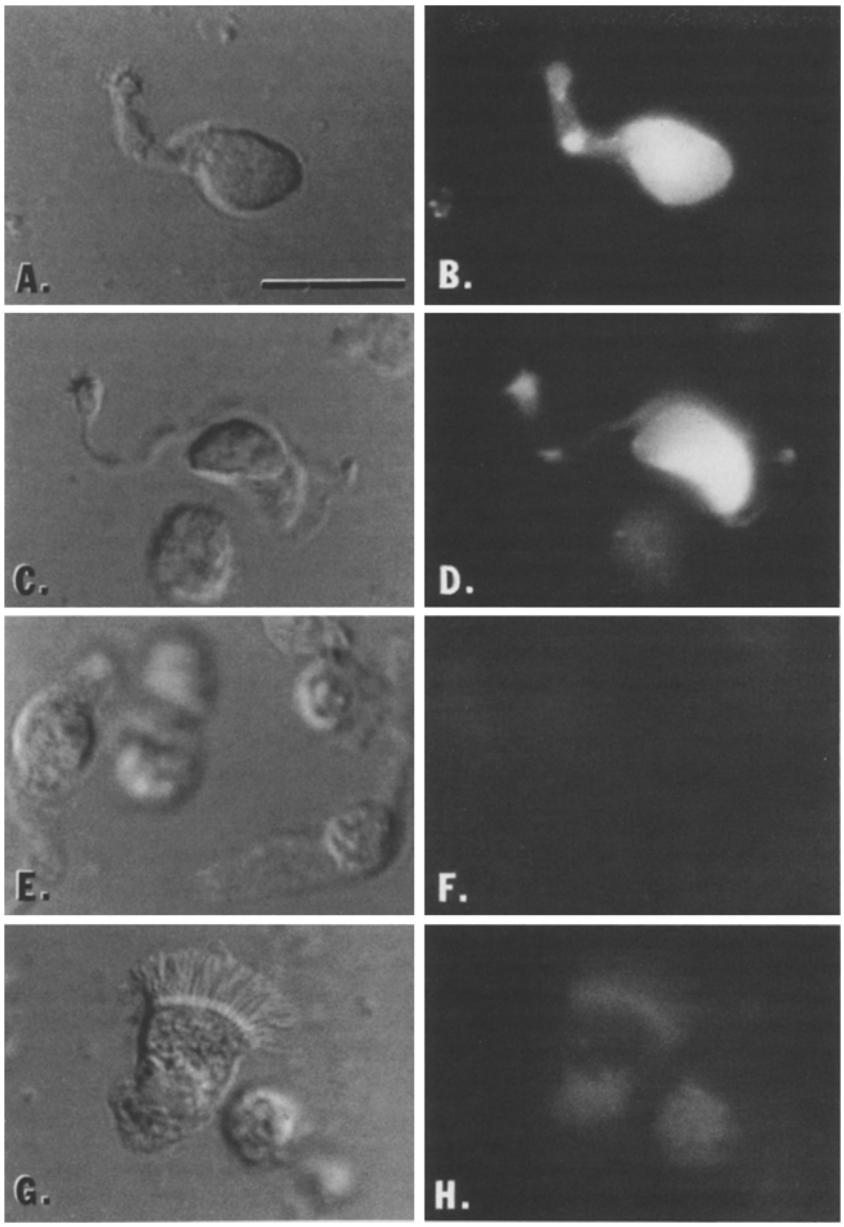

Fig. 4A–H
OMP labelling in isolated olfactory receptor neurons. Nomarski photographs of fixed, isolated cells (*left column*) and the same cells observed under fluorescent illumination (*right column*) show the specific labelling of the receptor neurons with goat anti-OMP serum. **A/B, C/D** Isolated olfactory receptor neurons. **E/F** Sustentacular cells. **G/H** Respiratory epithelial cell. *Calibration bar* in **A** is 10 μm and applies to all of the photographs

for isolating frog receptor neurons that avoided enzymatic treatment. Although the isolated cells appear to be viable, the yield was low and the morphology of the cells was altered. When isolated enzymatically (Kleene and Gesteland 1981), the viability of the frog receptor cells was questionable. Short-term culture of receptor neurons (Noble et al. 1984) is also hampered by low yield and questionable viability. Working on enzymatically treated cells, Anderson and Ache (1985) have recorded from lobster olfactory neurons using the patch-clamp technique. In situ the cells respond to chemical stimuli, but there are difficulties and uncertainties regarding cell identification and the degree of electrophysiological control due to the length of the dendritic processes. In contrast, the dissociated somata, which do not have any visible processes, lack odorant sensitivity.

In this paper we report a method for reliably producing large numbers of isolated olfactory neurons that retain their characteristic morphology, allowing them to be easily located and distinguished from other cell types in the preparation. These anatomical attributes provide the opportunity to focus on selected regions of the cell surface (e.g. terminal dendritic knob) during physiological experiments. The neurons were judged to be viable and intact for several hours by trypan blue exclusion and a variety of electrophysiological criteria which suggested that they had substantial membrane potentials, low levels of intracellular Ca^{2+}, and the ability to fire action potentials. Dissociated neurons also contained OMP, a cytosolic protein characteristic of mature receptor neurons.

The cells isolated by this procedure are amenable to study with the patch-clamp technique. High resistance "seals" of 1–10 gigohms have been formed between receptor cells and patch electrodes, allowing the activity of single ion channels to be monitored with a high degree of resolution (Figs. 2B; 3A, B). Several types of electrode glass (soft, borosilicate and aluminosilicate) and pipette solutions have been used to form seals, with an overall success rate of approximately

50–75%. Recordings have been made from membrane patches of the terminal dendritic knobs as well as the somata of the isolated receptor neurons. In addition, seals have been formed on receptor neurons that were isolated from nasal epithelium of embryonic and neonatal mice using this procedure (Fig. 3A) (Maue and Dionne 1986). Because of the prenatal change in the response of mammalian receptor neurons to odorants (Gesteland et al. 1980, 1982), comparing the membrane conductances in these neurons to those in neurons from adults may provide insight into the mechanism of discrimination as well as how it changes during development. Finally, we have also recorded from membrane patches on each of the nonneuronal cell types described here, although we have not studied them extensively.

This preparative method allows mammalian olfactory receptor neurons, isolated from animals of different ages, to be studied under well defined conditions with a technique suited to examining individual ion channels as well as the cellular responses to applied odorants (Maue and Dionne, in press). By doing so, it provides a powerful tool for understanding the function of the olfactory system.

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