Impaired Odor Adaptation in Olfactory Receptor Neurons after Inhibition of Ca^{2+}/Calmodulin Kinase II

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Odor adaptation in vertebrate olfactory receptor neurons (ORNs) is commonly attributed to feedback modulation caused by Ca^{2+} entry through the transduction channels, but it remains unclear and controversial whether this Ca^{2+}-mediated adaptation resides in the cAMP-gated channel alone or whether other molecules of the transduction cascade are modulated as well. Attenuation of adenylyl cyclase activity by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) has also been proposed as a mechanism for adaptation. To test this in intact ORNs, we have compared the properties of adaptation induced by a sustained (8 sec) or brief (100 msec) odor stimulus. Although adaptation induced by both types of stimuli occurs downstream from the odor receptors and is Ca^{2+}-dependent, only adaptation induced by a sustained pulse involves alterations in the odor response kinetics, consistent with a reduction in the rate of adenylyl cyclase activation. By disrupting CaMKII to block adenylyl cyclase attenuation using a specific peptide inhibitor of CaMKII, autocamtide-2-related inhibitory peptide (AIP), we show that this reaction is necessary for odor adaptation *in vivo*. With CaMKII disrupted, adaptation induced by a sustained stimulus is significantly impaired: the onset rate of adaptation is decreased by threefold, and the recovery rate from adaptation is increased by up to sixfold. In contrast, adaptation induced by a brief odor pulse is unaffected, demonstrating that the effect of AIP must be highly specific. The results indicate that CaMKII controls the temporal response properties of ORNs during odor adaptation. We propose that CaMKII plays a prominent role in odor perception.

Key words: olfactory adaptation; salamander; calcium signaling; cyclic nucleotide-gated channels; calcium/calmodulin kinase type II; receptor neurons; adenylyl cyclase

The mechanisms underlying odor adaptation in olfactory receptor neurons (ORNs) are fundamental for a complete understanding of the sense of smell. Like sensory neurons of other modalities, vertebrate ORNs adapt to ambient conditions by time-dependent modification in the sensitivity to a given stimulus, as seen in the decline of the sensory response during prolonged odor stimulation (Ottoson 1956; Getchell and Shepherd 1978; Firestein et al., 1990; Kurahashi, 1990). This process is commonly attributed to a feedback mechanism resulting from Ca^{2+} entry through the transduction channels causing modulation of the transduction machinery (Kurahashi and Shibuya 1990; Zufall et al., 1991; Kurahashi and Menini, 1997; Leinders-Zufall et al., 1998; Reisert and Matthews, 1998), but the precise mechanisms of adaptation are not well understood. Olfactory signal transduction involves the activation of a G-protein-coupled adenylyl cyclase/cAMP second messenger cascade leading to the sequential opening of Ca^{2+}-permeable cAMP-gated cation channels and Ca^{2+}-activated chloride channels (for review, see Reed, 1992; Ache and Zhainazarov, 1995; Restrepo et al., 1996). In an attempt to determine the molecular locus of adaptation, Kurahashi and Menini (1997) suggested that odor adaptation occurs entirely downstream from the adenylyl cyclase and consists essentially of a single step, Ca^{2+} modulation of the cAMP-gated channel, leading to the notion that olfactory adaptation is a simple process (Gold and Pugh, 1997). This model, however, has been challenged by a variety of *in vitro* biochemical studies demonstrating that odor-induced cAMP formation undergoes adaptation-like desensitization (Breer et al., 1990; Ronnett et al., 1991) and that elevated Ca^{2+} can attenuate adenylyl cyclase (Wayman et al., 1995; Boekhoff et al., 1996; Wei et al., 1996, 1998) and enhance phosphodiesterase activity (Borisy et al., 1992; Yan et al., 1995). Several Ca^{2+}-independent mechanisms have also been proposed to mediate adaptation, including odor receptor phosphorylation by protein kinase A (Boekhoff and Breer 1992) and G-protein coupled receptor kinase 3 (GRK3) (Dawson et al., 1993; Schleicher et al., 1993; Peppel et al., 1997).

These varied findings make further investigations necessary. Here we test whether attenuation of adenylyl cyclase by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is critical for odor adaptation. CaMKII is abundantly expressed in olfactory

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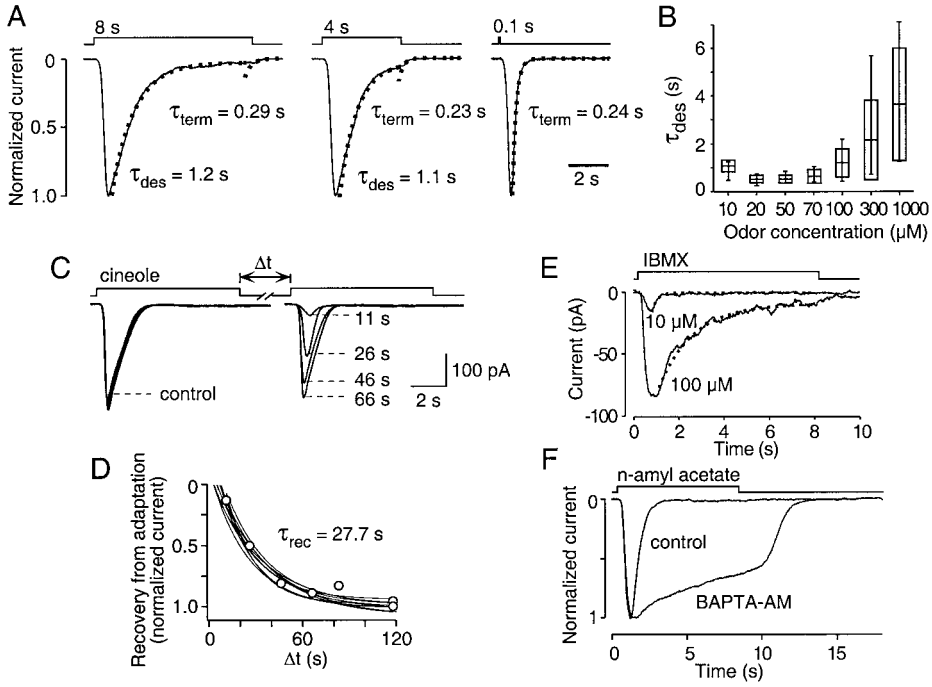

Figure 1. *A, B*, Analysis of the onset rate of odor adaptation derived from fits of the desensitization phases of odor-induced currents (*scaled*) to sustained odor steps (cineole, 70 μM) lasting for 8 or 4 sec, respectively. Single exponential functions are shown as *dotted lines* superimposed on the current traces; time constants τ_{des} are indicated. Desensitization is absent with a brief 100 msec stimulus; the decay reflects the termination time constant τ_{term}. *B*, Box plot illustrating the dependence of the onset rate of adaptation on the odorant strength (cineole). The *horizontal line* in each box reflects the mean; the *box* indicates the SD. Notches show the range of the data points. *C*, *D*, Analysis of the recovery rate from odor adaptation. Odor responses were induced by two identical 8 sec pulses (50 μM cineole) with varying interstimulus intervals (Δt) as indicated. The interval between two consecutive sets of paired pulses was 2 min. *D*, Plot of the peak amplitude of the second response as a function of Δt. Data points from 10 individual ORNs are fit with single exponential functions yielding an average recovery time constant τ_{rec} of 27.7 sec. *Open circles* reflect data points from the cell depicted in *C*. Responses are expressed as a percentage of the fully recovered amplitude. *E*, Odor receptor activation was bypassed by elevating cAMP levels via sustained IBMX pulses. IBMX-induced currents desensitized at a rate comparable with that seen with odor stimuli. IBMX-induced desensitization showed the same concentration dependence as odor-induced desensitization; with 10 μM IBMX desensitization occurred faster (τ_{des} = 0.22 sec) than with 100 μM IBMX (τ_{des} = 0.7 sec). *F*, Pretreatment of an ORN with the membrane-permeant intracellular Ca^{2+} chelator BAPTA AM largely eliminates adaptation. The fact that we did not observe complete elimination of adaptation reflects an insufficient buffering capacity of BAPTA AM; desensitization was completely abolished after removal of extracellular Ca^{2+} (data not shown).

cilia and inhibits olfactory adenylyl cyclase via Ca^{2+}/calmodulin-induced phosphorylation (Wei et al., 1996, 1998), but it is unknown whether this mechanism contributes to odor adaptation in intact ORNs. We report that odor adaptation induced by a sustained odor pulse is strongly impaired when CaMKII function is disrupted, whereas adaptation resulting from a brief odor pulse remains unchanged. These results represent the first evidence that CaMKII attenuation of adenylyl cyclase is necessary for odor adaptation *in vivo*. The fact that disruption of CaMKII impairs one form of odor adaptation but not another indicates that a single molecular step cannot be sufficient to explain all phases of odor adaptation.

MATERIALS AND METHODS

Preparation and recording. ORNs were acutely dissociated from the nasal epithelium of adult tiger salamanders (*Ambystoma tigrinum*) without the use of enzymes, closely following the methods described previously (Leinders-Zufall et al., 1996). Odor responses were recorded under voltage clamp by applying the perforated patch technique with amphotericin B to avoid effects from artificial Ca^{2+} buffering on the endogenous Ca^{2+} feedback system of the cells. Current recordings, data acquisition, and on-line analysis were controlled by an EPC-9 patch-clamp amplifier combined with the Pulse/Pulsefit software package (HEKA Electronic, Lambrecht/Pfalz, Germany) running on a Macintosh computer. The holding potential was −60 mV in all experiments. Focal stimulation of olfactory cilia was obtained by pressure ejecting the odor solutions from multibarrel glass pipettes (Leinders-Zufall et al., 1996). Odorant dose-response curves obtained with this method are in close agreement with previously described results in these cells (Firestein et al., 1993). Only ORNs that did not undergo long-lasting adaptation with the odor stimuli used here (Zufall and Leinders-Zufall 1997) were included in the analysis to avoid complications from the effects of cGMP-dependent adaptation.

Solutions and chemicals. ORNs were continuously superfused with Ringer's solution containing (in mM): 115 NaCl, 2.5 KCl, 1.0 $CaCl_2$, 1.5 $MgCl_2$, 4.5 HEPES, and 4.5 Na-HEPES, pH 7.6, adjusted to 240 mOsm. The pipettes contained (in mM): 17.7 KCl, 92.3 KOH, 82.3 methanesulfonic acid, 5.0 EGTA, and 10 HEPES, pH 7.5 (KOH), adjusted to 220 mOsm. All chemicals were obtained from Sigma (St. Louis, MO) if not otherwise stated. Odor solutions were prepared in Ringer's solution with <0.1% dimethylsulfoxide (DMSO) (v/v). 3-Isobutyl-1-methyl-xanthine (IBMX) was prepared in 10 mM stock solution containing 5% DMSO and diluted to the final concentrations with less than 0.3% DMSO. 1,2-bis-(2-Amino-phenoxy)ethane-*N,N,N′,N′*-tetra-acetic acid acetoxymethyl ester (BAPTA AM; Molecular Probes, Eugene OR) was prepared as described previously (Leinders-Zufall et al., 1998). Myristoylated (cell-permeant) autocamtide-2-related inhibitory peptide (AIP) (Myr-*N*-Lys-Lys-Ala-Leu-Arg-Arg-Gln-Glu-Ala-Val-Asp-Ala-Leu-OH) was obtained from Calbiochem (San Diego, CA) and prepared freshly before each experiment. AIP was applied to ORNs from a nearby puffer pipette. In control experiments (n = 3), we found that there was no difference in the effect of AIP irrespective of whether it was co-applied with protease inhibitors (leupeptin and bestatin, each at 100 μM). We therefore did not use protease inhibitors in these experiments.

Data analysis. All data analysis and calculations were performed using the Igor Pro software package (WaveMetrics, Lake Oswego, OR) running on Macintosh computers. Through this program user-defined functions in combination with an iterative Levenberg–Marquardt nonlinear, least squares fitting algorithm were applied to the data. To fit the decay phases of odor-induced currents we used single exponential functions. If not otherwise stated, data are expressed as means ± SD and number of observations (n). Statistical tests were performed with the use of Statview 4.02 software (Abacus Software, Berkeley, CA).

RESULTS

Onset and recovery kinetics of odor adaptation

ORNs were stimulated with maintained odor stimuli, and the resulting sensory currents were analyzed (Fig. 1). With an 8 sec odor stimulus (cineole, 70 μM) odor adaptation is evident in the progressive decline of the current despite the continued presence of the odorant (Fig. 1*A*). This adaptation (or desensitization) occurred with an onset time constant τ_{des} of 1.2 sec. After re-

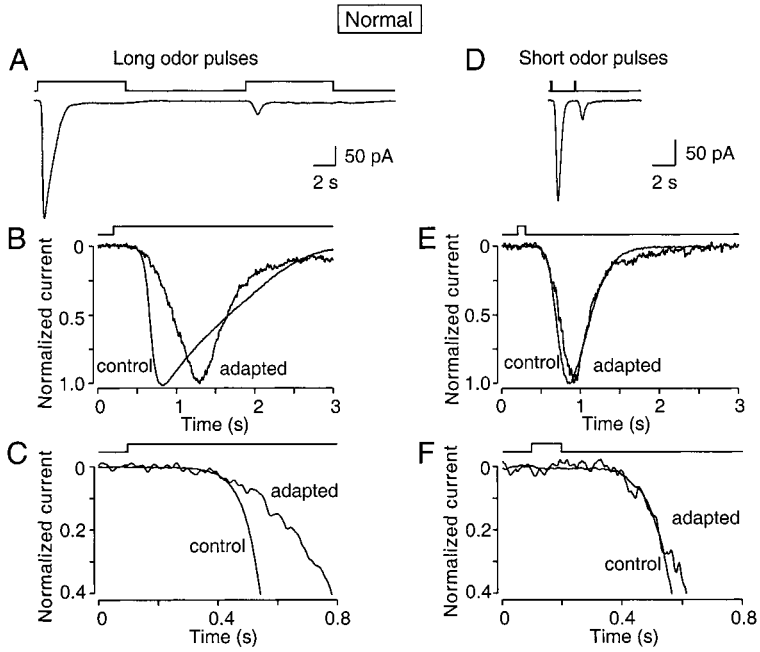

Figure 2. Adaptation caused by sustained odor pulses originates from a decrease in the slope of the rising phase and an accelerated falling phase of the responses. *A*, Responses to two identical 8 sec pulses of cineole (50 μM) that are paired with an interstimulus interval of 11 sec. *B*, To facilitate viewing of the kinetic changes induced by the conditioning pulse, the two responses from *A* are plotted at higher temporal resolution (scaled responses). *C*, Plot of the initial rising phase of the currents from *B*. Desensitization slowed the rate of activation of the current, increased the time to peak, and accelerated the decay rate. *D–F*, These kinetic changes did not occur when odor adaptation was induced by brief consecutive odor pulses leading to the same reduction in sensitivity as in *A*.

moval of the stimulus the current relaxed back toward the baseline, with a deactivation (or termination) time constant τ_{term} of 0.29 sec. The same kinetic rates were seen when the odor stimulus was applied for 4 sec. With a brief 100 msec odor pulse, desensitization was absent, and the current decay was determined entirely by the termination rate (Fig. 1*A*). Results from multiple experiments using 8 sec odor pulses are plotted in Figure 1*B*, illustrating that the onset rate of adaptation was dependent on the odorant strength becoming significantly slower with increasing odor concentrations. We therefore used only stimuli from the midrange of the odorant dose–response curve (50–100 μM) in the experiments described subsequently.

In addition to the onset rate, the recovery rate is an important parameter for the quantitative analysis of odor adaptation. The rate of recovery from adaptation was derived from fits of the recovery curve obtained from paired-pulse experiments. ORNs were stimulated with two identical 8 sec odor pulses (50 μM cineole) with varying interpulse intervals (Δt), separated by 2 min rest periods (Fig. 1*C*). In the example shown, the current decreased from a peak value of 330 pA to a plateau value of ~3 pA during odor exposure, reflecting a 110-fold reduction in transduction gain. This decline in sensitivity recovered nearly completely after an interpulse interval ≥66 sec as judged by the reappearance of the peak response (Fig. 1*C*). A plot of the peak response as a function of Δt yielded a recovery time constant τ_{rec} of 22.2 sec (Fig. 1*D*, *open symbols*). Closely similar results were observed throughout the experiments, with an average recovery time constant of 27.7 ± 5.4 sec (n = 10) (Fig. 1*D*). Thus recovery from adaptation to sustained odor pulses is significantly slower (fivefold to sixfold) than from adaptation induced by brief 100 msec pulses (Kurahashi and Menini, 1997; Leinders-Zufall et al., 1998).

Figure 1, *E* and *F*, illustrates that desensitization fulfills two important criteria necessary for an involvement of adenylyl cyclase attenuation by CaMKII in odor adaptation: (1) it occurs downstream from the odor receptors; and (2) it is Ca^{2+}-dependent. When we bypassed odor receptor activation by increasing cAMP levels through sustained application of the phosphodiesterase inhibitor IBMX (10 μM, n = 7), the current still desensitized at a rate comparable with that seen with odor stimulation (Fig. 1*E*). IBMX-evoked desensitization showed the same characteristic concentration dependence as odor-induced desensitization, occurring with a slower rate after increasing the strength of the IBMX stimulus (Fig. 1*E*). In a cross-desensitization paradigm in which the response to one odorant (cineole) was adapted by a different odorant (*n*-amylacetate), IBMX was sufficient to fully replace the effect of *n*-amylacetate (n = 3; data not shown). When we dialyzed ORNs with heparin (30 mg/ml) via the patch pipette for up to 30 min to inhibit receptor-specific kinases such as GRK3 (Schleicher et al., 1993), there was no measurable effect on the rate of desensitization (n = 4; data not shown). Desensitization was primarily eliminated by treatment of ORNs with the membrane-permeant intracellular Ca^{2+} chelator BAPTA AM (25 or 100 μM for 45 min, n = 5) (Fig. 1*F*), thus confirming that an increase in intracellular Ca^{2+} triggers adaptation resulting from sustained odor exposure (Kurahashi and Shibuya, 1990; Zufall et al., 1991).

Evidence that adenylyl cyclase activity is attenuated during adaptation

If adenylyl cyclase activity is attenuated significantly during odor adaptation, then this step should lead to distinct changes in the odor response kinetics; specifically the slope of the initial rising phase of the sensory current, which reflects the activity of adenylyl cyclase, should be reduced (Lamb and Pugh, 1992). In Figure 2*A–C* an ORN was stimulated with identical paired 8 sec pulses (50 μM cineole) separated by an interstimulus interval of 11 sec. The conditioning response caused a strong decrease in sensitivity of the test response (Fig. 2*A*). A plot of these odor currents at higher temporal resolution revealed that the activation kinetics of the second current were prolonged, whereas, at the same time, the rate of desensitization was increased relative to the control values (Fig. 2*B*, scaled). The time to peak increased twofold, from 0.43 sec (*control*) to 0.88 sec (*adapted*). The desensitization time constant decreased from 1.9 sec (*control*) to 0.4 sec (*adapted*). A plot of the rising phases of the responses on expanded time scale (Fig. 2*C*) revealed that the response delay (which reflects the

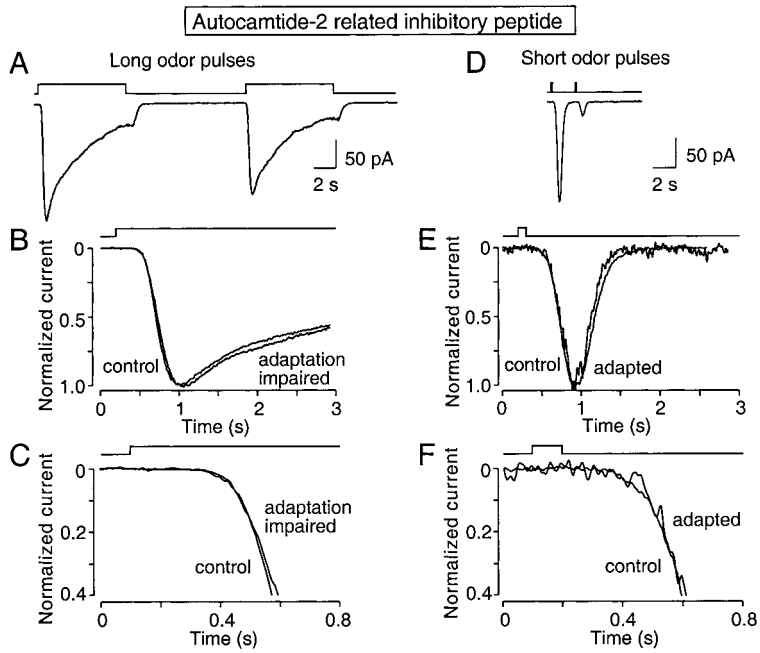

Figure 3. *A–C*, Impaired odor adaptation after treatment of the ORNs with the CaMKII inhibitor AIP (1 μM). *A*, When the adaptation paradigm of Figure 2*A* is repeated in AIP-treated ORNs, the onset rate of adaptation is slower, and the recovery rate is faster compared with untreated (normal) ORNs. *B*, *C*, The kinetic changes observed in untreated ORNs after adaptation are absent in AIP-treated ORNs. *D–F*, At the same time, there is no effect of AIP on adaptation induced by a brief odor pulse.

molecular steps between odor receptor activation and cAMP formation) was unchanged but that the initial slope of the rising phase was markedly reduced in adapted ORNs. These kinetic effects were reversible as the interval between the two pulses was increased to >60 sec (data not shown). Closely similar results were observed in a total of 10 ORNs. Thus desensitization reduced the amplitude of the responses by slowing the rate of activation and accelerating the decay rate.

Because these results differ from those of Kurahashi and Menini (1997), we next repeated this experiment by eliciting adaptation through a brief odor pulse, using the same adaptation paradigm as that of Kurahashi and Menini (1997) (Fig. 2*D–F*). The conditioning response caused a decrease in sensitivity of the test response comparable with that seen in Figure 2*A*. But unlike the results with sustained pulses there was virtually no change in the odor response kinetics under these experimental conditions in either the initial slope or in the rate of decay ($n = 11$) (Fig. 2*E,F*). Thus, although these data confirm those of Kurahashi and Menini (1997) for brief odor pulses, they also indicate that ORNs must use a variety of Ca^{2+}-dependent steps for adaptation, depending on the exact conditions of odor stimulation. In particular, the results do not support the conclusion that odor adaptation is a relatively simple process. Instead, they suggest that Ca^{2+}, in regulating odor adaptation, not only modulates the activity of cAMP-gated channels but also reduces the rate of adenylyl cyclase activation, and it may additionally modulate the activation of phosphodiesterase. The fact that we did not observe alterations in the response latency (for comparison, see Firestein et al., 1991) provides further evidence that G-protein coupling remains normal after adapting the cells by a sustained odor pulse.

Two forms of odor adaptation that depend differentially on CaMKII

To test directly whether adaptation depends on Ca^{2+}-mediated attenuation of adenylyl cyclase, we attempted to disrupt this molecular step. If the overall hypothesis is correct, then selective blockade of Ca^{2+}-dependent attenuation of adenylyl cyclase should impair adaptation caused by a sustained odor pulse but not by a brief pulse. Previously, it was reported that CaMKII-dependent phosphorylation causes attenuation of olfactory adenylyl cyclase (Wei et al., 1998). We therefore applied a potent and selective inhibitor of CaMKII, AIP (Ishida et al., 1995). AIP was chosen because of its lack of effect on other protein kinases and its established action on odor-induced cAMP transients (Wei et al., 1998). Also, unlike other inhibitors such as KN-62, AIP does not interfere with the Ca^{2+}/calmodulin site of CaMKII (Ishida et al., 1995), which is critical for avoiding potential side effects at the Ca^{2+}/calmodulin binding site of the cAMP-gated channels (Liu et al., 1994). When the adaptation paradigms outlined in Figure 2 were repeated with ORNs that were pretreated with myristoylated AIP (1 μM for 15–20 min), we observed three major effects on odor adaptation to sustained odor pulses. First, the onset rate of adaptation was markedly reduced (Fig. 3*A*). Second, the peak amplitude of the second response was much greater than in untreated ORNs, indicating that recovery from adaptation occurred at a faster rate (Fig. 3*A*). Third, the effects of adaptation on the odor response kinectics were largely abolished (Fig. 3*B,C*). By contrast, there was no detectable effect of AIP on odor adaptation induced by a brief pulse, providing an important control for the specificity of AIP (Fig. 3*D–F*).

Figure 4 shows the quantitative analysis of results from multiple experiments. At fixed odor concentration the time constant of desensitization to sustained odor pulses was increased by nearly threefold in AIP-treated neurons, from 1.2 ± 0.6 sec (*normal*, $n = 17$) to 3.2 ± 0.7 sec (*AIP*, $n = 10$; *t* test, $p < 0.001$) (Fig. 4*A*). This effect is sufficient to significantly impair odor adaptation: whereas it takes 2 sec on average to reduce the odor sensitivity to 50% of its peak value in normal (untreated) ORNs, it takes nearly 6 sec to achieve the same sensitivity reduction in AIP-treated ORNs. A plot of the time course of recovery from adaptation is shown in Figure 4*B*, illustrating that AIP decreased the time constant for recovery from adaptation induced by sustained pulses by sixfold, from 27.7 ± 5.4 sec (*normal*, $n = 10$) to 4.4 ± 1.5 sec (*AIP*, $n = 8$) (*t* test, $p < 0.01$). When we used a higher AIP concentration (10 μM), there was no further impair-

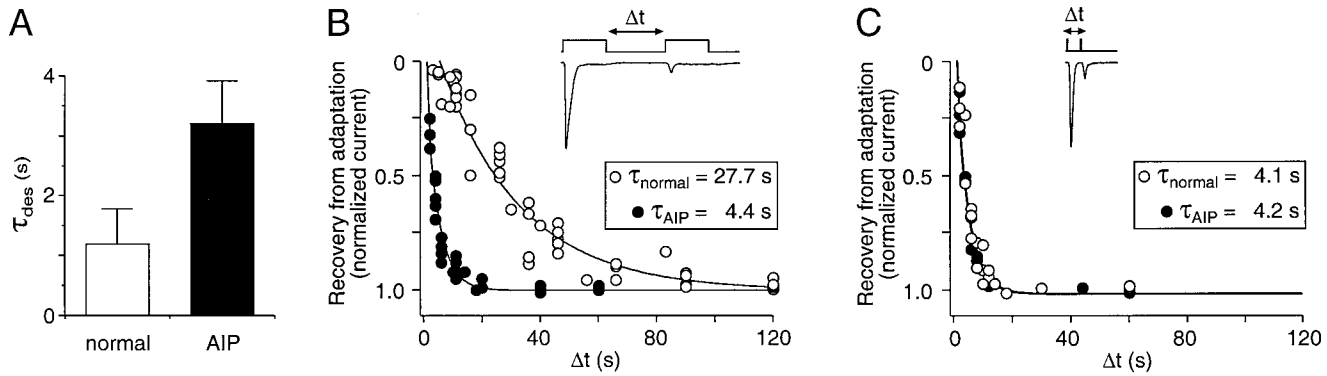

Figure 4. Analysis of the effects of AIP (1 μM) on odor adaptation using the same adaptation paradigms as in Figure 3. *A*, There is a significant difference in the onset rate of adaptation derived from single exponential fits of the desensitization time constant between normal and AIP-treated ORNs. Normal, τ_{des} = 1.2 ± 0.6 sec (n = 17); AIP, τ_{des} = 3.2 ± 0.7 sec (n = 10). *B*, The time constant of recovery from adaptation induced by a sustained odor pulse is reduced from 27.7 ± 5.4 sec (n = 10) in untreated ORNs to 4.4 ± 1.5 sec (n = 8) in AIP-treated ORNs. *C*, Recovery from adaptation by a brief odor pulse does not differ between normal and AIP-treated ORNs (*normal*, 4.1 ± 0.6 sec; n = 10; *AIP*, 4.2 ± 0.9 sec; n = 3).

ment of odor adaptation, indicating that a dose of 1 μM AIP was sufficient to cause a maximum effect (n = 3; data not shown). Recovery from adaptation induced by a brief odor stimulus did not differ between normal and AIP-treated ORNs (*normal*, 4.1 ± 0.6 sec; n = 10; *AIP*, 4.2 ± 0.9 sec; n = 3; t test, p = 0.95) (Fig. 4*C*).

DISCUSSION

This study provides strong support for the hypothesis that CaMKII-mediated attenuation of adenylyl cyclase activity is necessary for odor adaptation. Adaptation induced by sustained odor stimuli occurs downstream from the odor receptors and is triggered by Ca^{2+} entry. Consistent with a reduction in the rate of adenylyl cyclase activation, we demonstrate that adapted ORNs exhibit a markedly decreased slope of the rising phase of the odor responses. This adaptation-induced alteration in the response kinetics does not occur when ORNs are adapted by a brief odor stimulus that reduces the overall sensitivity to the same extent. To disrupt Ca^{2+}-mediated attenuation of adenylyl cyclase, we use a specific peptide inhibitor of CaMKII, AIP (Ishida et al., 1995), with established effects on odor-induced cAMP transients (Wei et al., 1998). Strikingly, only adaptation induced by sustained odor pulses is impaired after AIP treatment, whereas adaptation induced by brief odor pulses is not. Thus, the effects of AIP cannot be attributed to nonspecific actions on the cAMP signaling cascade. We therefore conclude that adenylyl cyclase activity is attenuated by CaMKII-mediated phosphorylation during a sustained odor stimulus. We demonstrate that this effect is critical for determining both the onset and recovery rate from adaptation. CaMKII is ideal for this function because, once activated, it can maintain its active state beyond the duration of the activating Ca^{2+} signal (for example, see Schulman, 1993). This is necessary because odor-induced Ca^{2+} rises in the olfactory cilia recover with a time constant of only a few seconds (Leinders-Zufall et al., 1998). Thus, if the kinetics of odor adaptation would be determined by Ca^{2+} alone, then the time course of recovery from adaptation should match the Ca^{2+} recovery. This, however, is not the case, as our results show.

The data enable us to estimate quantitatively the contribution of two distinct Ca^{2+}-dependent mechanisms to odor adaptation: modulation of cAMP-gated channels and adenylyl cyclase. If ORNs are exposed to a brief odor pulse, there seems to be no contribution of adenylyl cyclase. In this case, all adaptation depends on Ca^{2+} modulation of the cAMP-gated channel, because CaMKII disruption has no effect (Fig. 4*C*), and all aspects of adaptation can be mimicked by photolysis of caged cAMP (Kurahashi and Menini, 1997). If ORNs are exposed to sustained odor pulses, however, CaMKII inhibition of adenylyl cyclase becomes rate-limiting for recovery from adaptation (Fig. 4*B*). Adenylyl cyclase inhibition also contributes significantly to the onset rate of adaptation: with CaMKII disrupted the onset rate is diminished by threefold (Fig. 4*A*).

It has been thought that odor adaptation is caused by a single molecular step, Ca^{2+} regulation of the cAMP-gated channel (Kurahashi and Menini, 1997). The observations described in this paper are inconsistent with this notion. In fact, our results provide evidence that there are different kinetic forms of odor adaptation in single ORNs that are controlled by separate molecular mechanisms. Our study provides the first evidence that CaMKII function is necessary for determining the temporal response properties of ORNs during odor adaptation. Because temporal information may be an important part of the chemosensory code we hypothesize that CaMKII plays a prominent role in odor perception.

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