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Rapid application and removal of second messengers to cyclic nucleotide-gated channels from olfactory epithelium

(olfactory transduction/vertebrates/cAMP/kinetics)

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ABSTRACT **The last step in the second-messenger cascade mediating vertebrate olfactory transduction is the direct opening of a nonspecific cation channel by cAMP. The kinetic properties of this interaction are critical in determining the time course of the sensory response. To analyze these properties, excised inside-out membrane patches containing either the native channel from salamander olfactory-receptor neurons or a recombinant rat olfactory cyclic nucleotide-gated channel were exposed to short pulses of known concentrations of cAMP or cGMP to mimic a rapid and transient production of second messenger. Channel activity outlasted cyclic nucleotide pulses for several hundred milliseconds. This effect was due to an intrinsic property of the olfactory channel protein because it did not occur with cGMP-activated channels from retinal photoreceptors. Gating kinetics of the olfactory channel were both voltage and agonist dependent. These results demonstrate that the overall slow channel-gating kinetics could account for the difference in time course between the odor-induced changes in cAMP concentration and the subsequent sensory generator current.**

Odor-induced depolarization in vertebrate olfactory receptor neurons is at least in part the result of cAMP-dependent activation of a cation-permeable channel (1–3). Generation of the cyclic nucleotide is coupled to the presence of odor stimuli through a biochemical enzyme cascade that shares several features with that seen in visual, hormone, and neurotransmitter signal transduction (4, 5). In the olfactory system the occupied odor receptor activates a G protein, inducing the adenylate cyclase-catalyzed production of cAMP. The ion channel is directly gated by the cooperative binding of two to three cAMP molecules (1, 2, 6).

The factors that determine the time course of the olfactory response are not yet known. Measurements of odor-induced cAMP accumulation *in vitro* carried out on a subsecond time scale revealed a rapid and transient concentration increase of the second messenger that reached a peak within 50 ms of odor application (7). It has been a paradox, however, that the biochemically determined rate of cAMP production and removal is too fast to explain the overall slow time course of odor-induced whole-cell currents, which display decay time constants of several hundred milliseconds to 1 sec (8). If the cAMP production is transient, then the decay of the current would be expected to primarily reflect the distribution of channel lifetimes after removal of the agonist (9, 10). However, as measured in the steady state, channel lifetimes are quite brief, only ≈1.5 ms, on average (2).

We have analyzed this problem by applying brief pulses of known concentrations of cyclic nucleotides to excised membrane patches containing either native or recombinant cyclic nucleotide-gated channels (CNG channels) from olfactory-receptor neurons. We provide evidence that, after a brief pulse of agonist, channel activity can persist for hundreds of milliseconds, revealing an inherent channel property that could account for the difference in time course between the odor-induced changes in cAMP concentration and membrane conductance. Hence, the ion channel itself could serve to shape the odor response from the activity of the second-messenger cascade. Our results also suggest a surprising similarity of gating mechanisms between CNG channels and *N*-methyl-D-aspartate (NMDA)-receptor channels.

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MATERIALS AND METHODS

Isolation of Native CNG Channels. Olfactory-receptor neurons were freshly isolated from the nasal epithelium of the tiger salamander (*Ambystoma tigrinum*) by described methods (8). Cells were allowed to float freely in a Ringer's bath and to settle on plastic coverslips previously coated with poly-L-lysine hydrobromide (0.1 mg/ml). Olfactory neurons were clearly identifiable by their characteristic morphology, including a single thick dendrite ending in a knob-like swelling from which emanated a dozen or more thin cilia. For single-channel recordings, membrane patches were excised from the distal dendrite and dendritic knob of isolated olfactory neurons. We have previously shown that the CNG channels involved in odor transduction, present at high density on the cilia, exist at a much lower density on the outer dendritic membrane (2). Patches used in this study contained only a single CNG channel, as indicated by the observation that no superpositions of channel currents were detected during long stretches of recordings, even with saturating (millimolar) concentrations of agonist.

Isolation of Recombinant CNG Channels. cDNA clones containing a functional olfactory CNG channel from rat olfactory epithelium or a human rod photoreceptor CNG channel were supplied by R. Reed (Johns Hopkins University School of Medicine, Baltimore). The transfection procedure for both channels was identical and has been earlier described in refs. 6, 11. Briefly, transient transfection was accomplished by mixing expression vector DNA (5 μg) with 10 μg of Bluescript carrier DNA and Rous sarcoma virus-Tag (0.5 μg) in 250 mM $CaCl_2$. This material was added dropwise to 250 μl of 2× Hepes buffered saline, and the precipitate was then added to 20% confluent 293 human embryonic kidney cells (HEK cells) growing in Dulbecco's modified Eagle's medium/10% fetal calf serum in 5% CO_2 and allowed to incubate for 5 hr before washing with phosphate buffer. Electrical recordings were made after 2 days.

Abbreviations: CNG, cyclic nucleotide gated; NMDA, *N*-methyl-D-aspartate.

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Recording and Data Analysis. Patch pipettes with resistances of 10–15 MΩ after fire-polishing were coated along their shanks with Sylgard to reduce pipette capacitance. Specific care was taken to apply only a gentle suction to obtain GΩ seals. Inside–out patches were obtained by moving the electrode through the air–water interface. Currents were recorded with a List EPC 7 patch-clamp amplifier. Unless otherwise noted, records were low-pass filtered at 1 kHz and sampled at 5 kHz. Off-line analysis was accomplished by using a Hewlett–Packard HP 9800 computer system. Records for Fig. 4 were plotted directly from a digital oscilloscope. Peak and baseline currents were marked with cursors. The rise time of responses was determined as the difference between the times when the current reached 10 and 90% of the peak current.

Fast Application System. The fast application of agonists was performed by using the method originally developed by Franke *et al.* (12). Patches containing the ion channels were moved to a microchamber that was constantly perfused with intracellular control solution. Agonist containing test solution was delivered through a polyethylene tube drawn out to a 30-μm diameter and fixed to a piezoelectric crystal (Physik Instrumente, Waldbronn, F.R.G.). Applied voltage pulses caused the tube and the filament of solution flowing from it to shift by 20 μm and thus moved the interface of the two solutions across the tip of the patch pipette (see Fig. 1*A*). To obtain a sharp interface, which was the most important requirement for achieving fast and symmetrical solution exchange, the solution reservoirs were slightly pressurized to give an optimal flow rate of 100 μm/ms. Minimal solution-exchange time under these conditions, as measured with an open electrode, is 200 μs (13).

Solutions and Drugs. The Ringer's solution used for the salamander neurons contained 120 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM NaHepes, 5 mM glucose, pH 7.6. Recordings were performed in symmetrical, low-divalent-cationic solutions containing 117.5 mM NaCl, 1 mM $CaCl_2$, 10 mM EGTA, 10 mM NaHepes, pH 7.5, to give a free $[Ca^{2+}]$ of 10 nM. For the experiments with the recombinant CNG channels, symmetrical solutions contained 145 mM NaCl, 0.1 mM $CaCl_2$, 10 mM EGTA, 10 mM NaHepes, pH 7.6, to give a free $[Ca^{2+}]$ of 1 nM. All drugs were obtained from Sigma. Recordings were made at room temperature.

RESULTS

Nonequilibrium gating kinetics of native cyclic nucleotide-activated channels were investigated in inside–out patches from salamander olfactory dendrites (n = 14). Divalent-cation-free solutions were used to avoid Ca^{2+}-mediated desensitization (14, 15) and divalent-cation-induced channel block (16). Brief pulses of cyclic nucleotides (2–500 ms) were applied to these patches by rapidly moving the interface of two flowing solutions across the tip of the patch pipette (Fig. 1*A*). The main advantages of this arrangement are that both the application and the removal of the stimulus are rapid, and the concentration of agonist can be precisely controlled.

Fig. 1*B* shows the result of single activations of the CNG channel, in five consecutive traces, after a 120-ms pulse of 100 μM cAMP. After some initial delay, single-channel currents were activated and tended to group together in clusters (14). When the cAMP pulse was terminated by switching to control solution, channel activity outlasted the pulse and persisted for another 100–400 ms. Both during and after the pulse, clusters of channel openings separated by gaps of tens of milliseconds were detected. Under steady-state conditions and in the absence of desensitization (2), we would normally interpret the longest closings as incidents of the agonist coming unbound and channel closing, only to be

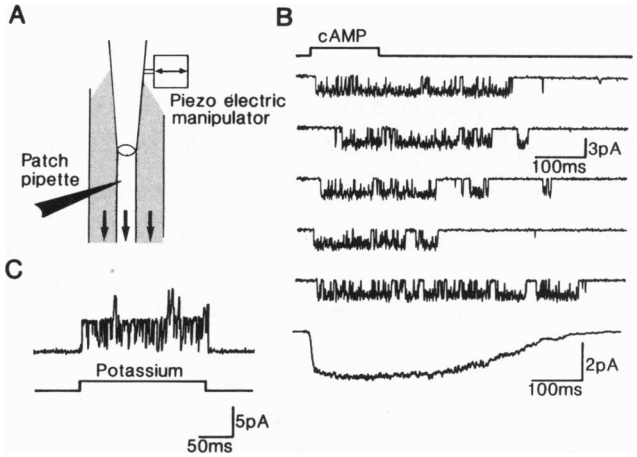

FIG. 1. CNG channel activity from freshly isolated salamander olfactory-receptor neurons persists after removal of agonist. (*A*) Diagram of the application system. Solution changes were accomplished by moving the sharp interface of two flowing solutions across the tip of the patch pipette (see *Materials and Methods*). (*B*) Five consecutive records of channel activity induced by a 120-ms pulse of 100 μM cAMP. Only one channel is present in the patch. The bottom trace is the ensemble average from 92 single applications. Holding potential was −60 mV. (*C*) In the same patch, with K^+ replacing Na^+ in the intracellular solution, the activation of K^+ channels (holding potential was 0 mV; no cAMP was in the solution) follows the pulse and displays no prolonged activity.

rebound moments later, causing a subsequent channel re-opening. Here, however, there was no new agonist to rebind, and so the re-opening of channels after longer closed times implied that the agonist remained bound, that the channel was in a closed but ligand-bound state, and that the channel openings and closings represented transitions between bound states. Further evidence for this view can be seen in the ensemble average of repeated activations of the single-channel currents (Fig. 1*B*, bottom trace) by the distinct plateau phase that continued for some time after the end of the stimulus pulse. After the plateau, the current decayed back to baseline along an exponential time course (τ = 65 ms in this experiment), now due presumably to ligand unbinding. The prolonged activation did not result from retarded solution exchange, as can be seen in Fig. 1*C*, where the activity of K^+ channels in the same patch ceased immediately at the end of a 180-ms pulse of solution containing 120 mM K^+ instead of Na^+.

To investigate further the activation and deactivation kinetics we turned to patches containing large numbers of the recombinant CNG channel from rat olfactory neurons transiently expressed in a human embryonic kidney cell line (6). The recombinant rat CNG channel exhibited behavior very similar to that seen in the native salamander channel (n = 21) (Fig. 2). Comparing the response elicited by a 300-ms pulse of either cAMP or cGMP (both at 1 mM), it can be seen that both agonists activated a nondesensitizing inward current of 185 pA in this membrane patch. This current corresponded to the simultaneous activation of ≈200 channels, as calculated from the 15-pS single-channel conductance of the native rat CNG channel (17). The response kinetics were agonist dependent; the cAMP-elicited current had a slower rise time and significantly shorter plateau current than that elicited by an identical pulse of cGMP. The 10–90% rise time (at 1 mM) was 6.5 ± 1.4 ms (SD, n = 7) for cGMP and 9.2 ± 2.5 ms (n = 8) for cAMP. In some patches the rise time was as much as 2-fold slower than these values, an effect we took to indicate restricted solution access within the patch. Patches exhibiting this behavior were not included in this study. The time to decay to 50% of the peak current after the end of the

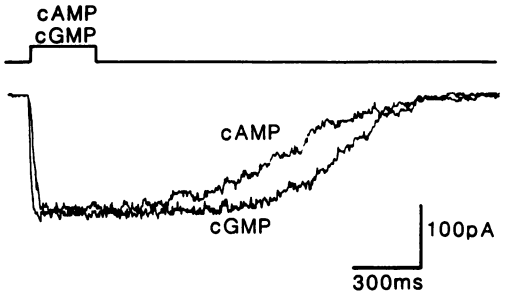

FIG. 2. CNG currents in an inside–out patch excised from 293 human embryonic kidney cells transfected with the rat olfactory channel clone. Responses elicited by 300-ms pulses of 1 mM cAMP or cGMP; holding potential was −60 mV. Note the lack of desensitization during the pulse of agonist. The response kinetics are agonist dependent (see *Results*). As in the native channel, the response persisted after the end of the pulse and decayed to baseline with a half-time of 919 ms (cAMP) and 1106 ms (cGMP).

pulse was 919 ms (cAMP) and 1106 ms (cGMP) in this experiment. These results suggest that the higher affinity of the channel for cGMP over cAMP (6) was obtained by both an increased forward rate and a decreased backward rate of cGMP versus cAMP. The rising phase of the cGMP response at 1 mM was likely to be almost entirely determined by the rate at which cGMP interacts with the receptor because a 10-fold increase in cGMP concentration could further shorten the response rise time to 2.5 ms (data not shown).

Channel-gating kinetics also displayed some voltage dependence (Fig. 3*A*). The current evoked by 1 mM cGMP was recorded at +60 mV (outward current) and −60 mV (inward current). At both potentials the same current amplitude was measured, indicating a linear current–voltage relation under the symmetrical divalent-cation-free conditions used here. However, at +60 mV the decay time to 50% of the peak current after the end of the pulse was 1300 ms, significantly longer than the 1020 ms required at −60 mV. By comparison, the rise time of the current was not voltage dependent (Fig. 3*B*), measuring 5.2 ms at both potentials. Similar results were obtained in five other experiments. Because K_m values for

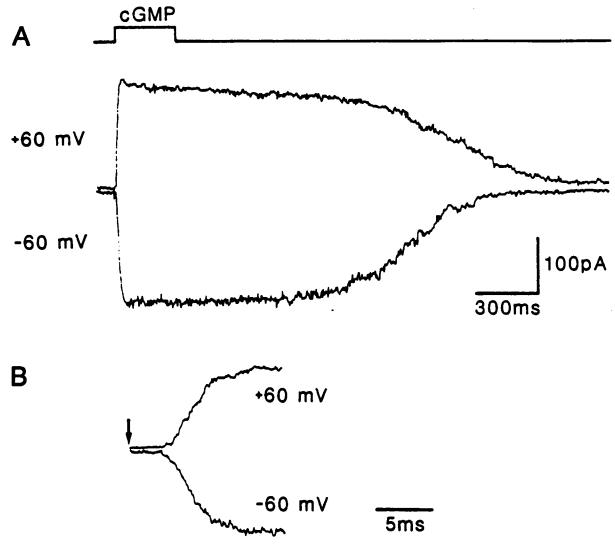

FIG. 3. The prolonged current shows voltage-dependent kinetics. (*A*) A 300-ms pulse of 1 mM cGMP was delivered to a patch held at −60 mV (inward current) and +60 mV (outward current). Time to decay to 50% of the maximum current after the end of the pulse was significantly longer at positive membrane potentials with 1300 ms at +60 mV compared with 1020 ms at −60 mV. (*B*) Rising phase of the cGMP-activated current at higher time resolution. The rise time was unaffected by membrane potential.

activation of CNG channels from dose–response curves are slightly lower at positive membrane potentials (17), these results agree with the idea that the entire voltage sensitivity for channel activation is in the rate constants for channel closing.

Because solutions are likely to equilibrate more slowly with an intact inside–out patch than with a bare pipette, more rigorous tests regarding the actual solution-exchange times under our experimental conditions were done. Fig. 4 shows an experiment in which we rapidly switched between solutions containing different ions as charge carriers. In the control (Fig. 4*A*) a 300-ms pulse of 1 mM cGMP was delivered at a membrane potential of +25 mV, eliciting a Na^+-driven outward current with the above-described sustained-activation pattern. Next, the patch was held in a solution where Na^+ had been replaced by choline (Fig. 4*B*). A current was activated by the same pulse of cAMP (in Na^+ solution), but at the end of the pulse the patch was switched back into the choline control solution. In this case the current decayed abruptly back to baseline, although the prolonged activation of the channel was still evident—now as a small Na^+ *inward* current due to the change in reversal potential under the asymmetric ionic conditions. Choline blocks the cAMP-gated channel, possibly by interacting with a binding site in the channel pore (note, for example, that the current rise time was strongly affected by choline in Fig. 4*B*). Due to the apparent strong interaction of choline ions with the channel molecule an absolute value of the solution-exchange time cannot be inferred from this experiment. Nonetheless, these experiments do show that a complete and effective solution exchange (between the cAMP/Na^+ solution and the choline-containing solution) has been obtained during the prolonged time course of channel activation. The remaining persistent activation must, therefore, have been due to an intrinsic property of the channel protein.

The effect of changing the duration of the second-messenger pulse at a fixed concentration (1 mM cGMP) is shown in Fig. 5. Pulses with durations between 2 and 100 ms could elicit the same maximal current amplitude (Fig. 5*A*). A pulse of just 2 ms was sufficient to activate the current maximally, even though this was significantly shorter than the rise time, suggesting that the activation kinetics of the channel are also slower than the concentration change of

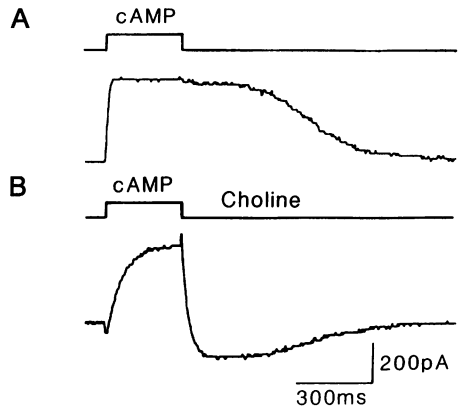

FIG. 4. Prolonged activation of olfactory CNG channels is not due to restricted diffusion in inside–out patches. The time course of the solution exchange at the patch was monitored by replacing Na^+ with impermeant choline ions. (*A*) Control outward current elicited by a 300-ms pulse of 1 mM cGMP at a holding potential of +25 mV. The prolonged plateau and slow decay are evident. (*B*) The stimulus solution is the same, but the background bathing solution contains choline instead of Na^+. The current decayed abruptly after the end of the pulse, but the persistent tail could now be seen as an inward current at the +25 mV holding potential (E_{Na} = +50 mV).

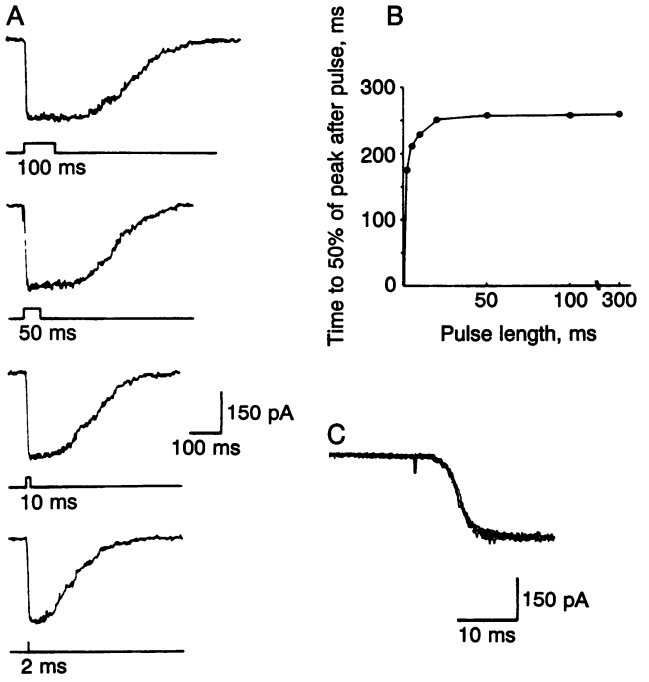

FIG. 5. Effect of pulse duration on current kinetics of the olfactory channel. (*A*) Currents elicited by application of 1 mM cGMP pulses of decreased lengths from 100 to 2 ms; pulse duration is indicated in the traces below the currents. All measurements were from the same patch; holding potential was −60 mV. The final current decay after the plateau currents could be fitted with a single exponential function that was independent of pulse duration (τ = 130 ± 18 ms). (*B*) Effect of pulse duration on plateau phase, quantified as the time to reach 50% of the maximal current after the end of pulse. For pulses ≥20 ms this time is independent of pulse length; for pulses <20 ms the sustained current after the end of the pulse is successively diminished and is virtually absent at the 2-ms pulse. (*C*) Rising phases of current records from *A* were plotted at higher-time resolution and on top of each other. Because the rise is the same for all agonist pulses and the rise time is concentration dependent at agonist concentrations up to 10 mM cGMP (unpublished data), the effective concentration reaching the inner face of the membrane patch must have been the same.

cyclic nucleotide. On the other hand, deactivation kinetics were strongly affected by the pulse length. Because the exponential portion of the decay remained constant, regardless of pulse length, we used the time to reach 50% of the maximum current after the end of the pulse as a measure of the deactivation kinetics. Fig. 5*B* illustrates that this time remained constant for pulses with a length between 20 and 300 ms, whereas pulses <20 ms successively reduced the time by shortening the plateau phase. This result indicates that the distribution between the various open and shut states of the channel molecule was not in equilibrium for exposure to agonist <20 ms. In Fig. 5*C* the rise times of the records shown in Fig. 5*A* are plotted at a higher time resolution. Decreasing the duration of the application pulse to 2 ms had no effect on the rise time, indicating that the effective concentration reaching the inside–out patch was the same at all pulses. This result suggests a full solution-exchange time in the range below 2 ms.

In photoreceptors a light flash results in the hydrolysis of intracellular cGMP, and the rise time of the light response is therefore related to channel deactivation kinetics. Given these functional properties, we hypothesized that the slow deactivation kinetics described above for the olfactory channel would be absent in the visual channel; this was indeed the case. Fig. 6 shows the response of an excised patch containing a recombinant human rod photoreceptor channel (11) to

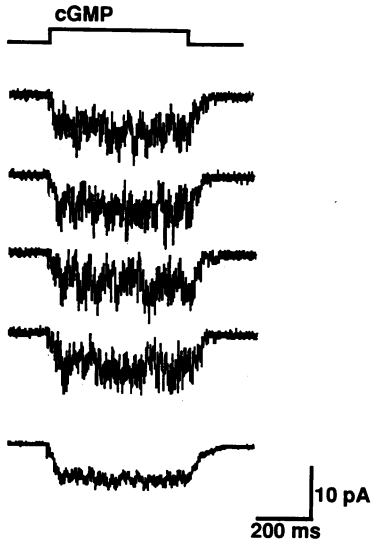

FIG. 6. Gating kinetics of photoreceptor CNG channels differ. Inside–out patch containing a recombinant human rod channel expressed in 293 human embryonic kidney cells. Four consecutive records of channel activity induced by a 500-ms pulse of 1 mM cGMP. In this case, the prolonged activation after the end of the pulse is absent. The bottom trace is the ensemble average from 10 single applications. Holding potential was −60 mV; signals were filtered at 2 kHz.

four consecutive single pulses of cGMP (1 mM). Cyclic nucleotide application elicited nondesensitizing inward currents of ≈10 pA due to the simultaneous activation of six to eight channels. However, unlike the olfactory CNG channel, the visual channel decayed abruptly back to baseline after the end of the pulse with a time constant of 26.5 ± 9 ms (n = 13). The slow rise (18) of the recombinant human rod channel, with a 10–90% rise time of 20–25 ms (at 1 mM cGMP), was consistent with its relatively high K_m value of 80 μM (11) compared with native photoreceptor CNG channels.

DISCUSSION

An important result of this study is that both the activation and deactivation kinetics of the olfactory CNG channels that are known to mediate olfactory transduction (2, 19) were much slower than rapid changes in cyclic nucleotide concentration. Although this result might be suspected from whole-cell recordings of the odor-induced current, these data point directly to the inherent channel kinetics as the main, if not single, determinant of the odor-response kinetics. This hypothesis contrasts with the view obtained from studies of phototransduction, where the rate constant for channel activation is at the diffusion-controlled limit, and the channel responds to the instantaneous cGMP level (18, 20). This difference between the two channels may relate to the different requirements of the two sensory modalities. The task of the olfactory receptor cell is to discriminate between a large number of different odor molecules. To achieve this broad specificity without loss of sensitivity it appears to use an intracellular amplification system that gains increased sensitivity at the expense of temporal resolution.

The slow decay of cyclic nucleotide-activated currents seemed surprising because individual openings of CNG channels are quite brief, ≈1.5 ms on average (2), and even individual bursts of openings are not much longer, containing two to three openings on average (21). However, preliminary analysis of the concentration dependence of channel gating under steady-state conditions yielded a strong similarity to the properties that have been described for NMDA-receptor

channel gating (22, 23)—namely, that an individual channel activation results in a complex cluster of openings that can last, on average, up to 100 msec (21). It is interesting to note that NMDA-channel activity also persists after short pulses of agonist and that this phenomenon has been implicated in the prolonged time course of NMDA-receptor-mediated postsynaptic currents (22, 24). This close similarity of CNG-channel activation to NMDA receptors is especially unexpected, because CNG channels have some structural similarities to the superfamily of voltage-gated channels (25).

At present, little is known about the molecular mechanism leading from the activation of the cyclic nucleotide-binding site to channel isomerization. It has been speculated that channel activation is a two-step process involving protein–protein interactions of the ligand-binding site with other parts of the channel molecule (26). This idea would be reminiscent of the "ball-and-chain" model of inactivation of Shaker K^+ channels (27), except that here the "ball" would serve channel activation in response to ligand binding. This idea allows the speculation that the plateau current after the end of the pulse, as seen here, is from such intramolecular interactions.

Our results, while not directly addressing the time course of cAMP-concentration changes during the odor response, are broadly consistent with an emerging picture of transient, rather than cumulative, activation of G protein signaling systems (28). In *Limulus* photoreceptors, for example, rhodopsin is inactivated within 60 ms of a light flash (29). Recently, negative feedback from activated effectors such as phosphodiesterase (vertebrate photoreceptors) or phospholipase C (m1 muscarinic receptors) has been shown to accelerate the inherent GTPase activity of G_α subunits, thereby shortening the cellular response to a stimulus (30, 31). Subsecond kinetic measurements of odor-induced accumulation of cAMP in cell-free preparations of olfactory cilia likewise show a rapid and transient time course: cAMP declines to baseline levels within 50–500 ms (7). In all of these cases the second-messenger cascade is only transiently activated, producing short-lived pulses of second-messenger molecules. From our results a short pulse of cAMP would be sufficient to elicit a prolonged activation of the CNG channels. This activation would enable the population of channels to act as an integrator, summing pulses of cAMP into a longer-lasting continuous-current response. In this view the slow inherent channel kinetics serve to optimize the ability of the channel to operate under intermittent agonist conditions.

Note Added in Proof. It has recently been shown (32) that the rapid liberation of caged cAMP by flash photolysis in intact olfactory neurons leads to a slowly developing current. This is consistent with the data presented here and with our proposition that the onset kinetics of the olfactory response are primarily set by the CNG channel and not by the stimulus-dependent production of cAMP.

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