Chem. Senses 27: 635–642, 2002

Comparison of Mechanical Agitation and Calcium Shock Methods for Preparation of a Membrane Fraction Enriched in Olfactory Cilia

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Abstract

Calcium plays an important regulatory role in olfactory signal transduction. Many investigations into the regulation of the olfactory signaling pathway have been performed using fractions enriched in ciliary membranes from olfactory sensory neurons. The traditional method of preparing ciliary fractions uses high calcium concentrations, thought to dislodge cilia from the dendritic knobs of the olfactory neurons in the nasal epithelium. However, calcium, an important second messenger in the odorant signaling cascade, modulates the activity of many enzymatic reactions in this cascade. Pre-exposure of cilia to high calcium concentrations may modify these signaling events. Therefore, we sought to develop a method of isolating cilia-enriched membranes that avoids exposing the cilia to high calcium concentrations. Our method of isolation, referred to as the mechanical agitation method, involves mechanical disruption and sonication of the olfactory epithelium to dislodge the cilia. To evaluate this method of cilia preparation, basal adenylyl cyclase activity, as well as forskolin- and odorant-activated adenylyl cyclase, were analyzed. Specific activity of adenylyl cyclase and protein yield were compared for the mechanical agitation and the high calcium preparations. Immunoblots were analyzed for the presence of transduction components enriched in olfactory cilia: adenylyl cyclase type III (ACIII), heterotrimeric G-protein subunit Gαolf and the 1C2 isoform of phosphodiesterase (PDE1C2). Based on these analyses, the ciliary fraction prepared by the mechanical agitation method appears to be very similar to that prepared by the high calcium method, with a higher yield.

Introduction

Signal cascade in olfactory sensory neurons

Our current understanding of olfactory signal pathways hypothesizes that odorants traverse the mucus layer of the nasal epithelium and then bind to G-protein-coupled seven-transmembrane receptors on the cilia of the olfactory neurons. Activated receptors then stimulate the Gαolf subunit of a heterotrimeric G-protein complex and thus activate adenylyl cyclase III (ACIII), and formation of the second messenger molecule, cAMP. cAMP stimulates the opening of a cyclic-nucleotide-gated channel on the plasma membrane, admitting both Na^+ and Ca^{2+} and depolarizing the cell. Elevated intracellular Ca^{2+} then induces the opening of calcium-activated chloride channels, further contributing to cell depolarization and generation of an action potential (Zufall and Leinders-Zufall, 2000).

Desensitization of the signaling mechanism is thought to involve the second messengers cAMP and calcium. cAMP-dependent protein kinase A (PKA), as well as G-protein-coupled receptor kinase type 3 (GRK3) have been reported to contribute to the desensitization of the receptor through phosphorylation (Boekhoff *et al.*, 1997). Calcium also has been implicated in desensitization via actions of CaMKII and calcium–calmodulin binding to the cyclic-nucleotide-gated channel. Electrophysiological recordings suggest that the rise in internal calcium levels leads to a decrease in the amplitude and duration of the odor-induced response (Kurahashi, 1989; Kurahashi *et al.*, 1990). Additionally, injections of EGTA into chemosensory neurons decrease the internal calcium levels, extending the duration of the odor-stimulated response (Kurahashi and Shibuya, 1990).

Biochemical analyses of the odor-stimulated response in enriched ciliary membrane preparations have focused on modulation of the activity of the adenylyl cyclase (AC) enzyme. Published reports with contradictory interpretations indicate that regulation is complex. Several investigations using cilia preparations presented data showing that increases in calcium concentration reduce the activity of adenylyl cyclase, in agreement with physiological desensitization studies (Shirley *et al.*, 1986; Sklar *et al.*, 1986; Boekhoff *et al.*, 1996). However, data from another study (Jaworsky *et al.*, 1995) suggested that the effects of calcium on adenylyl cyclase activity might be more dynamic than previously shown. This study indicated that intermediate calcium levels increase adenylyl cyclase activity, whereas high calcium

levels decrease adenylyl cyclase activity. On the other hand, calcium–calmodulin has been reported both to enhance the activity of adenylyl cyclase (Anholt and Rivers, 1990; Choi *et al.*, 1992) and to decrease the activity of adenylyl cyclase through calcium–calmodulin-activated CamKII (Wei *et al.*, 1998). It is possible that differing concentrations of calcium selectively affect various components of signaling associated with ciliary membranes and thereby modulate transduction in a complex manner.

All of the biochemical analyses discussed above were carried out with ciliary membrane fractions isolated by the calcium shock method, where treatment with a high calcium concentration under hypotonic buffer conditions is thought to free cilia from the olfactory neurons. In addition to the effects noted above, this treatment potentially could affect calcium-sensitive machinery such as calcium-activated proteases, calcium-mediated protein associations, and kinase and/or phosphatase activity. To obviate potential alterations by calcium of the basal state of the signaling system in ciliary membranes, we have developed a preparation that does not involve exposure to high calcium concentrations as a tool for further examining the role of calcium in the modulation of the signal cascade.

Materials and methods

Initially, we planned to develop methods for isolation of sealed ciliary vesicles from olfactory sensory neurons. In the course of these studies, we observed that preparations enriched with ciliary membranes could be prepared using isotonic rather than hypotonic buffer conditions and that high calcium was not necessary. Previous calcium shock preparations enriched in ciliary membranes were prepared using hypotonic buffer with high calcium concentrations (Pace *et al.*, 1985; Sklar *et al.*, 1986). We, however, used isotonic buffer in this preparation.

Isolation of a membrane fraction enriched in olfactory cilia from primary olfactory sensory neurons by the mechanical agitation method

Five CF-1 female mice, ranging from 3 to 10 weeks of age, were killed by cervical dislocation followed by decapitation. The buffer for the membrane preparations utilized HEPES instead of $NaHCO_3$ for buffering. The nasal epithelia were surgically removed and placed in 10 ml of solution A [145 mM NaCl, 5 mM KCl, 1.6 mM K_2HPO_4, 2.0 mM $MgSO_4$, 20 mM HEPES, 7.5 mM D-glucose, 6 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.1 mg/ml Pefabloc (Boehringer Mannheim), 1 μg/ml aprotinin, pH 7.4] on ice. EDTA (final concentration 1 mM) was added to the pooled epithelia (solution B) and the solution was rocked for 20 min at 4°C (setting number 4, intermediate speed on a 55S single platform rocker; Reliable Scientific). All subsequent steps were carried out at 4°C. The suspension was then centrifuged for 10 min at 1500 *g* and the supernatant (S1) was transferred to a separate tube. The remaining pellet was resuspended in 6 ml of solution B and sonicated at low power for 10 s using a microprobe (Micro Ultrasonic Cell Disrupter; Kontes). Tissue was allowed to settle to the bottom of the tube and the supernatant (S2a) was transferred to a new tube. The pellet was resuspended in 6 ml of solution B and sonicated again at low power for 30 s. After tissue settled to the bottom of the tube, the supernatant (S2b) was removed to a new tube. The final settled tissue ('deciliated epithelium') was resuspended in 3 ml of solution B and homogenized twice using a Brinkman Homogenizer (Polytron) for 5 s separated by a period of 1 min on ice to ensure that the solution remained cool. This homogenized solution and supernatants S2a and S2b were each centrifuged for 10 min at 1500 *g* to remove debris. Each of the supernatants from the homogenized tissue (S3) and supernatants S1, S2a and S2b was centrifuged at 43 140 *g* for 25 min at 4°C to collect membranes. Pellets were then resuspended in 200 (P1, P2A and P2B) or 400 μl (pellet P3) of solution B with 5% glycerol using a Teflon pestle, apportioned into small volumes and stored at –70°C. Protein was measured using the Lowry method (Lowry *et al.*, 1951), with bovine serum albumin as standard. In six experiments, the protein yield per mouse averaged 77 ± 17 μg for the P1 fraction, 164 ± 35 μg for fraction P2A, 133 ± 17 μg for the P2B fraction and 351 ± 107 μg for the P3 fraction.

Isolation of a membrane fraction enriched in olfactory cilia from primary olfactory sensory neurons by the calcium shock method

Ciliary membranes were isolated using minor modifications of the high calcium method previously described (Pace *et al.*, 1985; Sklar *et al.*, 1986). Cilia were dislodged from the epithelium by gentle agitation in a high calcium buffer and the remaining tissue was further disrupted by homogenization and centrifuged to generate a membrane pellet. Ten CF-1 female mice, ranging from 3 to 10 weeks of age, were killed by cervical dislocation followed by decapitation. The nasal epithelium from each was surgically excised and pooled with the others in 9 ml of solution C (120 mM NaCl, 5 mM KCl, 1.6 mM K_2HPO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 7.5 mM D-glucose, adjusted to pH 7.4). All steps were carried out at 4°C. The pooled tissue was allowed to settle to the bottom of the tube and the supernatant was discarded. The tissue was resuspended in 5 ml of solution C containing 10 mM $CaCl_2$ (solution D). Solution D was prepared by slowly adding $CaCl_2$ to a final concentration of 10 mM while gassing with 5% CO_2/95% air at 22°C to prevent Ca_2PO_4 from precipitating out of solution. Tissue was rocked for 20 min at 4°C (as in the mechanical agitation method) and then centrifuged at 7700 *g* for 5 min. This supernatant was reserved in a new tube for subsequent pooling with membranes dislodged in a second rocking step. The pellet was resuspended in 4 ml of solution D and rocked for a second time for 20 min. Supernatant from this step,

collected by centrifugation at 7700 *g* for 5 min, was added to the supernatant from the first calcium shock incubation. The combined supernatant was referred to as S2. The remaining pellet was resuspended in 3 ml of solution C, homogenized twice (as in the mechanical agitation method) for 5 s with an intervening 1 min cooling period on ice to prevent tissue from increasing in temperature. Centrifugation for 5 min at 7700 *g* yielded supernatant (S3). Both S2 and S3 supernatants were centrifuged for 15 min at higher speed (27 000 *g*) to generate pellets P2 and P3. The supernatants were discarded. P2 and P3 were resuspended in 200 and 400 μl, respectively, of TEM buffer (10 mM Tris–HCl, 3 mM $MgCl_2$, 2 mM EDTA, pH 8.0) with 5% glycerol, homogenized with a Teflon pestle, apportioned into small volumes and stored at –70°C. Protein was determined by the Lowry method (Lowry *et al.*, 1951), with bovine serum albumin as standard. In four experiments, the protein yield per mouse averaged 38 ± 14 μg for the P2 fraction and 261 ± 22 μg for the P3 fraction.

Immunoblot analysis

For immunoblotting, membrane proteins were separated on 8% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. Non-specific binding sites were blocked with 5% dry non-fat milk (Carnation) in TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl and 0.4% Tween 20) for 1 h at room temperature. The blots were incubated with primary antibody for 1 h at room temperature or overnight at 4°C. Blots were washed three times with TBST and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. The secondary antibodies used were HRP-conjugated anti-rabbit antibody (1:1000; Santa Cruz), HRP-conjugated anti-mouse antibody (1:2000; Santa Cruz) and HRP-conjugated anti-biotin antibody (1:2000; New England Biolabs). Blots were washed twice with TBST and once with TBS (10 mM Tris–HCl, pH 8.0, 150 mM NaCl). The NEN chemiluminescence system was used to monitor bound antibodies. Biotinylated mol. wt markers (New England BioLabs) were used for calibration.

Adenylyl cyclase assay

Adenylyl cyclase activity was assayed according to a modified version of the method described previously (Salomon, 1979). The reaction tube (total volume 50 μl/tube) contained: reaction buffer (20 μl), membranes (10 μl, 5 μg protein), stimulant (10 μl) and dilution buffer (10 μl). The final reaction mix contained 20 mM HEPES, 5 mM $MgCl_2$, 1 mM ATP-Na_2, 10 μM GTP-Na_2, 5 mM phosphocreatine, 85 U/ml creatine phosphokinase, 1 mM dithiothreitol, 1 μCi α-[^{32}P]ATP (30 Ci/mmol), pH 7.4, with or without a stimulant (odor mixture or forskolin) and 50 μM cAMP to inhibit breakdown of [^{32}P]cAMP by phosphodiesterase. In some experiments, 0.5 mM isobutyl-1-methylxanthine (IBMX), a non-selective phosphodiesterase inhibitor, was added to confirm that [^{32}P]cAMP breakdown was completely blocked. Addition of IBMX yielded the same results as excess cold cAMP, thereby indicating that cold cAMP is sufficient to block breakdown. Stimulants were odorant mixture (citralva, eugenol and D-carvone; final concentration of 0.1 mM each) or forskolin (final concentration of 5 μM), with a final ethanol concentration of 0.09 or 0.5%, respectively. Corresponding controls contained the same amount of ethanol. Reactions were incubated for 15 min at 37°C in a shaking water bath and terminated on ice by the addition of 10 μl of ice-cold 'stop' solution (5 mg/ml cAMP, 0.5 N HCl, [^{3}H]cAMP tracer, 1×10^5 d.p.m./sample to calculate recovery). cAMP was separated from the reaction mix on SpinZyme acidic alumina columns (Pierce Chemicals) according to manufacturer's instructions. Radioactivity of the eluates was determined by liquid scintillation spectrometry. The time course was linear for 15 min under these conditions at protein concentrations ranging between 1 and 5 μg.

Statistical analysis

Data were analyzed by two way, repeated-measure ANOVA. The within-subject factor was stimulus condition and the between-subject factor was preparation. Significance levels were set at $P \leq 0.05$.

Materials

Two-week-old female CF-1 mice were purchased from Charles River Laboratories. α-[^{32}P]ATP and 2,8-[^{3}H]cAMP were from Du Pont–New England Nuclear. Forskolin was purchased from Calbiochem. The odorant mix was composed of three different chemicals: D-carvone, eugenol (both from Fluka Chemicals) and citralva (International Flavors and Fragrances Inc.). The protease inhibitors chymostatin and Pefabloc were purchased from Boehringer-Mannheim. Aprotinin, leupeptin and pepstatin all were purchased from Sigma Chemicals. Affinity-purified rabbit polyclonal antibodies to adenylyl cyclase III and to Gαolf were purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibody to PDE1C2 was a generous gift from Dr Joseph Beavo, Department of Pharmacology, University of Washington, Seattle, WA. All other reagents were purchased from Sigma and were ultrapure.

Results

Two membrane preparations enriched in cilia from olfactory receptor neurons were evaluated: a newly developed 'mechanical agitation' method and the traditional calcium shock method. Preliminary experiments evaluated effects of sonication strength and time on the specific activity of adenylyl cyclase and the yield. The optimal conditions are reported here. Both methods of cilia preparation are outlined in Figure 1. In the mechanical agitation method, nasal epithelial tissue is rocked gently in HEPES/saline

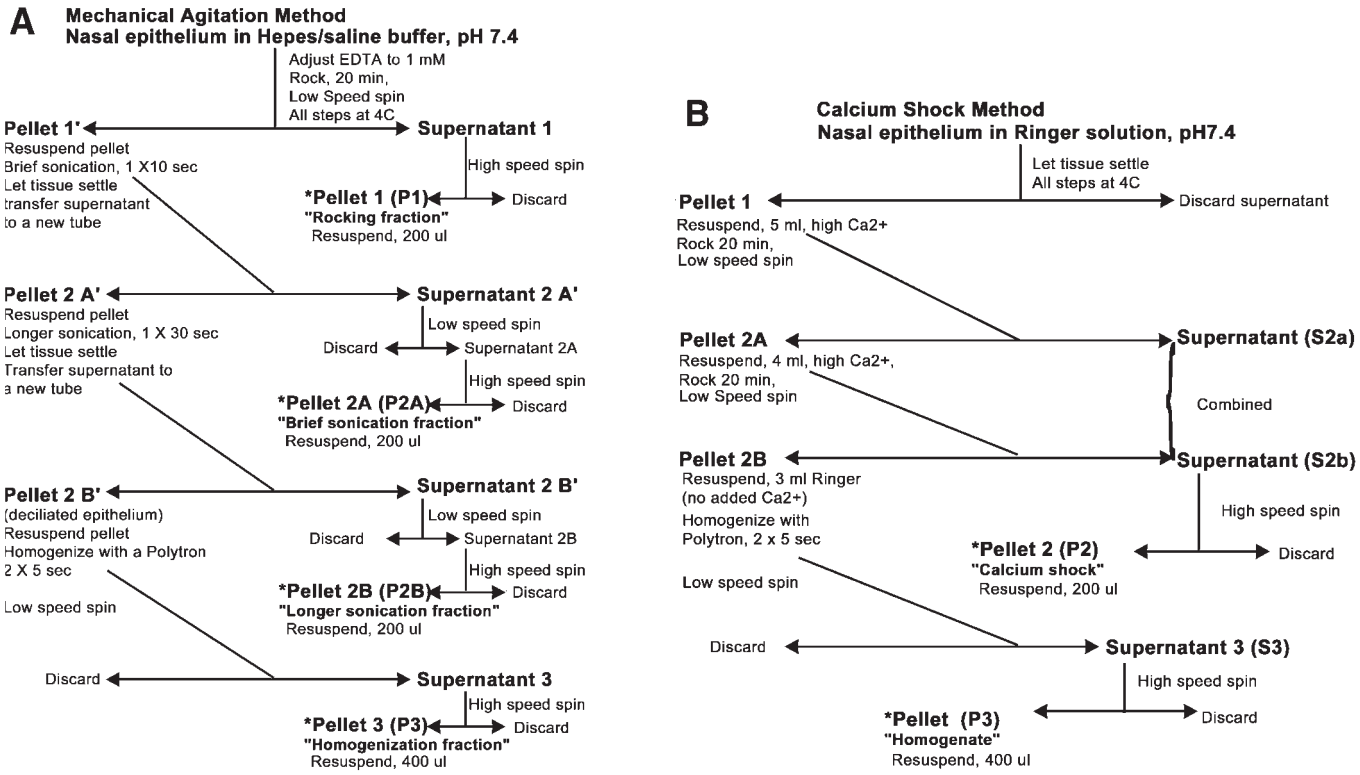

Figure 1 Purification schemes for isolating olfactory cilia from nasal epithelium. **(A)** Mechanical agitation scheme. All steps were carried out at 4°C. To remove debris, fractions were centrifuged at low speed (1500 *g*) for 10 min. To collect 'cilia', membrane fractions were centrifuged at high speed (43 140 *g*) for 25 min. All fractions were resuspended in HEPES/saline–EDTA buffer containing protease inhibitors (see Materials and methods). All final membrane pellets (*) were frozen at –70°C with 5% glycerol added. **(B)** Calcium shock scheme. All tissue pellets were resuspended in buffer D solution containing 10 mM $CaCl_2$, on ice unless otherwise indicated. To collect tissue fragments, fractions were centrifuged at low speed (7700 *g*) for 5 min. To collect 'cilia' and membranes, fractions were centrifuged at high speed (27 000 *g*) for 15 min. All fractions were resuspended in HEPES/saline–EDTA buffer containing protease inhibitors (see Materials and methods). All final membrane pellets (*) were resuspended into TEM buffer/5% glycerol with a Teflon pestle and stored at –70°C.

buffer with EDTA, followed by a centrifugation step. The resulting pellet fraction is further disrupted by two sonication steps and fractions are collected by differential centrifugation. In the calcium shock method, the nasal epithelium is gently rocked in a high calcium buffer followed by differential centrifugation to collect fractions. Fractions from these two preparations were biochemically characterized by comparing average protein yields, basal adenylyl cyclase activity, forskolin-stimulated adenylyl cyclase activity, odorant-stimulated adenylyl cyclase activity and by detection of ciliary marker proteins using immunoblot analysis.

Ciliary membrane enrichment in each fraction from both preparations was estimated by assay of odorant-stimulated adenylyl cyclase activity (an activity marker for ciliary membranes; Figure 2). Basal, forskolin-stimulated and odorant-stimulated adenylyl cyclase activity was observed in every fraction. Specific activity for odor-stimulated and forskolin-stimulated adenylyl cyclase activity was similar in the initial fraction of each preparation. Subsequent fractions had lower specific activity. There is no dramatic difference in fold-stimulation by odorant or forskolin across fractions, suggesting that components for stimulation are present in every fraction and that inhibitors are not concentrated in any particular fraction.

In Table 1, basal activity has been subtracted from stimulated activity to show net specific activity. For mechanical agitation, the results show that P1 specific activity was approximately twice that of P2A for the odor-stimulated fraction, indicating that P1 was more enriched for ciliary membranes. For calcium shock, the specific activity of the odor-stimulated and forskolin-stimulated fraction P2 was comparable to that for mechanical agitation. Although the average value of P2 appeared somewhat higher than the comparable fraction, P1, from the mechanical agitation method, ANOVA showed no significant difference between P1 of the mechanical agitation preparation and P2 of the calcium shock preparation.

The distribution of total cyclase activity from the mechanical agitation and the calcium shock procedure is outlined in Table 2. One-third of the total activity was recovered in the P1 fraction of the mechanical agitation procedure and

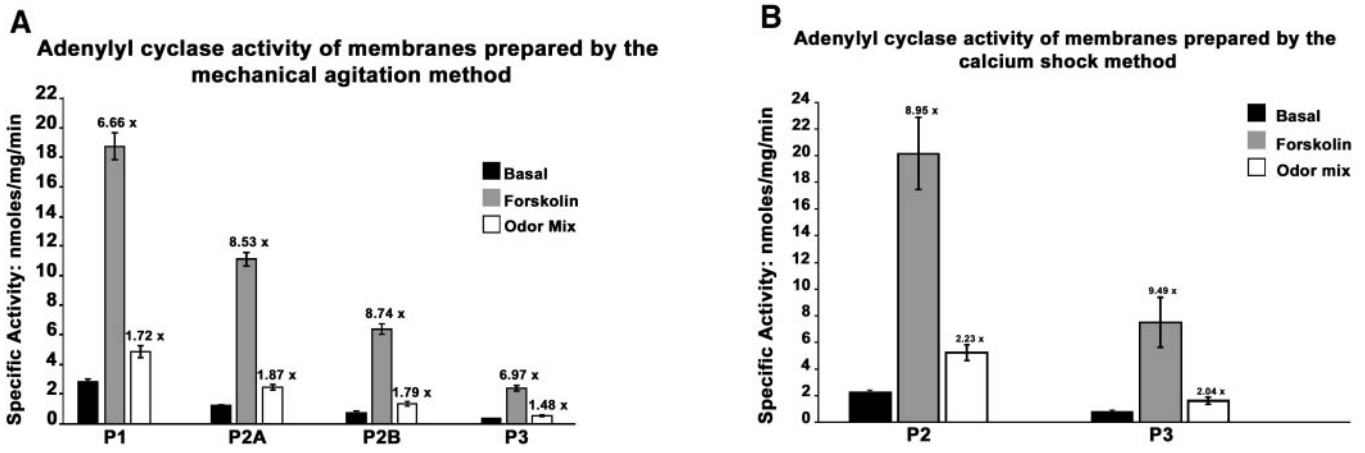

Figure 2 **(A)** Adenylyl cyclase assay of isolated membranes from mechanical agitation. Cyclase assay was carried out in the presence of 50 μM cAMP under basal conditions (black bars), with 5.0 μM forskolin (gray bars), or with an odorant mixture containing 100 μM each of citralva, eugenol and D-carvone (white bars). P1 = rocking step. P2A = first sonication. P2B = second sonication. P3 = homogenized (Polytron) deciliated epithelium. The specific activity of adenylyl cyclase is shown as nmol/mg protein/min. The average fold-stimulation compared to basal is reported above each column. The values are averages of six separate experiments ± SEM. Each data point was determined in duplicate. **(B)** Adenylyl cyclase assay of membranes isolated by calcium shock. Cilia membranes were assayed as in (A). P2 = membranes isolated by calcium shock. P3 = membranes isolated from residual epithelium by homogenization (Polytron, post-calcium). Data are represented as in (A). The values are averages of four separate experiments ± SEM.

Table 1 Distribution of adenylyl cyclase specific activity in isolated fractions

Method of isolation	Fraction		Odor-stimulated – basal specific activity ± SEM (nmol/mg/min)	Forskolin-stimulated – basal specific activity ± SEM (nmol/mg/min)
Mechanical agitation	rocking	P1	2.04 ± 0.17	15.92 ± 0.70
(n = 6)	brief sonication	P2A	1.13 ± 0.13	9.79 ± 0.40
	long sonication	P2B	0.57 ± 0.05	5.62 ± 0.29
	'deciliated membranes'	P3	0.17 ± 0.03	2.04 ± 0.18
Calcium shock	calcium shock	P2	2.97 ± 0.50	17.95 ± 2.60
(n = 4)	'deciliated membranes'	P3	0.82 ± 0.12	6.75 ± 1.76

The basal specific activity (nmol/mg/min) was subtracted from the odor- and forskolin-stimulated specific activity. SEM refers to standard error of the mean. There was a significant effect of stimulus, but no effect of preparation and no significant stimulus by preparation interaction.

the P2 fraction of the calcium shock procedure. Another 40% of the total activity was recovered in fraction P2A of the mechanical agitation procedure. However, the specific activity of the P2A fraction was only half that of the P1 fraction, thereby indicating that P2A was not as enriched for cilia as P1. Distribution of total activity across fractions indicated no evidence for separation of odor-stimulated and forskolin-stimulated components (Table 2). There did not appear to be any fraction in which odorant receptor-activated cyclase was enriched over another compartment containing cyclase uncoupled to receptor. The yield of odorant- and forskolin-stimulated adenylyl cyclase activity by the mechanical agitation method was significantly higher than the yield by the calcium shock method (ANOVA).

Molecular components of both preparations were characterized by immunoblot analysis of marker proteins of cilia. Immunohistochemical and immunoblot analysis from previous reports showed that ACIII, Gαolf and PDE1C2 are highly enriched in cilia structures (Yan *et al.*, 1995; Belluscio *et al.*, 1998; Schandar *et al.*, 1998; Wei *et al.*, 1998; Wong *et al.*, 2000). Immunoblot characterization of the mechanical agitation and the calcium shock fractions (Figure 3) shows that ACIII, Gαolf and PDE1C2 were enriched in the first fraction of membranes from both procedures. The levels of these proteins diminished in subsequent fractions. This result is consistent with the enzymatic analysis and indicates that the P1 fraction from mechanical agitation and the P2 fraction isolated by calcium shock were most enriched in ciliary membranes.

Discussion

Mechanical agitation is an effective way of preparing enriched fractions of olfactory cilia without exposing the tissue to high concentrations of calcium. This new method

Table 2 Distribution of adenylyl cyclase activity in isolated fractions

Method of isolation	Fraction		Odor-stimulated		Forskolin-stimulated	
			Activity yield ± SEM per animal (nmol/min)	Odor-stimulated percentage of total activity	Activity yield ± SEM per animal (nmol/min)	Forskolin-stimulated percentage of total activity
Mechanical agitation ($n = 6$)	rocking	P1	0.15 ± 0.006	33	1.20 ± 0.085	29
	brief sonication	P2A	0.18 ± 0.025	40	1.60 ± 0.155	39
	long sonication	P2B	0.06 ± 0.006	14	0.64 ± 0.051	15
	residual	P3	0.06 ± 0.012	13	0.71 ± 0.100	17
Calcium shock ($n = 4$)	calcium shock	P2	0.12 ± 0.033	35	0.72 ± 0.201	29
	residual	P3	0.22 ± 0.033	65	1.73 ± 0.398	71

Nasal epithelial tissue was extracted from five mice for the mechanical agitation procedure. Nasal epithelial tissue was extracted from 10 mice for the calcium shock procedure. Corrected specific activity (nmol cAMP/mg protein/min) multiplied by protein recovered (mg protein/fraction) equals total activity per fraction (nmol cAMP/min/fraction). This value was divided by the number of animals per preparation to calculate recovery yield per animal. SEM refers to standard error of the mean. There was a significant effect of stimulus and of preparation and there was a significant stimulus by preparation interaction.

of isolating membrane fractions enriched in cilia was compared to the calcium shock method, using isotonic buffer conditions for each preparation. We showed that membranes enriched with olfactory cilia can be isolated under isotonic conditions (a more physiological environment) without the use of high calcium concentrations. Maintaining a physiological environment may prevent perturbations of biochemical enzymes and pathways induced by hypotonic and high calcium buffer conditions.

The most active fractions from both preparations were equivalent in enrichment, as shown by several criteria: specific activity, fold stimulation and enrichment for specific ciliary marker proteins. In our hands, the mechanical agitation method produced a slightly better yield of odorant- and forskolin-stimulated adenylyl cyclase activity than the calcium shock method (Table 2).

The specific activity of adenylyl cyclase in fractions enriched in ciliary membranes varies according to animal species and across laboratories. These results are difficult to compare across studies because different odorants and concentrations were employed. The relevant studies also assayed cAMP production on a minute time scale and separated products by column chromatography. In our laboratory, fractions enriched for ciliary membranes that are isolated from mouse olfactory epithelial tissue by the mechanical agitation and the calcium shock preparations show similar basal (10 μM GTP; 2.81 ± 0.22 and 2.26 ± 0.15, nmol/mg/min, respectively) and forskolin-stimulated (18.73 ± 0.91 and 20.21 ± 2.71, nmol/mg/min, respectively) specific activity responses. For both preparations, odorant-stimulated activity exhibited a 1.7- to 2.3-fold increase over basal conditions. In agreement with these values, a study that used membranes from cilia dislodged by calcium shock from both male Sprague–Dawley rats and frogs (*Rana catesbeiana*) reported comparable values. For both frog and rat, the basal plus GTP activity ranged from 2 to 4 nmol/mg/min and 4 μM forskolin-stimlulated activity ranged from 18 to 24 nmol/mg/min. At maximum stimulation, the odorant (100 μM) activity was ~1.5- to 1.6-fold above basal plus GTP specific activity (Sklar *et al.*, 1986). Other publications from Lancet's laboratory using ciliary membrane preparations isolated by calcium shock from frog (*Rana ridibunda*) or rat (BN/Mai from Weizmann Institute Animal Breeding Center) report a higher basal and stimulated adenylyl cyclase specific activity. In frog, basal plus GTP activity is ~25 nmol/mg/min (Chen *et al.*, 1986) and maximal odorant stimulated activity at ~50 nmol/mg/min (Pace *et al.*, 1985; Chen *et al.*, 1986). In a separate study from this laboratory, even higher levels of basal and odorant-stimulated adenylyl cyclase activity have been reported (Pace and Lancet, 1986).

Calcium has been thought to be essential for dislodging cilia from olfactory neurons in epithelial tissue, but it remains unclear how exposure of olfactory epithelial tissue to high calcium concentrations affects the signaling process. Reasons to be concerned are that signal transduction is sensitive to dynamic changes in calcium. Calcium concentrations affect calcium-sensitive machinery, such as calcium-activated proteases, calcium-mediated protein association and kinase and/or phosphatase activity. Changes in calcium concentrations may alter covalent modifications, signal complex associations and/or location of signal components in cilia of olfactory neurons that potentially could alter the state of the isolated cilia. Therefore, calcium might be expected to alter the basal or regulatory state of isolated cilia because it affects so many signaling proteins in the cell.

In the majority of olfactory neurons, odor-stimulated olfactory receptors activate signal cascades that ultimately result in the increase of internal calcium concentrations.

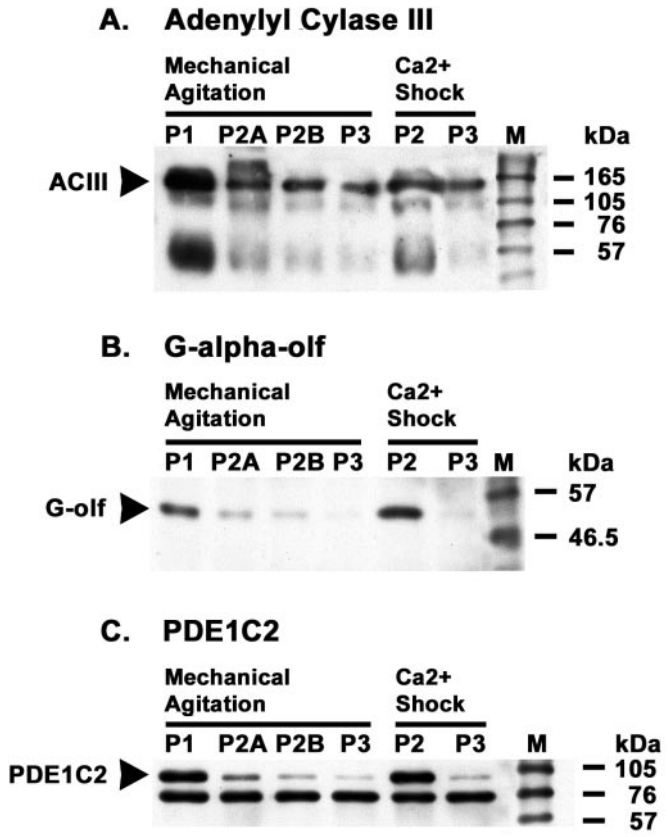

Figure 3 Immunoblot analysis of isolated membranes from the mechanical agitation and the calcium shock preparations using antibodies against ACIII, Gαolf and PDE1C2. Membrane fractions isolated from olfactory tissue by mechanical agitation or calcium shock were probed with antibodies to determine the enrichment of ciliary markers in a particular fraction of the preparation. **(A)** Fractions (1.5 μg of protein per lane) were probed with rabbit polyclonal antibodies to ACIII (1:200) from Santa Cruz. **(B)** Fractions (20 μg of protein per lane) were probed with rabbit polyclonal antibodies to Gαolf (1:200) from Santa Cruz. **(C)** Fractions (10 μg of protein per lane) were probed with rabbit polyclonal antibodies to PDE1C2 (1:10 000) from Dr Joseph Beavo. Fractions from the mechanical agitation procedure are labeled as follows. P1 = membranes from rocking. P2A = membranes from first sonication. P2B = membranes from second sonication. P3 = membranes from the homogenized deciliated epithelium. Fractions from the calcium shock procedure are labeled as follows. P2 = membranes from calcium shock. P3 = membranes from homogenized post-calcium treated epithelium. M = mol. wt markers.

Elevated cytosolic calcium levels lead to calcium–calmodulin inhibition of the cyclic-nucleotide-gated channel and also to activation of CaMKII. CaMKII phosphorylates and reduces the activity of adenylyl cyclase, suggesting an involvement with signal termination (Wei *et al.*, 1998). CaMKII activity also results in the phosphorylation of ERK I/II, leading to the stimulation of Cre-mediated gene transcription (Watt and Storm, 2001).

PDE1C2 also is calcium–calmodulin activated and breaks down the second messenger molecule cAMP to 5′-AMP, contributing to the termination of the signal event (Borisy *et al.*, 1992; Yan *et al.*, 1995). Thus, high calcium concentrations are intimately involved in the dynamics of cAMP signal transduction in olfactory cilia.

An additional reason for being concerned about the effect of high calcium on cilia is that some isoforms of G-protein-coupled receptor kinases (GRKs) are modulated by calcium. GRKs phosphorylate seven transmembrane receptors resulting in the down regulation of receptor activity. GRK3 has been localized to the cilia of rat olfactory neurons and appears to play a role in desensitization of olfactory receptors (Schleicher *et al.*, 1993). Calcium–calmodulin complexes inhibit GRK3, thereby potentially modulating odor receptor activity (Chuang *et al.*, 1996).

Protein kinase C (PKC) also has been implicated in odorant-mediated signaling and desensitization (Boekhoff and Breer, 1992; Boekhoff *et al.*, 1992; Schleicher *et al.*, 1993). In rat olfactory neurons, the γ, δ and λ isoforms of PKC have been localized to the sensory cilia by immunohistochemistry (Muller *et al.*, 1998). The γ isoform of PKC, a member of the 'classic' group, depends upon calcium for its regulation (Muller *et al.*, 1998). In frog olfactory epithelium, activators of PKC (PDBu and the diterpene mezerein) potentiated forskolin-induced cAMP accumulation, whereas inhibitors of PKC (Goe 6983, staurosporine and polymyxin) had no effect (Frings, 1993). Alterations of calcium concentrations also may alter PKC distribution (Almholt *et al.*, 1999) or perturb odorant-signaling.

Neural proteinases (calpains) are calcium-dependent cysteine proteases that should be considered in evaluating ciliary isolation protocols. Immunostaining of calpain I in rat brain showed high levels of expression in primary olfactory axons (Siman *et al.*, 1985). Calpains activate or alter the regulation of certain enzymes, such as protein kinases and phosphatases, by limited proteolysis and thus could potentially modify the state of proteins involved in odorant signaling (Molinari and Carafoli, 1997), although no such effects have been reported as yet.

Summary

We have developed an alternative method of isolating membranes enriched with ciliary markers that does not depend upon high calcium. Since calcium participates in multiple regulatory steps in olfactory neuron signaling, the possible effects of high calcium treatment warrant careful consideration. The data from these experiments indicate that high calcium is not essential for releasing cilia from olfactory neurons in the epithelial tissue. One apparent advantage of the mechanical agitation procedure is that the yield of functional membranes is somewhat higher than that of the calcium shock method, with comparable specific activity of adenylyl cyclase. Future studies will evaluate whether differing methods of isolation alter the dynamics of signaling and regulation.

Acknowledgement

This work was supported in part by a grant from DARPA (DAAK60-97-K-9502).

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Accepted June 6, 2002