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Simultaneous recording of $[Ca^{2+}]_i$ increases in isolated olfactory receptor neurons retaining their original spatial relationship in intact tissue

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A new method is described for isolating olfactory receptor neurons suitable for simultaneous recording of odorant responses in several cells. This method, called "tissue printing" by Cassab and Varner, was used to isolate cells for measurement of odorant-induced increases in cytosolic-free calcium concentration ($[Ca^{2+}]_i$) using the Ca^{2+} indicator dye fura-2. A large number of receptors could be isolated from a piece of olfactory epithelium (about 300 μm square), preserving their normal morphology and relative local topology to that in the intact olfactory tissue. The probability that there are one or more receptor cells with odorant-induced responses in $[Ca^{2+}]_i$ per preparation was 4 times higher with cells isolated by the tissue printing than with those obtained by the pipetting method. The responses of 2 receptor cells separated by 28 μm in the recording chamber differed for 2 odorants: isoamyl acetate and citralva. The method was useful for isolating receptor neurons without losing their morphological features and for investigating the spatial distribution of odorant responsiveness of each receptor over the olfactory epithelium.

Introduction

The patch-clamp recording technique (Hamill et al., 1981) has been used to study the electrophysiological properties of olfactory receptor neurons in frogs (Nakamura and Gold, 1987; Labarca and Bacigalupo, 1988; Suzuki, 1989; Trotier et al., 1989; Frings and Lindemann, 1990), salamanders (Trotier, 1986; Anderson and Hamilton, 1987; Firestein and Werblin, 1989), mice (Maue and Dionne, 1987a,b), newts (Kurahashi, 1989), catfish (Restrepo et al., 1990), etc. On the other hand, the development of optical recording techniques using voltage- or ion-sensitive dye provide a powerful tool for the spatial imaging or simultaneous recording of cellular responses (Grinvald, 1984; Kauer, 1988; Tsien, 1988; Ichikawa and Matsumoto, 1990). Measurements using the Ca^{2+} indicator dye, fura-2, showed that intracellular Ca^{2+} plays an important role in the olfactory transduction (Restrepo and Teeter, 1990; Sato et al., 1990, 1991). In order to achieve a higher S/N ratio in the optical recordings, an isolated cell is better than an intact or sliced tissue. The isolation of the neurons for patch-clamp recordings usually involved pipetting (triturating) the small pieces of the tissue after enzymatic treatment (e.g., in olfaction: Maue and Dionne, 1987b). This process, however, tends to damage olfactory receptor neurons because of

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their characteristic morphology, with cilia projecting from a knob at the end of a long dendrite. Most isolated receptor cells did not respond to odorants and usually lost their characteristic morphology (Dionne, 1989). Although the sensitivity and responsiveness to odorants are different from cell to cell and their distribution, which is termed "inherent" spatial patterning (Moulton, 1976), is heterogeneous in EOG recordings (MacKay-Sim et al., 1982), it is difficult to identify both the odorant responsiveness and the individual location of the receptor cells from the same topographical region of epithelium due to the lack of appropriate preparations.

Here we describe a new technique which permits efficient isolation of viable olfactory receptor cells from the same region of tissue. A similar technique called "tissue printing" has been used for the isolation of soybean seed coats (Cassab and Varner, 1987) and astrocytes from optic nerve (Barres et al., 1990) on the nitrocellulose paper for the immunocytochemical study or electrophysiological recording. We developed a tissue printing technique suitable for optical recordings, which involves rolling a piece of the tissue over a glass floor of the experimental chamber. This method resulted in a higher density of viable receptor neurons displaying odorant responses than the pipetting method. Moreover, this method was useful for simultaneously recording responses of several neighboring receptor cells in the intact tissue.

Materials and methods

Fig. 1 shows the procedure for preparing isolated olfactory receptor neurons. Wild bullfrogs (*Rana catesbeiana*), anesthetized with MS-222 (0.2 mg/g, i.p.; Aldrich, 886-86-2), were killed by double pithing. Olfactory epithelia were detached, then dissected from the cartilage, and kept in GIT medium (a culture medium, 398-00515, Nippon Pharmaceutical Co., Japan) containing 5% FBS (200-6140, Gibco) at 5°C.

The epithelium was sliced at about 300 μm vertically across the surface, and the olfactory mucus was removed from the epithelium by manipulation with solid needles and suction with a Pasteur pipette. The sliced epithelium was cut into small pieces at 200–500 μm. The enzymatic treatment was as follows: the pieces of epithelium without Bowmand's glands were put in calcium-free Ringer's solution (CFR: 110 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 3 mM sodium pyruvate, 10 mM glucose, 10 mM HEPES, pH 7.2) for 10 min, incubated in papain solution (Calbiochem: 1 mg/ml in CFR) for 5–10 min at 34°C, then kept in CFR for a few minutes and rinsed in normal Ringer's solution (NR: added 2 mM $CaCl_2$ to CFR).

One of the treated pieces was then transferred to a teflon recording chamber with a floor of cover glass (No. 0, Matsunami, Japan), the inside of which was coated with Con A (Sigma Type-V: 5 mg/ml in NR) to prevent the olfactory receptor neurons from being washed away by the flow of the bath solution during perfusion. The piece was slowly rolled over the glass floor by manipulating it with a micropipette, more elastic than a patch pipette, under a phase-contrast microscope. The rolling had to be done carefully so that the lateral side of the epithelial piece maintained gentle contact with the glass floor. Any excess pressure during rolling resulted in crushing the isolated cells between the glass floor and the piece of epithelium. This operation resulted in olfactory cells on the surface of the piece being isolated and continuously printed on the glass floor, preserving the relative local arrangement between cells. These printed cells were not dislodged by the fast flow of the bath solution (1.5 ml/min, perfused volume of the chamber was about 50 μl).

The solution in the chamber was replaced with GIT medium including 5 μM fura-2/AM (Molecular Probes) and the cells were incubated for 2 h at 25°C. Even after incubation, most of the cells maintained their characteristic morphology and ciliary motility.

The fluorescence at 510 nm, which was excited at 340 nm or 380 nm (150 W Xe lamp, Hamamatsu Photonics, Japan; invert microscope: TMD-EFQ, Nikon, Japan), was measured through a 40 × objective lens (CF fluor, Nikon) using a SIT camera (C-2400-08, Hamamatsu Photonics)

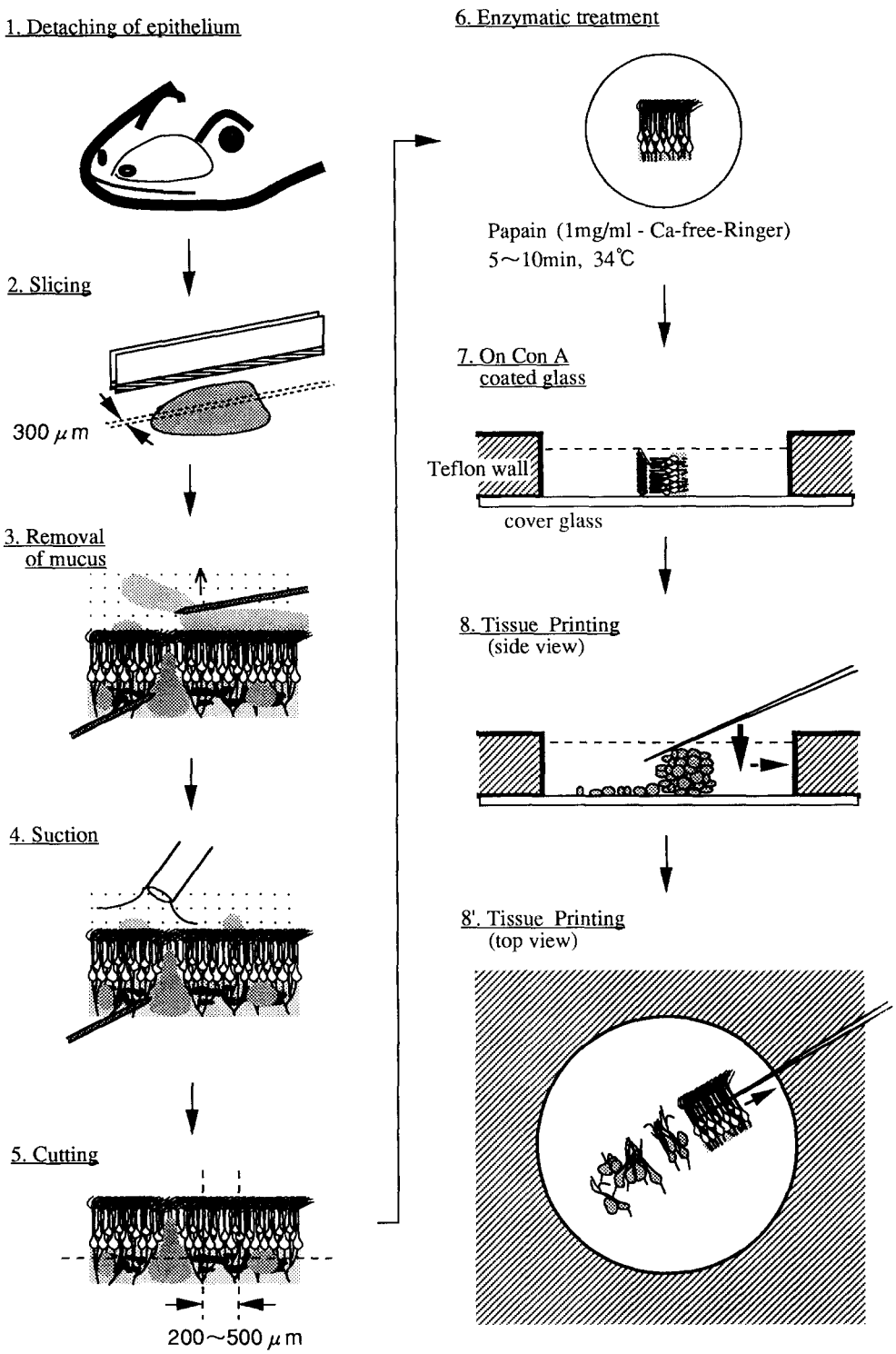


Fig. 1. Tissue printing method for isolation of olfactory receptor neurons.

and an image processor with 8-bit resolution (Argus-100, Hamamatsu Photonics) at 25°C. Data were recorded at 1/3-s intervals at 7 positions (each is the integrated value of 5×5 pixels (2×2.3 μm on a cell) in 8 video frames). The cytosolic free-calcium concentration ($[Ca^{2+}]_i$) was estimated from the ratio (340/380 nm, $R_{340/380}$) of the fluorescence intensities after the subtraction of the dark level. Free calcium concentration dependence of $R_{340/380}$ was measured in EGTA/Ca^{2+} buffers (calcium concentration: 0–1 mM) containing 5 μM fura-2, 110 mM KCl and 10 mM MOPS (pH 7.2). Citralva (100 μM CT; 15767–8, Aldrich) and isoamyl acetate (10 mM AM; Wako Chemical, Japan) were used as odorants, and forskolin (2 μM FK; F-6886, Sigma) as an adenylate cyclase activator. They were dissolved in NR (contained 0.8 mM ethanol in FK) and were applied to the cells by the perfusion of the bath solution using a peristaltic pump (Microperpex, Pharmacia LKB).

Results

Isolated olfactory neurons by tissue printing

Figs. 2 and 3 show isolated olfactory cells on a primaria dish (3802, Falcon) and on a Con A-coated glass, respectively. Continuous rolling in the tissue printing allowed isolated cells to be arranged in a long band. In the both preparations, numerous olfactory receptor cells had normal shapes: cell bodies of about 10 μm diameter attached to dendrites of 10–60 μm length with

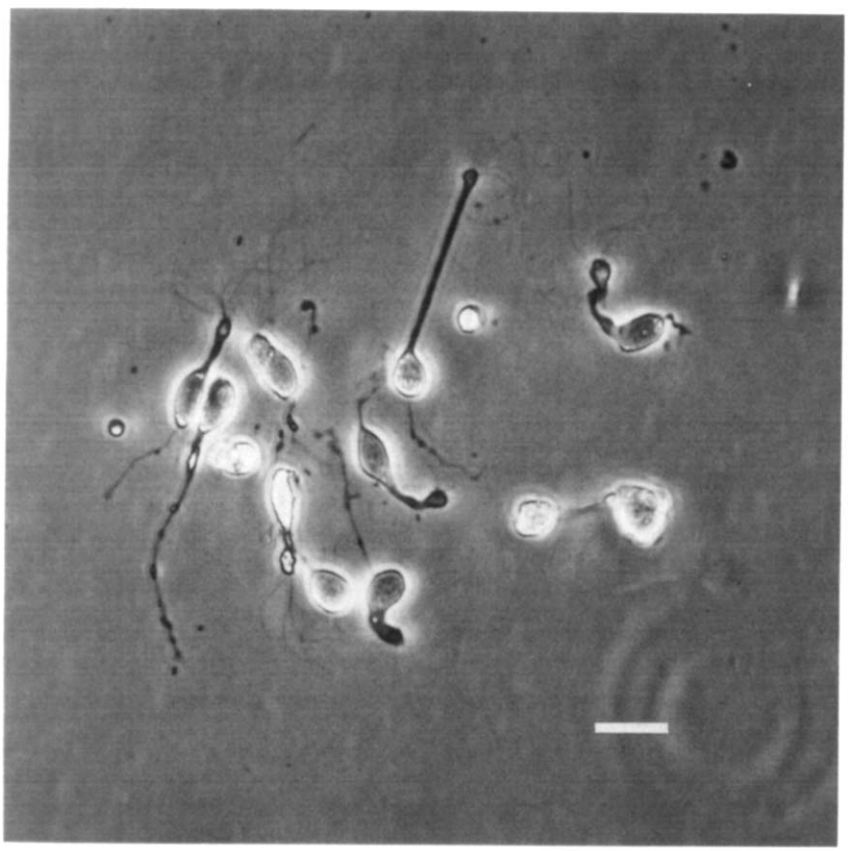


Fig. 2. Olfactory cells isolated on a primaria dish. Typical receptor cells of a frog showing the long dendrite, cilia and axon. In addition to the receptor cells, supporting cells were present on the floor of a dish. Calibration bars = 20 μm.

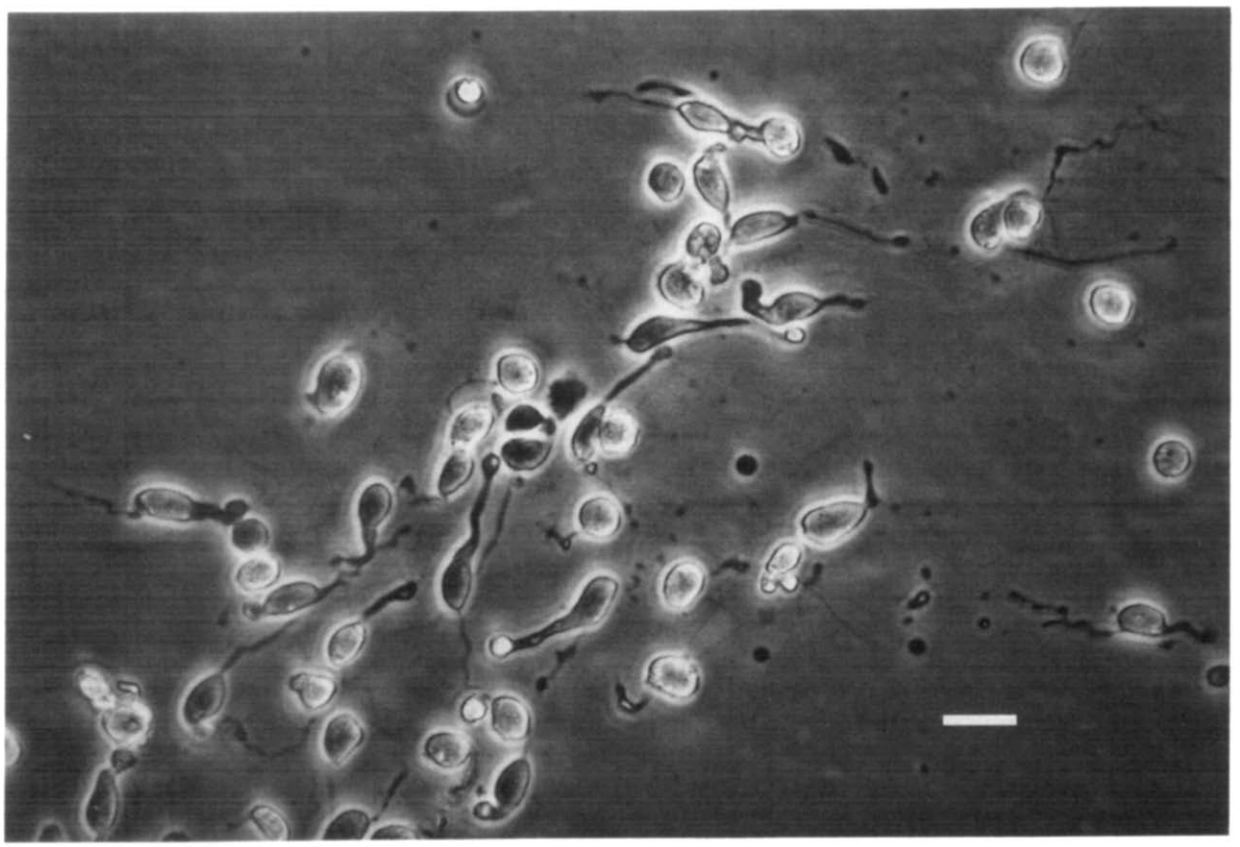


Fig. 2 (continued).

several cilia projecting several tens of micrometers from the knob. The cilia beat asynchronously. Olfactory receptor neurons could be easily distinguished from supporting, basal, and other cells by their characteristic morphology.

Fura-2 loading

Fura-2 was loaded into the cell body more readily than into other parts of the receptor. However, overall efficiency of loading fura-2 into the receptors was not very high, particularly in those receptors with normal morphology. Fluorescence intensity at the cilia was too weak to be measured. However, in every preparation made by the tissue printing method, we obtained some receptors from which the fluorescence intensity changes corresponding to $[Ca^{2+}]_i$ increases could be measured.

Responsiveness

We recorded responses of olfactory receptor neurons with motile cilia. Odorant or FK stimulation transiently increased $[Ca^{2+}]_i$ in many of these

TABLE 1

COMPARISON OF THE NUMBERS OF PREPARATIONS INCLUDING ODORANT RESPONSIVE RECEPTOR OBTAINED BY THE TISSUE PRINTING METHOD WITH THOSE BY THE PIPETTING METHOD

Isolation method	Number of preparations		Total number of odorant responsive receptors
	Total	Including odorant responsive receptor	
Tissue printing	17	11 (64.7%)	15
Pipetting	151	24 (15.9%)	28

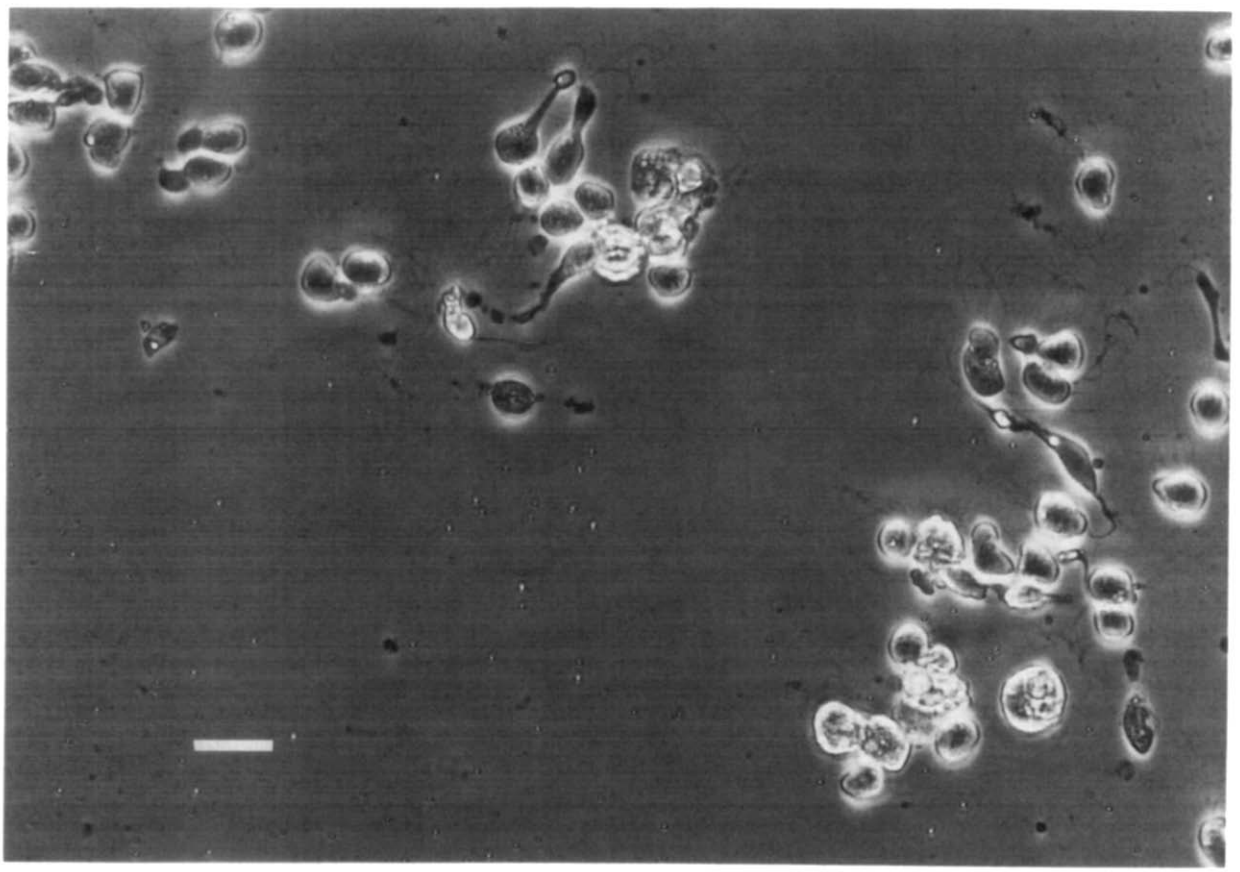


Fig. 3. Olfactory cells isolated on a recording chamber. Isolated olfactory receptors were attached at high density on the Con A-coated glass floor of a recording chamber for $[Ca^{2+}]_i$ measurement. Calibration bar = 20 μm.

TABLE II

COMPARISON OF THE ODORANT RESPONSIVITIES OF OLFACTORY RECEPTORS OBTAINED BY THE TISSUE PRINTING METHOD

In the upper row, responsivities are classified by the responses to citralva (CT) and isoamyl acetate (AM), and the values are the number of receptors. In the lower row, receptors are classified by the responses to odorants and forskolin

Total	CT (+) AM (−)	CT (−) AM (+)	CT (+) AM (+)	CT (−) AM (−)
63	8 (12.7%)	3 (4.8%)	4 (6.3%)	48 (76.2%)
	Forskolin (+)		Forskolin (−)	
	AM or CT (+)	AM or CT (−)	AM (+)	AM or CT (−)
	14 (22.2%)	20 (31.7%)	1 (1.6%)	28 (44.4%)

* (+): responsive; (−): unresponsive.

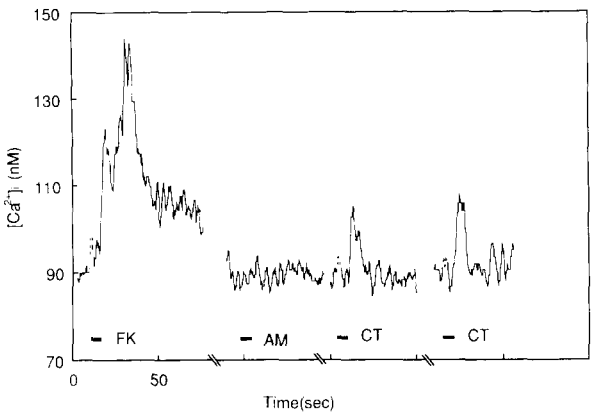
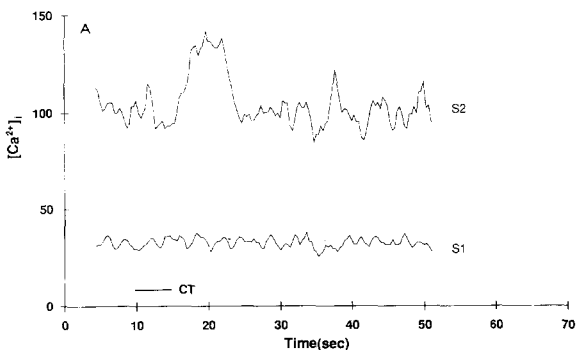


Fig. 4. Intracellular calcium increases by odorant and FK stimulations. Representative responses evoked by different stimuli (2 μM forskolin (FK), 10 mM isoamyl acetate (AM) and 100 μM citralva (CT)) are shown. A response following FK stimulation was the largest in the amplitude. This cell did not respond to AM. The responses to CT were reproducible as shown in the right 2 traces. Each stimulus was applied by perfusion with 5-s duration as indicated by the bar. The dotted lines indicate artifacts evoked by the air between the different solutions during the perfusion.

receptors. With tissue printing, the probability of obtaining preparations with olfactory receptors responsive to odorants was 64.7%, which was about 4 times as high as that for the pipetting method (Table I).

Responsiveness to CT, AM and FK was tested in 63 isolated olfactory receptor cells (Table II). Thirty-four (54%) responded to FK and 15 (23.8%) to CT and/or AM. The odorant responsivities were as follows: 8 (12.7%) responded only to CT, 3 (4.8%) only to AM, and 4 (6.3%) to both CT and AM. Fourteen of these cells responded to both FK and odorants, and 1 receptor responded to AM but not to FK. Twenty receptors did not respond to odorant but did respond to FK.

Fig. 4 shows typical $[Ca^{2+}]_i$ responses in a receptor. The responses to CT were reproducible as shown in the right 2 traces. In most receptors, FK induced larger $[Ca^{2+}]_i$ increases than CT or AM. The onset of the odorant-induced responses had about a 5-s latency. The duration of the increase in $[Ca^{2+}]_i$ following 5–10 s stimulation lasted for 10–20 s, or more in some cases. The increase in $[Ca^{2+}]_i$ after FK was similar to that produced by odorants in the time course of the rising phase, but the latency was several seconds longer and the decay was slower than for the odorant-induced $[Ca^{2+}]_i$ increase.



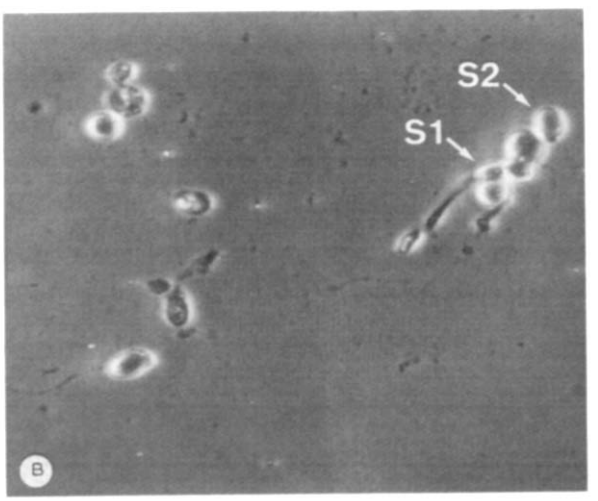


Fig. 5. Responses of 2 receptors, S1 and S2, following CT stimulation. a: intracellular calcium increases following CT stimulation. $[Ca^{2+}]_i$ increase showed that the receptor, S2, responded to CT. The stimulus duration was 5 s as indicated by the bar. b: phase-contrast image of the recorded olfactory receptor neurons. S1 and S2 were around a supporting cell and close to each other with a distance of 28 μm center-to-center on the chamber.

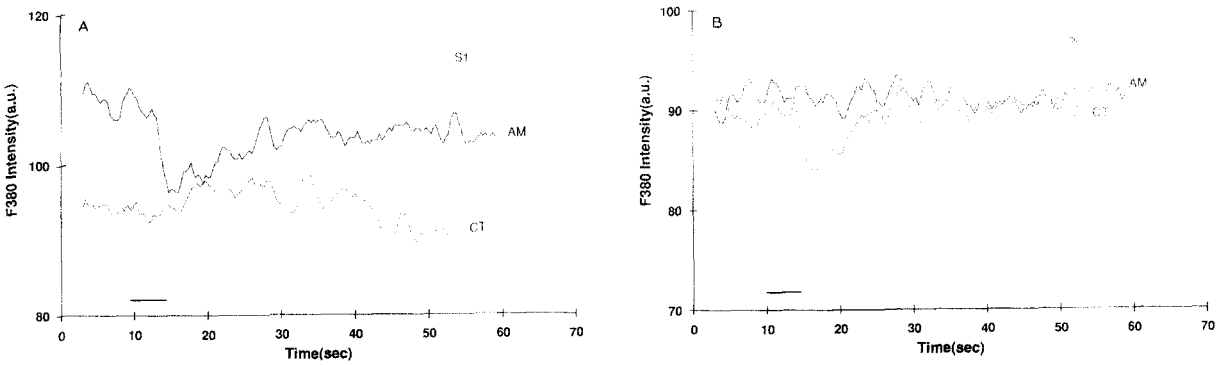


Fig. 6. Different responsivities of S1 and S2. S1 and S2 were the same as in Fig. 5. As fluorescence intensities were measured at 380 nm excitation, its decrease corresponded to the $[Ca^{2+}]_i$ increase. a: in S1, $[Ca^{2+}]_i$ increase was induced by AM but not by CT. b: on the contrary, in S2, it was induced by CT but not by AM. These responses indicated different odorant responsivities in the 2 olfactory receptors.

Simultaneous recording

We found 2 neighboring receptors, S1 and S2, which showed different responses to CT and AM (Figs. 5 and 6). Fig. 5a shows that CT did not increase $[Ca^{2+}]_i$ in S1 but did produce an increase in S2. Fig. 6a and 6b show the responses of the 2 receptors to AM stimulation, respectively, in fluorescence intensity at 380 nm excitation, where the decrease corresponds to the $[Ca^{2+}]_i$ increase. The responses to CT are also shown in the same figures for comparison. These data mean that S1 responded to AM selectively, while S2 responded to CT selectively. These 2 receptor cells were adjacent to a supporting cell and approximately 28 μm from each other (Fig. 5b).

Discussion

Tissue printing

In contrast to receptor cells isolated by tissue printing, most of the receptors obtained by the pipetting method from *Rana catesbeiana* lost their long dendrites and some appeared as round cells with several motile cilia. We also observed that many "printed" olfactory receptors retained segments of axon longer than the neurons isolated by pipetting. One of the reasons for these differences is probably that receptor cells isolated by pipetting suffered strong stress at the edges of the dendrite, resulting from friction against the inside wall of the pipette. Also adhesion of the dendrite to the glass floor might be weaker in preparations prepared by pipetting than those obtained by tissue printing, thus allowing the dendrite to shorten freely.

Because tissue printing arranged the isolated receptors in the same relative relationship to other cells as in the lateral side of the epithelium, their relative position to and their distances from each other reflected the characteristics of the intact tissue. Although some receptors in the chamber probably preserved the distances in the intact tissue, others were changed to varying degrees. In our experiments, where the size of a piece of tissue was about 300 μm, the continuous region of isolated cells on the glass floor was up to a few hundreds micrometer in length. We speculate that the receptors, at least within several tens of micrometers, roughly kept their position relative to that in the intact epithelium.

Responsiveness

All but 1 odor-responsive receptor also responded to FK. This supports the hypothesis that olfactory signal transduction is mediated by an adenylate cyclase cascade. The different time course of the response to FK from that to odorants might reflect different activation/inactivation properties of the adenylate cyclase between FK and the specific G protein.

In the tissue printed preparations, 44.4% of the receptors did not show $[Ca^{2+}]_i$ increases to either odorant or FK. This population was almost the same as the percentage of survivaling cells at 5 h after plating (Maue and Dionne, 1987b), unresponsive receptors might have been damaged during isolation. This may have resulted from (1) chemical damage by the enzyme used in the isolation procedure, (2) mechanical damage during tissue printing, (3) inhibition of receptors by Con A, and (4) lack of appropriate environment (e.g., supporting cells, mucus, etc.). Although Con A might inhibit cellular responses to some degree, this inhibition should have been minimized by the continuous perfusion with fresh NR. We think that inhibition by Con A is not a serious problem because others have obtained responses from receptors plated with Con A (Maue and Dionne, 1987a; Kurahashi, 1989). If the unresponsive receptors were not damaged, they might have cAMP-independent responses, cytosolic Ca^{2+}-independent responses, or resistance to forskolin. More than one-half of the FK-responsive receptors did not respond to both CT and AM. Perhaps these cells are responsive to other odorants. A receptor which was AM-responsive and FK-unresponsive might have cAMP-independent response, e.g., use a different second messenger.

Simultaneous recording

The tissue printing method provides a novel preparation the individual cells of which appear to keep some of their relative spatial relationships as in the intact epithelium. This merit, which the pipetting method does not have, is useful for investigating the distribution of odorant responsivity over the olfactory epithelium.

In one case, we found that 2 neighboring receptor cells responded differentially to 2 odorants of different qualities. Some researchers have argued that the distribution of odorant sensitivity in the EOG was in several separate regions of the epithelium with some overlapping (MacKay-Sim et al., 1982), but the details of the distribution of the individual cells with different odorant sensitivities remains unclear. This question, together with that of nerve projection to the olfactory bulb, is involved in the mechanism of olfactory discrimination and might be elucidated through measurements of odor-response properties in many groups of neighboring receptors. The tissue printing technique may provide a method for confirming the local distribution of receptor neurons identified their odorant responsivities.

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