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TIME COURSE OF THE MEMBRANE CURRENT UNDERLYING SENSORY TRANSDUCTION IN SALAMANDER OLFACTORY RECEPTOR NEURONES

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SUMMARY

1. Odour elicited currents in freshly isolated olfactory receptor neurones were analysed using the whole-cell patch-clamp technique. Brief pulses (35–50 ms) and steps (100 ms–5 s) of odour solution were delivered by pressure ejection from a nearby micropipette.

2. Pulses of odour solution directed at the cell induced an inward depolarizing current of 50–750 pA leading to the generation of action potentials. The *I–V* relation for this current was linear over the range −60–+20 mV and showed a reversal potential of +5 mV. The magnitude of the current increased with stimulus strength, for a given pulse duration, over approximately one decade of concentration change.

3. Pulses of odour solution focally delivered to the cilia elicited a large response, but those directed toward the soma did not. Conversely pulses of K^+ solution at the cilia failed to evoke any response while those directed at the dendrite and soma elicited an inward clamp current. This provides direct evidence that odour sensitivity is localized mainly to the cilia and possibly the distal dendrite.

4. The odour elicited current activated with a long latency of 150–600 ms after the odour solution arrived at the cell. This latency, as well as the time-to-peak and the rise half-time, were relatively independent of stimulus concentration, changing less than 25% over the entire concentration range of stimulus sensitivity. These observations are consistent with the participation of a second messenger system in olfactory transduction.

5. For brief stimulus pulses less than 100 ms, the stimulus diffused away before the odour response current reached its peak value, so that the peak and decay of the odour response occurred in the absence of significant odour stimulus. The time course of the current decay was fitted by a single exponential with a time constant that was concentration dependent, varying from 0·8 to 1·3 s.

6. For longer steps of stimulus presentation, up to 1 s, the magnitude of the response current became a function of the duration of the pulse as well as the stimulus concentration, indicating that the transduction process involved an integrating step. This is consistent with the idea that the odour elicited current is the

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result of the summation of many smaller unitary events. From responses to weak stimulation an integration period of 700–1000 ms was calculated.

7. During prolonged steps of maintained stimulus presentation (> 5 s) the odour elicited current was transient. After reaching a peak in less than 1 s the current remained at or near this level for an additional 1–2 s and then decayed exponentially with a time constant of 4–6 s. In about 40 % of the cells the decay was to a tonic level of about 30 % of the peak current.

INTRODUCTION

It is now generally believed that olfactory transduction occurs through the interaction of odour molecules with membrane bound receptor proteins (Lancet, 1986), in analogy with other receptor–ligand interactions. Additionally, all the elements of a cyclic AMP based second messenger system have been identified in olfactory neurones (Jones & Reed, 1989; Nakamura & Gold, 1987; Pace, Hanski, Salomon & Lancet, 1985; Sklar, Anholt & Snyder, 1986) further indicating a similarity in mechanism between olfactory and other signal transducing systems (i.e. photoreception (Yau & Baylor, 1989) and chemical synaptic transmission (Levitski, 1988; Kaczmarek & Levitan, 1987)).

Crucial to understanding the physiological mechanisms of this process is a direct measure of the sensory response of individual cells to a well-defined stimulus, but this has proved difficult in olfactory receptors (cf. Getchell & Shepherd, 1978*a*). One problem is that both the timing and intensity of the stimulus are difficult to control since the nasal epithelium is covered with a layer of mucus through which the odour molecules must diffuse to the putative receptor sites. Further complications arise because, in addition to the sensory conductance, these cells possess a family of voltage-gated conductances which convert the receptor potentials into action potentials (Firestein & Werblin, 1987*a*; Trotier, 1986; Suzuki, 1989). This makes it impossible to measure the pure sensory response without clamping the membrane potential to subthreshold voltages. The inaccessibility of the cells in the epithelium has proved to be a serious obstacle to these experiments as well.

Recently the whole-cell patch clamp has been successfully utilized to voltage clamp individual olfactory receptor neurones, both in an epithelial slice (Firestein & Werblin, 1989; Trotier, 1989) and in isolated cells (Firestein & Werblin, 1987*a*; Trotier, 1986; Kurahashi, 1989), enabling odour induced currents to be measured. Some preliminary measurements of the time course and intensity of the odour induced current have also been obtained (Firestein & Shepherd, 1989*a*). From these studies it appears that the odour elicited current is an inward cationic current which reverses near 0 mV and is graded with stimulus intensity (Firestein & Werblin, 1989; Kurahashi, 1989).

Still lacking are data regarding the precise time course of the odour response. This information is important since kinetic measurements could give useful clues to the molecular mechanisms underlying the regulation of the sensory membrane conductance. They will also be essential to further studies of the actions of second messengers and the mechanisms of adaptation and desensitization. The primary aim of this paper is to provide such measurements and to suggest certain constraints which they place on possible models of the transduction process.

METHODS

Animals and preparation

Olfactory receptor neurones were isolated from the nasal epithelium of land phase adult salamanders, *Ambystoma tigrinum*. The animals were housed in a terrarium with mud and water at room temperature and were fed live earthworms. This level of care was found to be important in recovering sufficient numbers of cells with robust odour responses. Prior to the experiment animals were cooled to 4 °C and decapitated. The nasal cavities were opened and the flat sheets of nasal epithelium on the dorsal and ventral walls were dissected free of the underlying cartilage in single pieces. The tissue was placed in a cold Ringer solution and left in the refrigerator (4–6 °C) for at least 1 h. This had the effect of partially solubilizing the mucus, making it easier to dissociate cells with intact cilia. Normal Ringer solution contained (mM): NaCl, 120; KCl, 2·5; $CaCl_2$, 4; $MgCl_2$, 0·2; HEPES, 5; glucose, 5; pH 7·6. Cells could be maintained in good condition in this environment for at least 8 h.

Immediately prior to recording, one piece of the dissected epithelium was placed in 1·5 ml of Ringer solution and cut into small pieces with a dissecting scissors. No enzymes or proteases were added. The pieces were drawn into a pasteur pipette (tip fire-polished to approximately a 0·5 mm diameter opening) and gently triturated until the solution appeared cloudy. After waiting for the remaining large pieces to settle to the bottom (about 5 min) approximately 1 ml of the solution was pipetted onto a glass cover-slip. The cells settled onto the surface of the slide in 20–30 min. No attempt was made to adhere the cells to the glass surface. Debris in the solution was removed by a few minutes of perfusion with Ringer solution but otherwise it was found that constant perfusion of the bath was not needed. From visual inspection the cells appeared to be free of mucus and other debris and it was not necessary to 'clean' their surfaces in order to obtain 10 GΩ seals.

Recording

Olfactory neurones are particularly well suited for the whole-cell patch-clamp technique. Their simple geometry includes a large cell body (12–20 μm in diameter), and a single dendrite 2–3 μm in diameter and 2–100 μm in length *in situ* (Rafols & Getchell, 1983), with a tendency for the mean dimensions to be thicker (2–5 μm) and shorter (2–50 μm) in our dissociated cells. Diffusion between the soma and cilia is rapid; when the patch pipette was filled with a 1% Lucifer solution, fluorescence could be seen in the cilia within 3–4 min of patch rupture. Electrotonic coupling between the soma and cilia is also close; in our cells with input resistances of 3–5 GΩ (cf. Firestein & Werblin, 1987*a*) we estimate that the length of the dendrite plus cilia is 0·1–0·3 of a characteristic length constant for the equivalent cylinder (Pongracz, Firestein & Shepherd, 1989).

During the course of the experiments described here the series resistance was checked frequently to be sure it was less than 10 MΩ. For most of the experiments, excepting those concerning response localization, we chose cells with dendrites less than 20 μm long. Thus space clamping is more favourable than in most neurones and should be well within the bounds required to analyse the slowly activating sensory current.

Recordings were made with a List EPC-7 patch clamp using patch electrodes of borosilicate glass (TW-150 from WPI, New Haven, CT). Electrodes were manufactured on a Narashige PP83 puller using a double stage pull. The standard electrode solution contained (mM): KCl, 125; $MgCl_2$, 2; $BaCl_2$, 0·1; EGTA, 2; ATP, 1; GTP, 1; HEPES, 5; pH 7·6. With calcium buffered so strongly we found that inclusion of 100 μM-$BaCl_2$ aided in forming a gigaseal. At this concentration there was no measurable effect on the odour elicited current, the voltage-gated currents or the membrane resting potential.

Data were recorded directly to the hard disc of an AST brand IBM compatible PC AT computer using the FASTLAB software package (INDEC Inc, Sunnyvale, CA) and Tecmar D/A interface. The records were filtered at 3·5 kHz with a low-pass 8 pole Bessel filter and sampled at between 100 and 1000 points s^{-1}. Analysis was performed on the digitized data using several commercial software packages which are noted in the appropriate sections of the text.

Stimulus delivery and calibration

The odour stimuli were delivered by pressure ejection from a micropipette with a lumen 1·5–2 μm in diameter. Pressure pulses were regulated by a Picospritzer (General Valve, Fairfield, NJ) that was controlled from within the computer program. The ejection pipette was placed, under visual

control, within 10–50 μm of the cell. Cells and pipette were visualized using an Olympus inverted microscope with a 40× LWD objective and 10× eyepiece (total magnification = 400×). The objective and condenser were fitted with Hoffman Modulation Contrast optics.

The stimulus for all experiments consisted of a mixture of three odours, acetophenone, isoamyl acetate and cineole. Since there is no way to tell *a priori* what odour or odours a particular cell may respond to, this mixture was used throughout to increase the likelihood of recording from a responsive cell. Most odorous substances are small hydrophobic molecules with limited miscibility in water, which places limits on the stimuli that could be used. The three odours selected for the mixture were intended to be representative of several odour 'qualities', and also to be sufficiently soluble in water to attain appropriate concentrations. They are among those commonly used in olfactory studies (see Sicard & Holley, 1984) and also are among those known to activate a ciliary adenylate cyclase (Sklar *et al.* 1986). Using the formula weight, density and solubilities (The Merck Index, 1983) a 10^{-2} M stock solution of each odorous substance was prepared. All further dilutions and mixtures were prepared from these stock solutions. In general, the pipette concentration was 0·1 or 1 mM of each odour substance.

The duration of the odour pulses could be varied from 10 ms to several seconds. Very brief pulses (15–50 ms) were used to approximate a near-instantaneous stimulus, for the purpose of analysing response latency and other response kinetics. In addition this brief duration minimized response desensitization (Getchell & Shepherd, 1978*b*). Longer duration steps (0·5 s or longer) were used to analyse further the time course and adaptation of the response. Stimulus intensity was controlled by the ejection pressure, which was varied between 0·5 and 10 lbf in^{-2} (3·5–67 kPa).

The problem of monitoring the stimulus is a difficult one in olfactory studies (Getchell & Shepherd, 1987*a*). Previously we have described a method of estimating the odour concentration at the cell membrane by including an elevated concentration of K^+ in the stimulus mixture (Firestein & Werblin, 1989). In this method the magnitude of the clamp current that flows in response to a pulse of 100 mM-K^+ solution is used as a measure of the concentration of solution (including both K^+ and odours) delivered to the cell by that pulse. This method provides an accurate monitor of the stimulus delivered to the receptor neurone.

Isolated cells have offered several advantages for the control and monitoring of the stimulus over *in situ* preparations. The cells were entirely in an aqueous solution (Ringer) so that there was no phase boundary through which the odour molecules had to partition for access to the ciliary membrane. Many of the factors introduced by complex diffusion paths (e.g. through a mucus layer) were therefore avoided.

Similarity of movement of odour molecules and K^+ ions

For the purpose of monitoring the stimulus time course, we assume that within the ejected solution the odour molecules move at approximately the same rate as do the K^+ ions, and that the solution reaches the entire cell membrane, including odour sensitive and K^+ conductance regions, at approximately the same time. These assumptions are reasonable for the following reasons.

For the compact cells with short dendrites the volume of the cell was several orders of magnitude smaller than the volume of ejected solution (picolitres *vs.* nano- or microlitres). The stimulating pipette could be placed within 10–25 μm of the cell so that an ejected solution would rapidly arrive at and completely engulf the cell (Fig. 1). We checked this by including the dye Fast Green in the ejected solution and noted that a bolus of solution several times larger than the cell was ejected and almost instantaneously surrounded the entire cell. Only when the pipette was moved further than 35 μm away from the cell could a spreading of the solution be detected.

Since the arrival of the solution at the cell surface was by pressure ejection, diffusion effects were minimized during this time. The K^+ current began within a few milliseconds of the electrical activation of the pressure-controlling solenoid and reached a peak magnitude in another 20 ms. These times are much faster than could be accounted for by diffusion. The K^+ response could thus be considered a reasonably faithful representation of the time course of arrival of the odour molecules as well, since the force of the ejection was likely to be more than sufficient, over these short distances, to overwhelm the differences in diffusion coefficients between K^+ ions and odour molecules.

Although the pressure was applied only briefly for the pulses, the stimulus solution disappeared more slowly. We assume that the decay of the K^+ current represented mainly passive diffusion of K^+ ions away from the cell. Since the K^+ ions and the small, low-molecular-weight odour molecules

have diffusion coefficients within the same order of magnitude ($1–2 \times 10^{-5}$ cm^2 s^{-1}; see below), it is likely that they diffused away with similar time courses. This estimate for the diffusion coefficients of small odour molecules is reasonable (see Results).

It is possible that odour–receptor interactions could have retarded diffusion of the odour molecules, but there are reasons for believing that this is not a significant effect. The volume of the

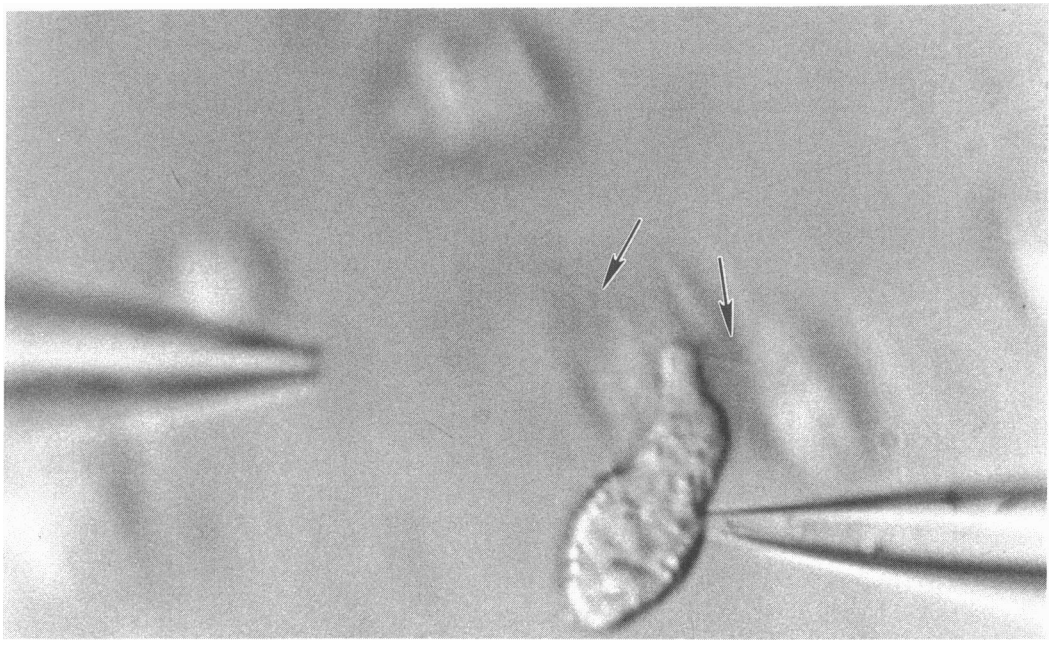

Fig. 1. Photomicrograph of an isolated receptor neurone attached to the tip of a patch pipette. This cell was morphologically compact (compare it to the cell shown in Fig. 4, for example) and was typical of the type chosen for these experiments. Two of the cilia, which at 0·25 μm in diameter are near the limit of resolution, are marked by arrows. The stimulus carrying pipette is on the left. Note that it can be brought quite close to the cell and that a bolus of solution forcefully ejected from the pipette would rapidly engulf the cell. The optics included a 40 × objective with Hoffman modulation optics. Scale bar = 20 μm.

bath into which bulk diffusion occurred was several orders of magnitude greater than that of the ejected solution (millilitres compared to nanolitres), so that the K^+ current was at least a good measurement of free (unbound) odour concentration in the region of the cell. The relatively low affinity ($K_D = 10^{-6}$ M) measured for these receptors (Firestein & Werblin, 1989) makes it probable, by the law of mass action, that the change in bound concentration closely followed the change in free concentration. Similarly, odour molecules are not likely to remain adsorbed or trapped in the lipid plasma membrane since the affinity between the hydrophobic odour molecules and the lipid is rather weak (Hornung & Mozell, 1981). Further, any error associated with the difference in K^+ and odour diffusion is likely to be restricted to the early part of the stimulus pulse when the concentration is highest. As the concentration of free odour falls below the K_D one would expect any effects due to odour–receptor, or odour–membrane, interactions to be minimized. These

considerations provide the basis for using the K^+ current as a reasonably accurate and direct monitor of the pulsed odour stimulus.

The decay of the K^+ current often overlapped the onset of the odour elicited current. A function describing the K^+ current decay, i.e. the stimulus solution decay, would permit the subtraction of the K^+ current from the overall current, revealing both the complete time course of the stimulus decay and the early part of the odour current. Since the decay of this current was due to diffusion we tried a simple exponential fit and found that this provided an adequate description. With the program ENZFITTER (Biosoft, Inc., Cambridge, UK) it was possible to determine acceptable values for the time constant from just the first 250–300 ms of the current trace. With these values and the measured peak value the entire curve could be generated. This procedure was checked by successfully applying it to families of K^+ current from three cells.

RESULTS

General characteristics of the odour response

In this study ninety-five cells were successfully voltage clamped by the whole-cell patch method. Of these, fifty-four gave a measurable and repeatable response to odour pulses. The other thirty-nine cells showed no detectable response to the odour pulses, although they possessed a full complement of voltage gated conductances (Firestein & Werblin, 1987*a*), a normal response to K^+ stimulation, and appeared morphologically normal. In cells that did not respond to odours there was no sign of a pressure artifact from the stimulus pulse; the current trace was completely flat even when stimulus pulses were > 500 ms and 10 lbf in^{-2}.

We first examined the membrane potential changes in response to a brief pulse of odour for comparison with intracellular recordings from *in vivo* preparations (Trotier & MacLeod, 1983). As illustrated in Fig. 2*A* (top trace) the response began with a graded depolarization; after a rapid rise to threshold an action potential was generated, followed by a second smaller spike and then a series of membrane oscillations. This behaviour approximates that of cells in more intact preparations except that long spike trains are not found, presumably due to the absence of the axon. It bears a close similarity as well to the response to a depolarizing (positive) input current (Trotier & MacLeod, 1983; Masukawa, Hedlund & Shepherd, 1985). An example of this is shown in Fig. 2*B* for comparison.

The main focus of our analysis was the inward cationic current underlying the graded depolarization elicited by the odour pulse as measured under whole-cell voltage clamp. The *I–V* relation for this current, shown in Fig. 3*A*, was linear in the physiological (mean resting potential $= -56 \pm 5$ mV S.D., $n = 12$) range of -60 to $+20$ mV and reversed at $+5$ mV. The time course of the current was not affected by the holding potential. Figure 3*B* shows the relation between stimulus intensity and current magnitude for a typical cell. As we found in receptor cells in epithelial slices, the current magnitude was dose dependent and the dynamic range of the cell was rather narrow, covering approximately one log unit of concentration change. Thus the same general characteristics prevailed in the isolated olfactory neurones used in this study and those from the more intact slice preparation (cf. Firestein & Werblin, 1989).

The peak amplitude of the responses to the stimuli used in this study ranged from 30 to 700 pA. The rising phase of the responses tended to follow a sigmoidal time course, whereas the decay was generally exponential. In about 20% of the cells the

onset and decay were nearly symmetrical. Occasionally ($n = 5$) we found cells which responded with a large (> 400 pA) current to the first pulse of odour but were completely unresponsive to further stimulation, even after waiting for 5 min.

Aside from these abrupt changes, there were sometimes smaller changes in subsequent responses, due presumably to 'wash-out' effects. For the most part this

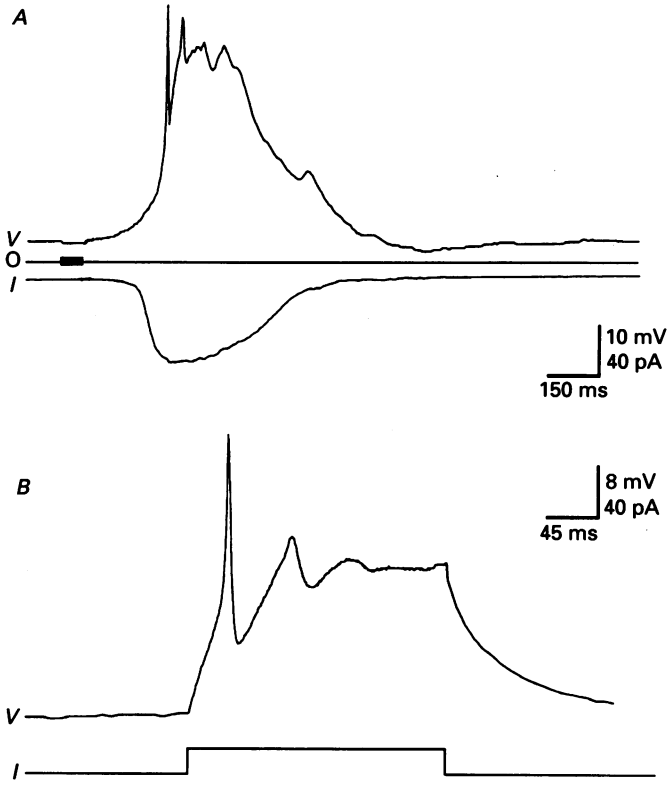

Fig. 2. Responses of an olfactory neurone to odour and current. *A*, the top trace (*V*) is the voltage response to a 50 ms pulse of the odour mixture (middle trace, O) recorded under current-clamp conditions. Lower trace (*I*) is the current response, recorded under voltage clamp, to an identical stimulus pulse. *B*, voltage response (*V*) to a 20 pA depolarizing current (*I*) step in the same cell as *A*. The single large spike followed by a smaller spike and a series of membrane oscillations is typical for isolated olfactory neurones without their axons. For all traces membrane potential (V_m) or holding potential (V_h) is -60 mV.

was not a serious problem; repeatable odour responses have been obtained in some cells for more than 60 min, including as many as 50 stimulus pulses. Typically a cell gave reproducible responses for 30–40 min depending on the duration and timing of the stimuli.

Despite this degree of response heterogeneity, which could have been due in part to the use of a mixture of odours as the stimulus, the odour elicited currents all appeared to be due to a kinetically distinct conductance. There was no evidence, at membrane holding potentials ranging from -45 to -80 mV, of an outward current component in response to odour pulses. Nor did the odour stimulus appear to

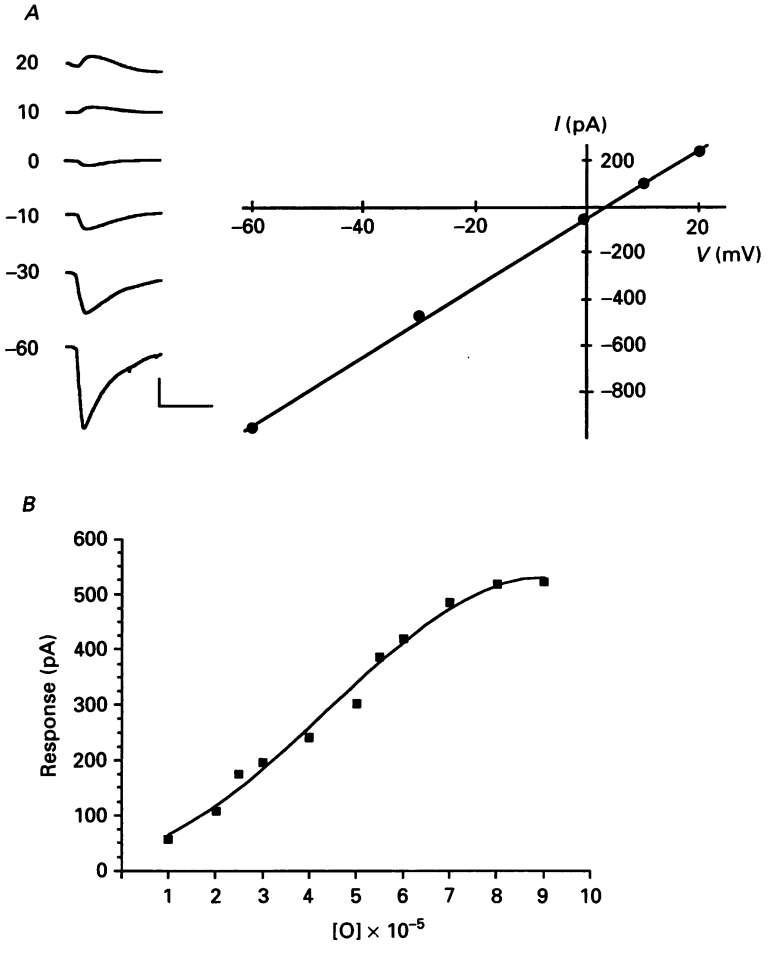

Fig. 3. General characteristics of the odour response in isolated cells. *A*, the current–voltage relation is linear through the physiological range of membrane potentials and reverses near +5 mV. The normal resting potential for these cells is around −55 mV. On the left are representative current traces with the holding potential at which each trace was recorded. The stimulus was a 50 ms pulse of the odour mixture at 0·1 mM. Scale bars for the traces are 100 pA and 4 s. In these experiments CsCl replaced KCl in the pipette (intracellular) solution to block the effects of K^+ currents. Holding potentials were established for several seconds before the stimulus was delivered in order to inactivate the Na^+ current. *B*, dose-response curve for a typical isolated cell. The concentrations were estimated by the method of Firestein & Werblin (1989). The pipette contained the odour mixture at 1 mM but the cell response appeared to saturate near 0·1 mM. The narrow dynamic range (about a decade of concentration change from threshold to saturation) and the sensitivity range are both similar to that found in cells from epithelial slices.

produce any direct effects on the various somatic voltage-gated currents which were checked periodically during the course of an experiment by applying a series of depolarizing pulses to the cell.

Localization of the response

The cells which responded to odour had six to twelve (occasionally more) cilia which ranged in length from 20 to 60 μm. In approximately two-thirds of these cells the cilia were motile, their movements ranging from a slow, regular waving motion to a more rapid but asynchronous 'flicking' movement. In some cells the cilia appeared to extend rigidly from the dendritic knob. Cells with motile cilia (of either the slow or rapid type) were preferred for recording on the assumption that this was an indication of better physiological condition. However, in many cases the cilia gradually lost their motility during the course of the experiment with no detectable change in the odour response. Recordings were made from an additional eight cells without observable cilia for purposes of calibrating the K^+ response and none of these had any response to the odour pulses. This is consistent with the notion that the sensory response is generated in the cilia.

This notion was tested directly in a number of isolated cells which possessed long, thin dendrites (50–100 μm length and 2·5 μm diameter). In these cells the cilia and soma were sufficiently separated so that the odour pulse could be restricted to either region. When the odour pulse was directed toward the region of the cilia and distal dendritic knob (Fig. 4*A*), a large inward current was observed (Fig. 4*B*). By contrast, when the stimulus was directed toward the region of the soma and proximal dendrite (Fig. 4*D*), little or no current response was seen (Fig. 4*E*). When the ejection pipette contained 100 mM-KCl there was only a small, delayed current from cilia directed pulses (Fig. 4*C*) while a large, fast current was elicited by pulses directed at the soma (Fig. 4*F*).

We rarely saw a pressure artifact in the current trace, but in the response to the pulse directed toward the soma shown in Fig. 4*E* there was a small pressure artifact which served to mark the arrival of the solution at the soma. Nearly 1 s later a small inward current developed. In the experiment the dye was seen to engulf the cell rapidly and then slowly diffuse away. The delayed current response appeared to be the result of diffusing odour molecules reaching the ciliary region of the cell.

The delay of the response provided an opportunity to estimate the diffusion coefficient for the small odour molecules. The distance from the centre of the soma, which we take to be the approximate centre of the pulsed bolus of odour solution, to the cilia was 90 μm. The normal response latency (i.e. not due to diffusion), taken from the record in Fig. 4*B*, was about 200 ms. Therefore the time required for odour molecules to diffuse a distance of some 90 μm was 0·8 s. This permitted us to estimate the diffusion coefficients of the odour molecules in solution (cf. Hille, 1984, pp. 154) from,

$$r^2 = 6Dt, \tag{1}$$

where r^2 is the mean square displacement in three dimensions of the molecule, D is the diffusion coefficient, and t the time in seconds. From this we calculated the diffusion coefficient of the odour species to be $1{\cdot}7 \times 10^{-5}$ cm^2 s^{-1}. This is in reasonable

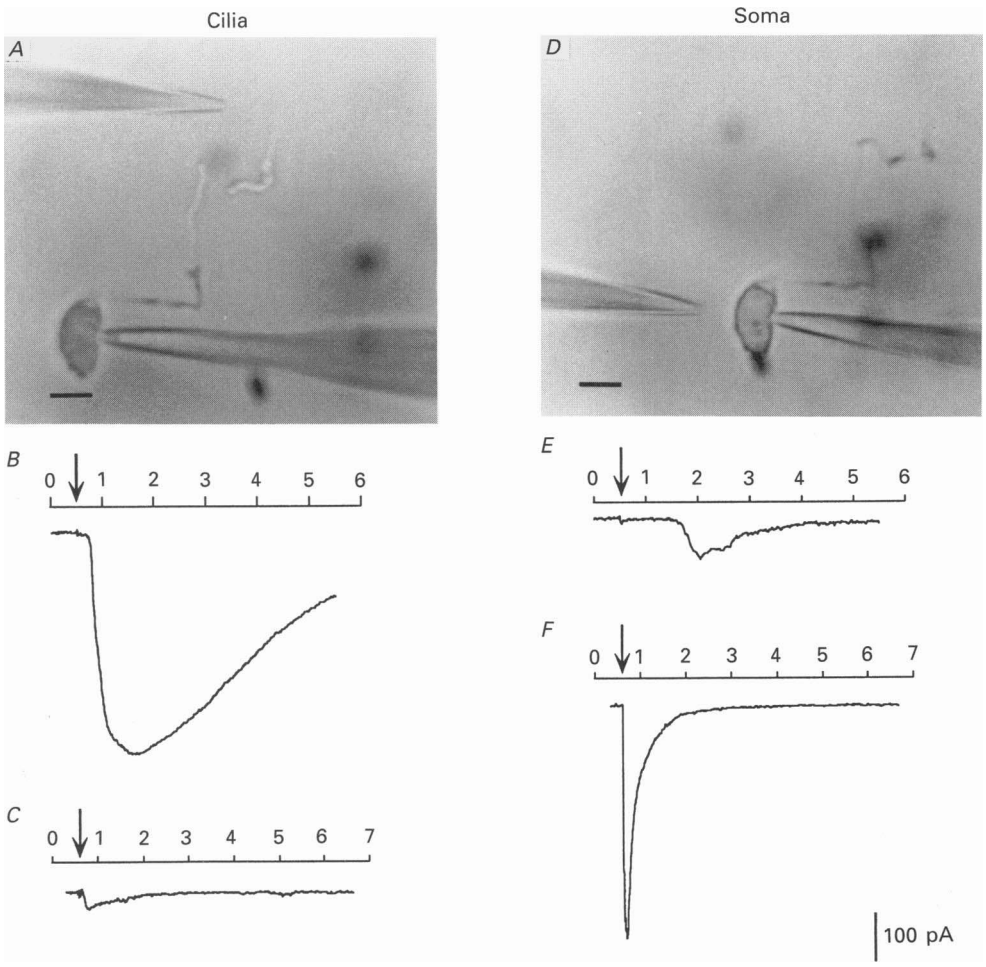

Fig. 4. Segregation of the response to odour and K^+ in different regions of the cell. Due to the length of the dendrite in this cell (~ 200 μm), it was possible to direct the stimulus either to the distal dendrite and ciliary region or to the soma. In *A*, note that the soma lies behind the tip of the pipette and away from the direction in which the stimulus solution was being directed, while in *D*, the odour solution was ejected directly at the soma. Scale bar is 15 μm. *B*, a 35 ms, 3 lbf in^{-2} pulse (arrow) of 0·1 mM odour solution directed at the cilia resulted in a large inward current. For a similar pulse directed at the soma (*E*) there was no response for nearly a second, during which time some of the odour presumably diffused to the ciliary region. Conversely when a pipette which contained only 100 mM-KCl was similarly positioned there was virtually no response from pulses directed at the ciliary region (*C*) while pulses directed at the soma (*D*) elicited a large current response (*F*). $V_h = -65$ mV.

agreement with previous estimates based on the size and weight of these substances (Getchell, Margolis & Getchell, 1984; Tucker, 1963). It is also within the range of other small non-electrolyte molecules (Hille, 1984, p. 172).

We carried out four additional experiments with cells possessing long dendrites. In all cases, the results were the same: the cilia gave the largest response, usually

saturating at more than 250 pA, and the soma gave the smallest response, usually less than 25 pA. The transition appeared to occur in the region of the dendritic knob and distal part of the dendrite. These experiments indicate that the site of sensory transduction is primarily, possibly exclusively, limited to the ciliary region and perhaps the most distal part of the dendrite.

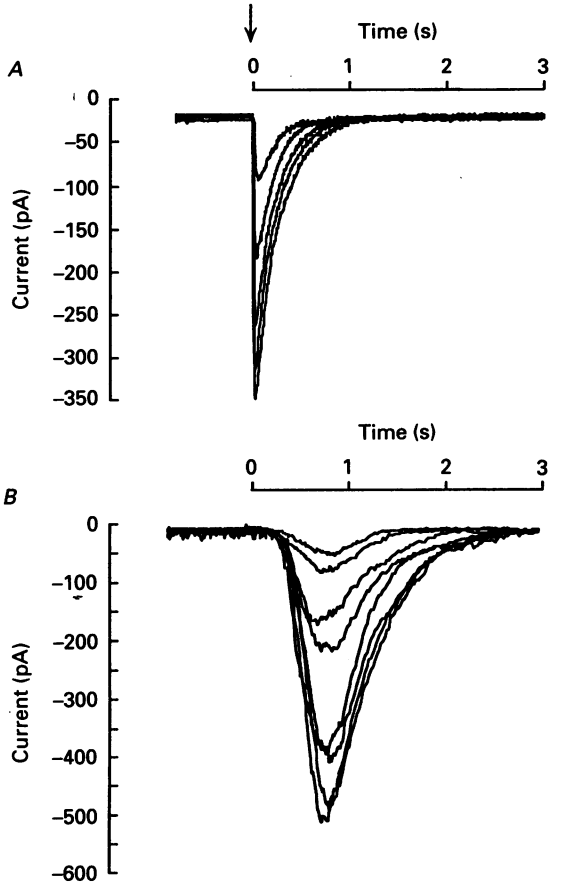

Fig. 5. Time course of the cell responses to identical pulses of K^+ and odours differed by an order of magnitude. *A*, family of responses to a 35 ms pulse of 125 mM-KCl at 1–6 lbf in^{-2} from a pipette placed 30 μm from the cell. The arrow marks activation of the solenoid controlling the pressure valve. The K^+ current activated within 10–20 ms of switching the pressure on and rose to a peak within another 40 ms. By contrast the current elicited by the odour stimulus delivered from an identically placed pipette (but at different cell) activated after nearly 400 ms and required an additional 400–500 ms to reach peak amplitude. $V_h = -60$ mV.

Time courses of the stimulus pulse and the sensory response

By mixing the odours in a 100 mM-potassium solution we were able to monitor not only the amplitude of the stimulus pulse but also the time course of the stimulus solution at the cell surface. This was possible because the cell response to the elevated K^+ was very rapid compared with its response to the odour molecules, even though

both the K^+ ions and the odour molecules arrived at the same time. This can be seen clearly in Fig. 5. Thus the clamp current which flowed in response to the sudden increase in extracellular K^+ began within 10–20 ms from the activation of the pressure-controlling solenoid and reached its peak amplitude within another 20–40 ms. The response to odour molecules pulsed from an identical pipette situated the same distance from the cell did not begin for at least 250 ms and reached its peak amplitude after some 500 ms. While the time course of both responses can be varied by changing the position of the ejection pipette the odour response was always an order of magnitude slower than the K^+ elicited current. On the basis of these properties it appeared justified, for the purposes of carrying out a kinetic analysis of the onset and rising phases of the odour response, to approximate the onset of the stimulus pulse as an instantaneous step to its peak value.

The time course of the K^+ current decay, described in the Methods, could be well approximated by a single exponential. It is due, primarily, to diffusion into the bulk medium. The effect of the stimulus decay on the shape of the odour response will be treated later.

Responses to brief pulses of odour

With brief pulses of odour stimulation (< 50 ms) we could explore some kinetic features of the odour current over a range of stimulus and response amplitudes. The families of responses shown in Fig. 6 were representative of the recordings analysed. Six other cells were treated similarly and less complete data from an additional twelve cells will also be noted.

The currents shown in Fig. 6 are taken from two cells which represent the most commonly encountered response types. In both cases the stimulus consisted of brief (35 ms) pulses of odour at increasing pressures. The symmetrical shape of the response in the cell of Fig. 6*B* is noteworthy. Responses to weak stimulation tended to be symmetrical in all cells, but in 80% of the cells responses to stimulation at higher concentrations became asymmetrical, as typified by the responses shown in Fig. 6*D*. The peak became sharper and the decay slower as stimulus concentration increased. The largest current shown in these records was the maximal current that could be achieved. Stronger pressures elicited larger K^+ currents but no greater odour current. The maximum odour elicited currents, in all the cells recorded, ranged from 330 to 535 pA (mean = 445 pA, $n = 12$).

Neither response type, the symmetrical or asymmetrical, could be definitively correlated with increased or decreased sensitivity to the stimuli presented, but it was our impression that cells with symmetrical type responses tended to respond at lower odour concentrations and gave larger maximal current responses. As can be seen from inspection of the traces, most of the differences in these response types were in the peak and decay phases of the response. The main features of the onset phase were more consistent.

Onset kinetics

Perhaps the most notable kinetic feature of the odour response was the latency from stimulus arrival to the onset of the odour elicited current. The arrows in Fig. 6 mark the arrival of the stimulus, i.e. the start of the now subtracted K^+ current. The latencies ranged from 425 to 570 ms (Fig. 6*A* and *B*) and 180 to 280 ms (*C* and

D) over the full range of stimulus strength, from threshold to saturation. For the population of measured cells, the latencies ranged from 150 to 660 ms, with a mean of 350 ms ($n = 10$).

In the cells depicted in Fig. 6 the latency did not vary appreciably with increases in concentration, becoming only 25–30% shorter over the full response range. In

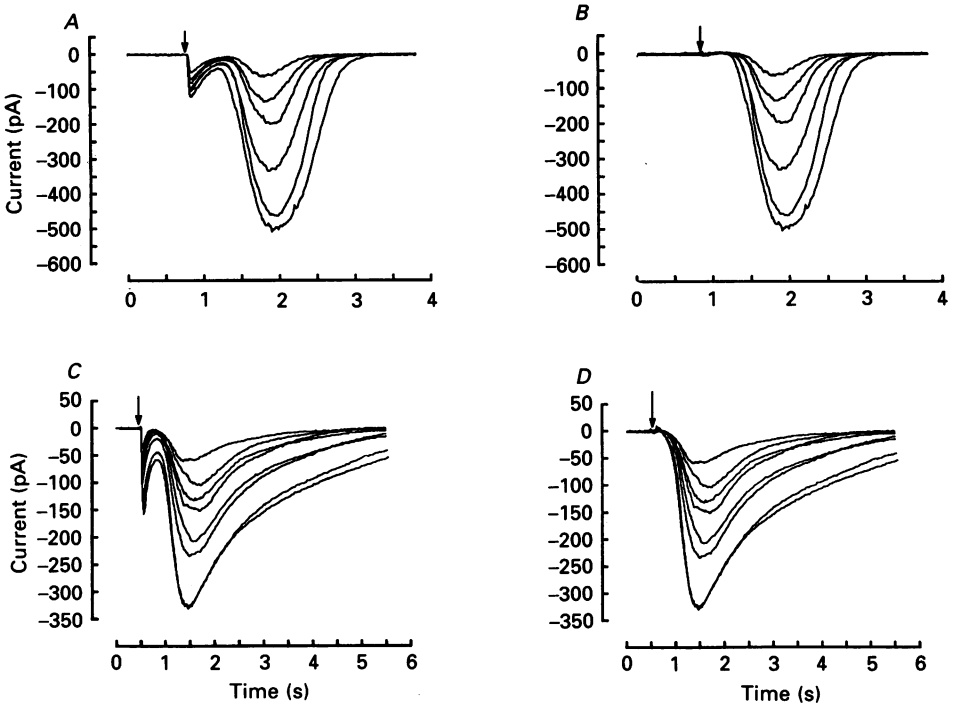

Fig. 6. Families of superimposed responses to 50 ms pulses of solution (arrows) containing 1 mM-odour mixture and 100 mM-KCl. *A*, a cell of the symmetrical response type with responses to both K^+ and odour. *B*, the same cell with the K^+ elicited currents subtracted out. *C*, a cell of the asymmetrical response type with both K^+ and odour elicited currents. *D*, as for *C* with the K^+ current subtracted. Note the differences in both the time and response scales between the two cell types. $V_h = -60$ mV. Arrows mark the arrival of the solution at the cell surface.

some cells the latency displayed a stronger concentration dependence, but even at its most labile it never varied by more than 40% over the full response range.

Two other parameters describing the kinetics of the onset phase, the time-to-peak (t_p) and the half-time-to-peak ($t_{\frac{1}{2}}$), were measured. For the cell of Fig. 6*B* all three values are plotted *versus* concentration in Fig. 7*A*, and in Fig. 7*B* the fractional change is plotted as a function of stimulus concentration. Similar to the latency, neither of these two parameters showed strong concentration dependence, although from cell to cell there was a considerable variability. For example, the range of t_p for this population of cells was 0·75–1·2 s (differing by 42%), but for any individual cell the decrease in t_p with increasing concentration, up to a saturating concentration, was less than 30%.

A similar treatment of the other cell type, that of Fig. 6*D*, is shown in Fig 7*C* and *D*. For this cell the values of the three parameters and the fractional change are plotted *versus* normalized response instead of stimulus concentration. This emphasizes that for the low and mid-range responses the onset kinetics varied very

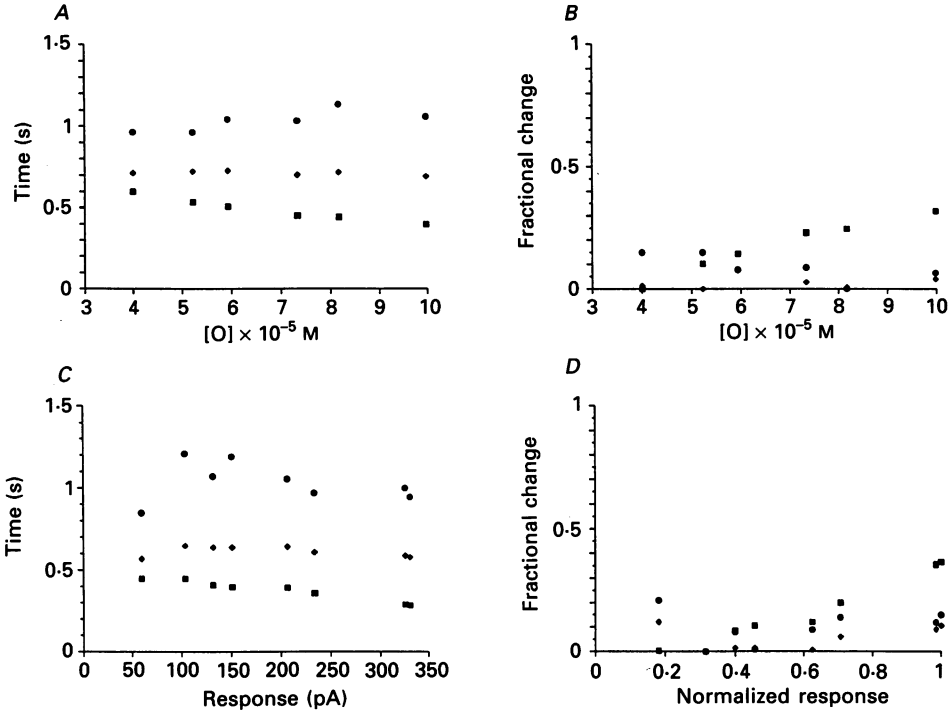

Fig. 7. The kinetic parameters latency (■), half-time (◆) and time-to-peak (●) plotted against the magnitude of the stimulus (*A*) or response (*C*) are shown for the two representative cells of Fig. 6. *A*, for the symmetrical response type of cell the kinetic parameters are plotted *versus* stimulus concentration. *B*, the fractional change in these kinetic measures over the concentration range tested. This was arrived at by dividing the kinetic value at a given stimulus concentration by the largest (i.e. slowest) value over the entire range (usually the value for the weakest stimulus). *C*, for the non-symmetrical cell of Fig. 6*C* and *D* the kinetic values are plotted *versus* response amplitude rather than stimulus magnitude. *D*, the fractional change is also plotted *versus* response amplitude.

little; only for the largest, saturating responses was there an appreciable decrease in the latency, half-time and time-to-peak.

Since these parameters were fairly constant over a normal concentration range, it appeared that the rate of activation of the sensory conductance, i.e. the rate of rise in the current to its peak amplitude, would also be constant over the response range of the cell. This is brought out in Fig. 8 where the responses of the two representative cells have been normalized and replotted. For low and mid-range concentrations the activation time courses of the responses were nearly identical. Even at saturating concentrations the increase in rate of rise was less than 10%. This relative

concentration independence of the onset kinetics was a general finding among all the cells from which we recorded.

Kinetics of the termination phase

From records such as those shown in Fig. 6 it appears that the stimulus had decayed to less than 5% of its peak value by the time the sensory current reached

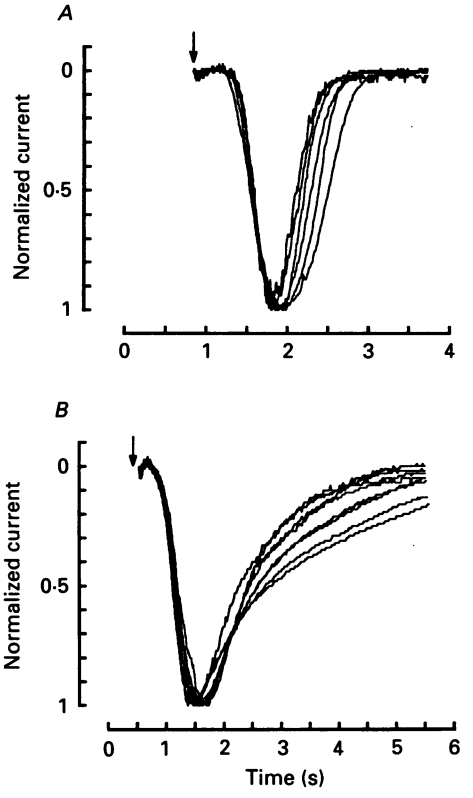

Fig. 8. When all the responses are normalized to the same peak amplitude their onset time courses are nearly identical. Arrows indicate the arrival of the stimulus. For all but the largest responses to saturating stimulus concentrations the traces in both cell types overlap during the activation phase of the response. $V_h = -62$ mV.

its peak value. For weak pulses the stimulus had virtually disappeared by the time of the peak of the odour current. This can be seen more clearly in Fig. 9 where the K^+ currents for three pulses (weak, medium and strong) from the cell of Fig. 6*B* are shown with their respective odour elicited currents. Even for the largest of the odour elicited currents the stimulus amplitude had decreased by nearly two orders of magnitude, to less than 5% of its peak value.

Since the odour current has been shown previously to saturate within a log unit of concentration change (Firestein & Werblin, 1989) it seems unlikely that the stimulus concentration represented by the tail of the decaying K^+ current was sufficient to maintain the odour response. It appears, therefore, that the odour current decays

from its peak value in the virtual absence of stimulus, and that the current relaxes back to baseline under conditions which approximate a steady state of essentially negligible stimulus concentration.

For the most common response type, with non-symmetrical time courses, the decay phase could be well fitted by a single exponential. This can be seen in Fig. 10

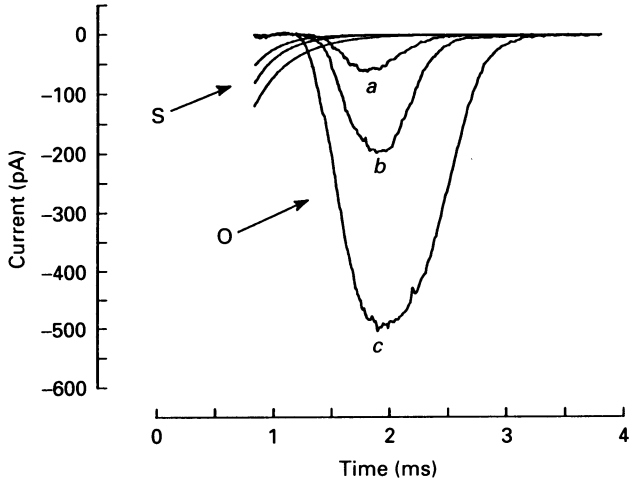

Fig. 9. Comparison of the decay of the stimulus to the time course of the odour elicited current. Three responses, to weak (*a*), medium (*b*) and strong (*c*), stimulus concentrations are shown. The traces labelled with an S are the K^+ elicited currents, normally subtracted out, for these responses and they mark the time course of the stimulus solution. The odour elicited responses (O) activate as the stimulus concentration is decreasing and reach a peak when the stimulus is less than 5% of its maximal intensity. Decay of the odour current occurs in the complete absence of stimulus. $V_h = -60$ mV.

where three responses, from the low, mid and high range, are shown with a dashed curve representing a single exponential function overlaid. The time constants for these three curves are 0·839, 0·999 and 1·34 s. The time constants for the whole family of responses are plotted as a function of stimulus concentration in Fig. 10*B*.

In order to obtain the close fits such as those seen in Fig. 10*A* we excluded the actual peak of the response as well as some portion of the early part of the decay. This can be seen in the figure as a departure of the theoretical curve from the recorded trace. The response peak is the result of a complex interplay between activating and terminating processes. Excluding this period from the analysis permitted us to describe most of the decay phase with a simple function.

Responses to maintained steps of odour

In our initial experiments only brief pulses of odour were used in order to avoid complications due to the effects of adaptation; the concentration of the stimulus was varied by adjusting only the pulse pressure. The responses to maintained stimuli were explored by increasing the duration of the odour pulse with the pressure held constant. Two time ranges, 100–1000 ms and 5–10 s, were used for exploring different characteristics of the response to sustained stimulation.

Many cells which appeared unresponsive to brief pulses of the stimulus mixture

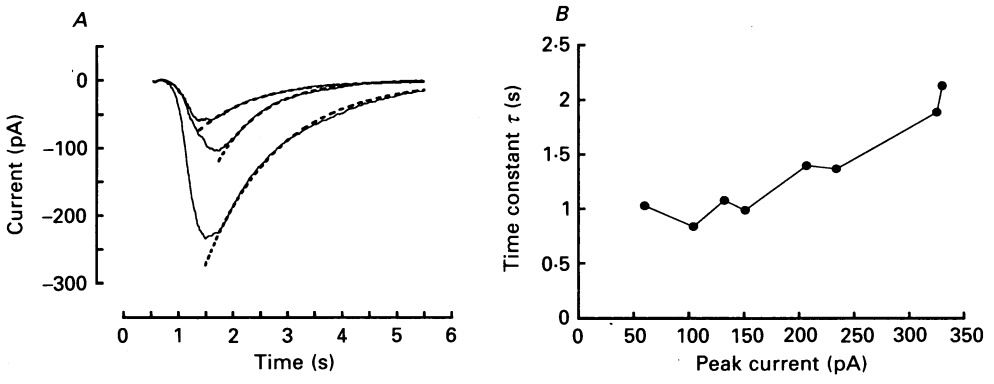

Fig. 10. The decay of the non-symmetrical response type can be well fitted by a single exponential. *A*, three responses to weak, medium and strong, but not saturating, stimulation are shown with exponential curves fitted to the decay phase using the program ENZFITTER (dashed lines). In order to obtain these reasonable fits it was necessary to omit the actual peak of the responses and the dashed lines can be seen to deviate from the response near the peak. $V_h = -60$ mV. *B*, the decay time constants are plotted against the response amplitude and concentration. They are mildly concentration dependent except for the responses to saturating stimuli which are much slower. The concentration of odour which produced these responses ranged from $4{\cdot}2 \times 10^{-5}$ to $4{\cdot}6 \times 10^{-4}$ M.

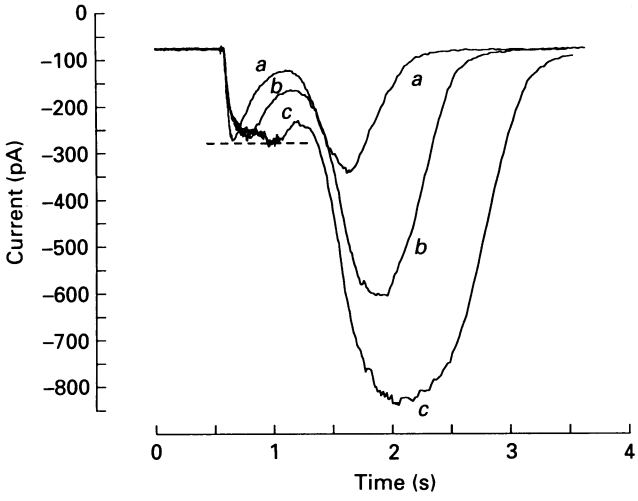

Fig. 11. Three superimposed responses to peak stimulus concentration *versus* stimulus flux. The stimulus, represented by the K^+ currents (the first downward deflection in the traces) was delivered in pulses of increasing duration while the pressure was adjusted to maintain the same peak concentration (dashed line). The odour elicited response currents followed the integral of the stimulus and not the maximal concentration. Pulse duration for the steps was: *a*, 50 ms; *b*, 200 ms; *c*, 500 ms. $V_h = -59$ mV.

would respond to the longer steps, even though the maximum concentration attained during the brief pulse was greater than that for the longer step. This suggested that the sensory response was a function of the stimulus duration as well as its intensity.

This possibility was tested in the experiment of Fig. 11. The stimulus consisted of

the normal odour mixture with 100 mM-K^+. The pulses of odour were varied in duration while the pressure was adjusted to produce the same maximum concentration of stimulus. Since the K^+ current is a measure of the stimulus concentration, the integral under the curve represents the number of molecules in a

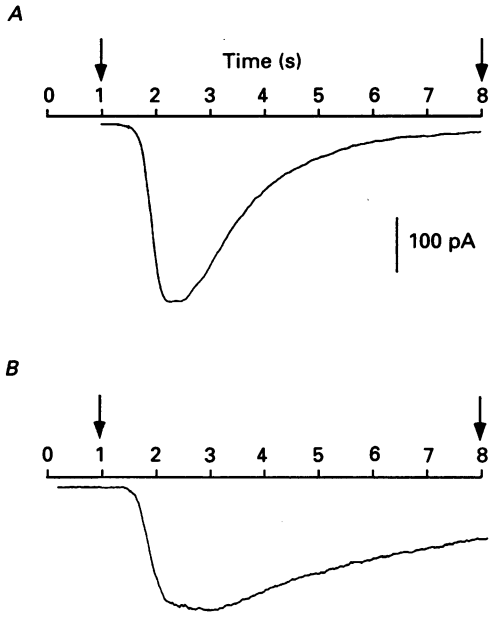

Fig. 12. Adaptation during a maintained step of stimulus lasting 7 s. The odour mixture, at 0·1 mM, was continuously ejected from the pipette at 3 lbf in^{-2} during the period between the arrows. *A*, an example of the more rapidly desensitizing response which occurs within 0·5–1·5 s of the peak response and continues along an exponential time course. *B*, the more protracted type of desensitization in which the response remains at a peak plateau for 1–2 s and declines exponentially to a steady level about 30% of the maximal amplitude. $V_h = -61$ mV.

given time which reach the cell. In the three records of Fig. 11 the steps were 50, 200 and 500 ms in duration. The peak K^+ current, and therefore the peak odour concentration, was the same for all three steps, but the integrals of the K^+ response were quite different. Clearly the amplitude of the odour elicited current followed the integral and not simply the peak concentration. These results suggest that the receptor neurone not only measures concentration but integrates concentration information over some time period. We were unable to determine directly the time period over which this integration might occur because an integral for the K^+ current could not be calculated for steps over 700 ms. In experiments without K^+ in the stimulus solution, responses failed to increase for stimulus steps longer than 900–1000 ms. However since there was no K^+ it was impossible to be sure that the peak concentration was the same for all stimulus presentations. There was no clear contribution from adaptation effects until the stimulus steps were increased to 2 s.

The second time range we explored, using stimulus steps between 2 and 10 s, was that in which responses showed adaptation. Examples of two cells are shown in Fig.

12. In both cases the stimulus solution, 0·1 mM odour mixture without K^+, was directed at the cell for 7 s at a pressure of 3 lbf in^{-2}. The odour elicited current activated after a normal latency of between 300 and 400 ms and reached a peak within another 500 ms. It remained at this peak for 0·5–1·5 s and then began to decline along an approximately exponential time course. Although we have only exposed a few cells to this treatment ($n = 11$) we found that most ($n = 7$) are similar to the cell of Fig. 12*A* in which the current declined to very near the baseline with a time constant of from 1 to 2 s. The remaining cells decayed more slowly and reached a sustained plateau about 0·4–0·5 of the peak amplitude. Cells treated in this way recovered their normal response to brief pulses within 30 s.

DISCUSSION

We have used several strategies to overcome the difficulties associated with delivering a known stimulus to the receptor neurone and measure the time course of the odour elicited current itself. Most important among these was the use of isolated cells which were cleaned of mucus and maintained in a Ringer bath. Previously we have reported odour elicited currents from cells in epithelial slices (Firestein & Werblin, 1989). By several physiological measures the isolated cells are comparable to the cells in slices. The reversal potential near +5 mV, the magnitude and shape of the cationic current and the range of odour sensitivity are all similar for cells from both preparations, suggesting that the physiological condition of the isolated cells is equivalent to that of cells in the more intact slice preparation.

Transduction occurs in the olfactory cilia

The specialized ciliary structures emanating from the knob at the distal end of the dendrite have been presumed, largely on the basis of anatomical (Menco, 1980; Reese, 1965) or indirect physiological evidence (Getchell *et al.* 1984; Simmons & Getchell, 1981), to be likely site of a putative odour receptor. We have noted here, and previously (Firestein, Werblin & Shepherd, 1988), that cells without cilia never responded to odour stimulation. Kurahashi (1989) using punctate stimulation around an isolated olfactory neurone, saw higher sensitivity to odours at the apical end of the cell. However, the short dendrite of the cell and the high concentration of odour used did not permit a clear spatial separation of regions of the cell.

The results of our localized odour ejection experiments provide direct evidence that the odour response is localized to the cilia and perhaps the distal portion of the dendrite. By contrast, the response to 100 mM-KCl appeared restricted to the dendrite and soma. The total surface area of the cilia is comparable to that of the soma so it appears that the differential responses to odour and high K^+ reflect differences in the properties of the two membranes. The various voltage-gated currents responsible for action potential generation are unaffected by the absence of cilia (Firestein & Werblin, 1987*a*), presumably because these conductance channels are restricted primarily to the soma and possibly the main dendritic shaft.

There is thus a clear segregation of function between the cilia and soma/dendrite compartments, with the cilia acting in the specialized role of signal transduction and the soma serving to regulate the resting potential and generate action potentials.

This organization is similar to that seen in other types of sensory cells. Rod photoreceptors devote the outer segment (a modified cilium) to transduction, whereas voltage gated conductances are present only in the inner segment (Bader, Macleish & Schwartz, 1979; Baylor, Lamb & Yau, 1979). Similarly in hair cells of the cochlea, the mechanosensitive transduction channels are at the tips of the stereocilia (Hudspeth, 1982), whereas the voltage-gated channels which act to 'tune' the cell are in the soma membrane (Lewis & Hudspeth, 1983).

Responses to brief pulses of odour

The activation of the odour elicited current was characterized by a latency ranging from 175 to 600 ms. Previously latencies of this order of magnitude have been measured in single unit (Getchell & Shepherd, 1978*a*) and intracellular (Trotier & MacLeod, 1983) recordings. However, from those measurements it was not possible to distinguish whether the source of the latency was due to diffusion of odour molecules in the mucus, intracellular steps in transduction, or electrotonic conduction from cilia to axon (cf. Getchell *et al.* 1984). The data presented here indicate that at least a large proportion of this latency is due rather to intracellular processing steps.

The K^+ current was shown to be a reasonably accurate representation of the time course of the stimulus, appearing within a few milliseconds of the arrival of the stimulus solution at the cell membrane. The latency from that time to the activation of the odour elicited current must, therefore, be due to processes within the sensory neurone. Since the membrane voltage was clamped and the series resistance was low enough to reasonably ensure isopotentiality, there was no significant electrotonic contribution to the latency.

The response latencies under these conditions were similar to the shortest latencies reported when relatively strong odour stimuli are delivered to the mucus surface of a more intact preparation (e.g. 200–500 ms). This suggests that diffusion in the mucus can be relatively rapid and that the cilia bound receptors are readily accessible to the diffusing odour molecules. This could also account for the correlation between concentration and latency seen in preparations with the mucus layer present (i.e. diffusion is faster at higher concentration) in contrast to our data which tend not to demonstrate this dependence (see below).

There is increasing evidence that a second messenger system could be responsible for the delayed activation of the odour conductance (for review see Lancet, 1986). We have recently found that 3-isobutyl-1-methylxanthine (IBMX) prolongs the response, and that various non-hydrolyzable cyclic nucleotides and nucleotide phosphates affect the odour current (Firestein & Shepherd, 1989*b*). The data presented here are consistent with this body of work, all of which strongly suggest a scheme in which a G-protein-mediated intracellular cascade underlies transduction through the production of cyclic AMP.

The concentration independence of the onset kinetics may also be consistent with the involvement of a second messenger. One interpretation of this independence is that the odour–receptor interaction is not a rate limiting step, and that the production of some intermediate product, or products, is required for the activation for the conductance. Co-operative interactions at one or more steps in a possible cascade are indicated by Hill coefficients of 1·7 for the activation of the cyclic AMP dependent ciliary channel (Nakamura & Gold, 1987), and 2·7 from the dose–response

relation for the odour induced current (Firestein & Werblin, 1989). Thus the activation of the odour elicited current could be directly dependent on the accumulation of a message substance and only weakly affected by the more rapidly equilibrating odour–receptor binding reaction.

The lack of a concentration effect on the latency may also be related to the relatively narrow dynamic range over which these receptors appear to operate. A stimulus change of about one order of magnitude, sufficient to drive the cell from threshold to saturation, may not be large enough to produce a significant effect on the latency. By this reasoning the narrow operating range would be due to a high gain amplification system, all of which was subsequent to the G-protein. A possible candidate for this might be the olfactory adenylate cyclase, which has been determined by Sklar *et al.* (1986) to be among the most active form known in neural cells. Thus the loading of the G-protein, probably the main determinant of the latency, could remain relatively unaffected by the small changes in concentration, while the cyclase produced the high gain and narrow dynamic range.

The activation of the odour current is significantly slower than responses in either photoreceptors or hair cells. In the case of hair cells the latencies are in the microsecond range and it is improbable that a second messenger system is involved (Hudspeth & Lewis, 1988). Photoreceptors, which make use of a cyclic nucleotide second messenger cascade (Yau & Baylor, 1989), operate somewhat faster than olfactory neurones when corrected for temperature (Baylor, Nunn & Schnapf, 1984). Latencies for turtle cone responses to flashes of light range from 10 to 50 ms and are strongly dependent on stimulus intensity; the latency decreases by 80% over the full stimulus range (Baylor & Hodgkin, 1973). Measurements of the light induced outward current in toad rods show similar latencies and an activation time course that is strongly dependent on light intensity. This suggests that, although there may be some analogies to be drawn between these two sensory cell types, there must also be some fundamental differences in the mechanisms which lead to the sensory current activation.

Termination of the odour response

Another remarkable feature of the response to brief pulses of odour molecules was that the stimulus solution had diffused away to well below threshold concentrations by the time the current had reached its peak amplitude. This is a further indication of the probable involvement of a second messenger system mediating signal transduction. It is hard to imagine a model for direct gating of the odour channel by the stimulus molecules when the response current is increasing while the stimulus concentration is decreasing or absent.

Since the odour elicited current decays in the virtual absence of stimulus, the process is a good approximation of a relaxation to equilibrium (i.e. zero concentration). The single exponential decay of the odour current indicates that it is a first order process. The time constant (1/rate constant of the reaction) is concentration dependent, becoming longer with increasing response amplitude. This concentration dependence could arise from several sources, since the decay is presumably due to the hydrolysis of one or more products in a second messenger cascade.

Responses to maintained steps of odour

Response integration

It is common to equate olfactory stimulus magnitudes with concentration, defined simply as the molarity of the delivered stimulus solution. This is not entirely correct, however, since the stimulus is delivered for a finite time period. A more appropriate measure might be molecular flux, i.e. the product of concentration and duration. Our results with longer duration steps of odour support this notion; the magnitude of the odour elicited current is a function of the stimulus duration as well as the peak concentration.

In order to measure flux and not simply concentration, the receptor neurone must integrate stimulus information over some time period. From experiments with stimuli of identical peak concentrations but increasing duration, it appeared that this integration period could be around 750–1100 ms. More exact measurements could not be made since the K^+ current stimulus marker interfered with the odour response at these long times.

Adaptation

The responses to 7–10 s long steps of maintained odour stimulus were transient, reflecting a process of adaptation in the receptor neurone. In a study of receptor adaptation using extracellular single unit recordings, Getchell & Shepherd (1977) described two distinct phases of the response to 10 long exposures to odours. An initial phasic burst of spikes followed in 1–2 s by a sustained tonic level of spiking at about 50% of the peak phasic burst frequency. A similar pattern of spike generation has been recorded intracellularly (Trotier & MacLeod, 1983) and with the patch clamp (Trotier, 1986; Firestein & Werblin, 1987*a*, *b*) in response to electrical stimulation (depolarizing current pulses), leaving some ambiguity as to whether the effect is due to desensitization to the stimulus or to some property of the voltage-gated currents underlying action potential generation (Firestein & Werblin, 1987*b*).

The results presented here indicate that a combination of these processes is at work. The odour induced current could be described as having two phases, an initial rise to a brief plateau amplitude which was maintained for 1–2 s, followed by an exponential decay. The decay phase was variable; in some cells the decay was not complete but appeared to remain at a level about 30% of the maximum for the duration of a 7 s pulse. In most cells this tonic phase was not observed and the decay was complete within 5–6 s. Thus, desensitization of the transduction process appears to occur more rapidly than adaptation as measured by spike frequency. Apparently some residual excitability remains in the nerve membrane after the sensory current has disappeared.

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