Hippocalcin in the olfactory epithelium: a mediator of second messenger signaling

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Abstract

Intracellular Ca²⁺ plays an important role in a variety of second messenger cascades. The function of Ca²⁺ is mediated, in part, by Ca²⁺-binding proteins such as calmodulin, calretinin, calbindin, neurocalcin, recoverin, and visinin-like proteins (VILIPs). These proteins are highly expressed in rat olfactory receptor neurons (ORNs) and are localized to distinct intracellular regions. In the present study, we have identified another Ca²⁺-binding protein, hippocalcin, in the rat olfactory epithelium (OE). Olfactory/brain hippocalcin shows high sequence homology with hippocalcins expressed in mice and humans. Hippocalcin was predominantly localized to the olfactory cilia, the site of the initial events of olfactory signal transduction, and was found to regulate the activity of ciliary adenylate cyclases (ACs) and particulate guanylyl cyclases (GCs) in a Ca²⁺-dependent manner. These data indicate that hippocalcin is expressed in rat ORNs, and is likely to regulate second messenger cascades in a Ca²⁺-dependent manner.

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Ca²⁺ regulates diverse cellular functions in a variety of cell types, including neurons. In neurons, Ca²⁺-dependent regulation is involved in a number of critical neuronal processes such as excitation, neurotransmitter release, synaptic plasticity, and gene transcription [1–4]. In most cases, Ca²⁺-binding proteins regulate these Ca²⁺-related functions (see a review [5]). Olfaction is an excellent example of a signaling system that utilizes Ca²⁺ in a number of ways (see reviews [6,7]). In response to odorants, ORNs display a rapid and transient increase in intracellular Ca²⁺ levels. Briefly, transduction begins when odorants interact with specific receptors present on the cilia of ORNs. These G-protein-coupled receptors activate ACs to produce cAMP (Fig. 1). As intracellular cAMP levels increase, olfactory cyclic nucleotide-gated channels (oCNCs) open to allow an influx of Na⁺ and Ca²⁺, leading to the generation of an action potential, which transduces signals to the olfactory bulb. Later, Ca²⁺ is pumped out of ORNs by Na⁺/Ca²⁺ exchangers and Ca²⁺-ATPases present in the cilia and dendritic knobs, thus maintaining Ca²⁺ homeostasis and returning the cell to electrical neutrality [8–10] (Fig. 1). Other second messengers, such as cyclic GMP, are also produced by odorant stimulation [11–13].

Olfactory signal transduction pathways are modulated by changes in intracellular Ca²⁺ concentrations (see reviews [6,7]), and specific Ca²⁺-binding proteins mediate these changes. Thus, identification and characterization of the Ca²⁺-binding proteins within the olfactory system can provide important information about the complex signal transduction involved in olfaction. To date, olfactory tissues have been reported to contain

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Abbreviations: AC, adenylate cyclase; GC, guanylyl cyclase; GCAP, guanylyl cyclase-activating protein; NCS, neuronal Ca²⁺ sensor; OE, olfactory epithelium; ORN, olfactory receptor neuron; PKG, cGMP-dependent protein kinase; VILIP, visinin-like protein.

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a variety of Ca\(^{2+}\)-binding proteins, including calmodulin [14], calretinin [15], p26olf [16], calbindin-D28k [15], neurocalcin [17,18], recoverin [18], VILIP [19], and guanylyl cyclase-activating protein 1 (GCAP1) [13]. We have identified another Ca\(^{2+}\)-binding protein, hippocalcin, in rat OE.

Hippocalcin is a Ca\(^{2+}\)-binding protein of the neuronal Ca\(^{2+}\) sensor protein (NCS) family. It was first thought to be expressed exclusively in the hippocampus [20], but later was found in other brain regions, including the neocortex, caudate-putamen, taenia tecti, claustrum, olfactory tubercle, anterior olfactory nucleus, and the granule cell and glomerular layers of the olfactory bulb [21]. It has a primary structure containing three putative Ca\(^{2+}\)-binding sites (EF-hands) and a N-terminal myristoylation site. Several studies have revealed that myristoylation is necessary for its Ca\(^{2+}\)-dependent membrane association [20,22,23]. The N-terminal myristoyl moiety on hippocalcin interacts with lipid bilayers and facilitates interaction with other membrane proteins [20]. Hippocalcin is also expressed in Drosophila melanogaster and is found primarily in the brain [24]. The fact that hippocalcin is expressed throughout the central nervous system of adult flies, particularly in the neuropil, suggests that hippocalcin is perhaps involved in synaptogenesis. Similar implications are suggested by studies of post-natal developmental expression of hippocalcin in rat retina. These studies demonstrate that compared to other Ca\(^{2+}\) sensor proteins, hippocalcin is expressed later in development at a time when synaptogenesis is taking place [25]. Here, we demonstrate another important role for hippocalcin in ORNs, modulation of second messenger signaling.

**Materials and methods**

**Antibody.** Rabbit anti-hippocalcin antibodies were a gift from Dr. M. Kobayashi (Toho University School of Medicine, Tokyo, Japan).

**Calculation of the phylogenetic tree.** The phylogenetic tree was constructed from aligned sequences using the ClustalW program by the neighbor-joining method [26] using the Neighbor program in the PHYLIP package (version 3.6a, 2002, distributed by Dr. J. Felsenstein, Department of Genetics, University of Washington, Seattle, WA). Gaps were removed from the calculations. The evolutionary distances of the sequences were estimated based on Dayhoff’s PAM matrix [27] using the program of Protdist of the PHYLIP package (version 3.6a). Bootstrap re-samplings were performed in the Seqboot program of the PHYLIP package (version 3.6a). Accession numbers of amino acid sequences are indicated in the figure legend.

**Cilia preparation.** Cilia were prepared as previously published [13,28]. Briefly, male Sprague–Dawley rat nasal turbinates were dissected, pooled, and washed in EDTA (2mM) in Ringer’s solution (112mM NaCl, 3.4mM KCl, 2.4mM NaHCO\(_3\), and 2mM Hepes, pH 7.4) at 4°C. The tissue was centrifuged at 5000 g for 5 min, and the pellet was re-suspended in deciliation buffer. The bathing medium was supplemented with CaCl\(_2\) to a final concentration of 10mM and agitated gently end-over-end for 20 min at 4°C. The deciliated epithelium was removed by centrifugation for 5 min at 1500 g. The supernatant containing isolated cilia was centrifuged for 10 min at 12,000 g and the final cilia pellet was re-suspended in 20 l of 10mM Tris–HCl, 3mM MgCl\(_2\), and 1mM EDTA, pH 8.0, and stored at –80°C before use.

**Cyclase assay.** Cyclase assays were performed as previously described [29] with some modifications. The reaction was initiated by adding olfactory cilia (5µg) and purified hippocalcin (40 µM) to the stimulation solution containing (final concentration): 1.3mM \([\gamma-^{32}\text{P}]\text{ATP (19,000–22,000 dpm/mmol; New England Nuclear), 50mM Hepes (pH 7.8), 60mM KCl, 20mM NaCl, 10mM MgCl}_2, 0.4M EGTA, an appropriate concentration of CaCl}_2 (0.1–10µM free Ca}^{2+}\), and 1mM 3-isobutyl-1-methyl-xanthine (IBMX). Free Ca\(^{2+}\) was calculated using the MaxChelator program (www.stanford.edu/~cpatton/webmaxx2.htm). The reaction was terminated after 2 min stimulation.
cAMP formation was measured with a scintillation counter [30–45]. For guanylyl cyclase (GC) assays, 1.3 mM [γ-32P]GTP (19,000–22,000 dpm/nmol) was used and cGMP [30] formation was measured after 10 min stimulation.

Expression and purification of hippocalcin. The entire coding region of hippocalcin was sub-cloned into pET17b (Novagen, Madison, WI, USA) using HindIII and BamHI sites, and expressed in BL21 (DE3) co-transfected with a pBB131 construct containing the sequence for N-myristoyl transferase. Bacterially expressed hippocalcin was purified according to the instructions for insoluble proteins (T7 Tag Purification Kit; Novagen).

Immunohistochemical analysis. Immunohistochemistry was performed following the Vectastain ELITE protocol (Vector Laboratories). Tissue sections were permeabilized for 1 h in PBS containing 0.1% Triton X-100 and blocked for 1 h in PBS containing 1% BSA and 4% normal serum. Section slides were then incubated overnight at 4°C in PBS containing the rabbit anti-hippocalcin antibody at a dilution of 1:500. The next day, slides were rinsed with PBS and incubated with Vectastain (Vector Laboratories) biotinylated secondary antibody (1:500). The next day, slides were rinsed with PBS and incubated with Vectastain (Vector Laboratories) biotinylated secondary antibody (1:1000) for 30 min, and then incubated in 0.5% H2O2 for 10 min at room temperature. Slides were then incubated in the avidin–biotin reagent for 30 min, rinsed in PBS, and developed using 0.25 mg/ml diaminobenzidine (Sigma) in 50 mM Tris, pH 7.4. For pre-absorption of anti-hippocalcin antibody, the rabbit anti-hippocalcin antibody was incubated with purified hippocalcin (1.5 μg) on an orbit rotator overnight at 4°C and used as a primary antibody.

Gel electrophoresis and immunoblot analysis. Whole rat adult nasal epithelium and tissues were homogenized in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (v/v) deoxycholic acid, and 0.1% SDS) and incubated on ice for 30 min. The extracts were cleared by centrifugation for 15 min in an Eppendorf microfuge at 13,000 rpm at 4°C. Supernatants (40 μg of protein per gel lane) were subjected to SDS–PAGE on a 4–15% gradient gel. Protein content was measured using Bradford assay with BSA as a standard. The separated proteins were transferred to PVDF membrane and the membranes were probed with rabbit anti-hippocalcin antibody. Horseradish peroxidase-conjugated goat anti-rabbit IgG (Roche Molecular Biochemicals) was used as a secondary antibody at 1:5000 dilutions. Bands were visualized by enhanced chemiluminescence (Amersham–Pharmacia).

Isolation of the hippocalcin cDNA clone. Total RNA was purified from OE tissues by the acid guanidinium thiocyanate phenolchloroform extraction method [46]. For cDNA synthesis, polyadenylated RNA was further isolated using mRNA purification kit (Invitrogen). An oligo(dT)-primed rat OE cDNA library was constructed in UniZAP XR (Strategene) from poly(t)+ RNA isolated from rat OE tissues by using a ZAP-cDNA synthesis kit (Strategene).

Degenerate oligonucleotide primers were designed based on conserved sequences of GCAP Ca2+-binding protein family members from various species. Hippocalcin was isolated by reverse transcriptase-polymerase chain reaction from the rat OE cDNA library using sense primer (5'-CATATGGGCAAGCAGATAGCAAGC-3') and anti-sense primers (5'-AAGCTTCAACTGAGGAGCT-3'). Multiple DNA fragments were acquired through PCR and cloned into pSTBlue-1 (Novagen, Madison, WI, USA) using the Perfect Blunt Cloning Kit. Sequence analysis was used to identify full-length clones that were then sub-cloned into a pET17B expression vector.

Phosphorylation and dephosphorylation of cilia fractions. Phosphorylation reactions were performed according to the method of Moon et al. [13], with modifications. Protein kinases and phosphatases were obtained from Promega (Madison, WI). Isolated cilia fractions (200 μg) were prepared in a reaction buffer (200 μl) containing 50 mM Tris·HCl, pH 7.5, 10 mM MgCl2, 0.4 mM EGTA, 10 μg/ml calmodulin, 1 mM LiCl, 1 mM 8-Br-cGMP, and 1 mM ATP.

For phosphorylation of cilia fractions, cAMP-dependent protein kinase catalytic subunit (120 U), cGMP-dependent protein kinase (300 U), and Ca2+/calmodulin PK (CaMK, 1 U) were added and incubated for 30 min at room temperature. The cilia fractions were centrifuged for 2 min at 12,000g. The resulting pellet of cilia fraction was re-suspended in cyclase assay solution and assayed for AC activity.

For dephosphorylation of cilia fractions, protein phosphatases 2A and 2B were used. Cilia fractions were incubated with protein phosphatase 2A (2.5 U) and protein phosphatase 2B (0.5 U) for 30 min at room temperature. Then, the cilia fractions were centrifuged, re-suspended in cyclase assay solution, and assayed for AC activity.

Control cilia fractions were kept for 30 min at room temperature and assayed for AC activity.

Statistical analysis. Apparent cyclase activities under various free Ca2+ concentrations and dose–responses of hippocalcin protein were analyzed statistically, using one-way ANOVA. Post testing was performed comparing all values to control (Dunnett method). Statistical significance is shown in each figure.

Results and discussion

Hippocalcin was identified in a rat OE cDNA library generated using poly(A)+ RNA isolated from rat OE. The complete rat hippocalcin sequence (GenBank AY442172) contained an open reading frame of 579 bp (193 amino acids). The rat hippocalcin showed high amino acid sequence homology to mouse and human hippocalclins from brains (100% and 99%, respectively). Sequence analysis of hippocalcin compared to VILIP-1, VILIP-2, and VILIP-3 showed that hippocalcin had the highest sequence homology to VILIP-3 (95%; Fig. 2A), but showed less homology to other VILIPs (66%; Fig. 2A).

Phylogenetic analysis of Ca2+-binding proteins suggests that the ancestors of NCSs were first duplicated into the ancestors of the GCAP subfamily and the other NCSs (Fig. 2B). High clustering probabilities of NCSs (100%) and GCAP1s (99.7%) substantiate this possibility. The ancestors of the other neuronal Ca2+ sensors diverged to form recoverin, VILIP-1, VILIP-2, VILIP-3, hippocalcin, and neurocalcin subfamilies. Hippocalcin is phylogenetically similar to members of the VILIP family, which are found in various regions of brains and known to be involved in cellular signal transduction [47]. Olfactory hippocalcin is 100% identical to the brain hippocalcin. Another OE Ca2+-binding protein, p26olf, is more closely related to calbindin/calretinin than to NCSs.

To investigate the role of hippocalcin in olfactory signal transduction, we determined the intracellular localization of hippocalcin in adult rat OE by immunohistochemical analysis. Hippocalcin immunoreactivity was predominantly localized to the apical layer of the OE, where the olfactory knob and cilia of mature ORNs reside (Fig. 3A), suggesting that hippocalcin may be involved in initial olfactory signal transduction or adaptation. Signal was not detectable in soma or axons of ORNs. When the antibodies were pre-absorbed with purified hippocalcin proteins, the immunoreactivity of hippocalcin in the ciliary layer was greatly reduced (Fig. 3B, right panel). Immunoblot analysis showed that hippocalcin is also
expressed in the olfactory bulb though at lower levels than in the OE (Fig. 3C), indicating additional roles for hippocalcin in olfactory processing.

Olfactory tissues express a variety of Ca\(^{2+}\)-binding proteins in distinct subcellular locations. Calmodulin is expressed in olfactory cilia, cytoplasm, and axon fibers [18,48]. Calretinin is expressed in rat apical dendrites and axon fibers of ORNs [18]; p26olf in frog olfactory cilia [16]; calbindin-D28k in external fiber bundles of ORNs [15]; neurocalcin in the cell body, cytoplasm, and axon fibers of ORNs [17,18,49]; VILIP in rat olfactory cilia and dendritic knobs [19]; and GCAP1 in rat olfactory cilia [13]. The cellular localization of these Ca\(^{2+}\)-binding proteins may be closely related to their functions in the ORNs. Thus, our immunohistochemistry data suggested that hippocalcin may be involved in initial olfactory signal transduction or adaptation, as proposed for other Ca\(^{2+}\)-binding proteins localized to the olfactory cilia.

We tested whether hippocalcin could affect second messenger signaling in olfactory cilia. Since N-terminal myristoylation is necessary for the interaction of...
hippocalcin with other membrane proteins [20], we expressed both hippocalcin and N-myristoyl transferase in bacteria. After over-expression in bacteria, hippocalcin was purified to apparent homogeneity (Fig. 3D). Using purified hippocalcin and isolated olfactory cilia, we examined the effects of hippocalcin on cyclase activities. We first determined the dose–response of AC activity to hippocalcin at a free Ca\(^{2+}\) concentration of 10nM (Fig. 4A). At this free Ca\(^{2+}\) concentration, AC activity was increased as the concentration of hippocalcin protein was raised. Under these conditions, the maximum effect of hippocalcin was achieved at 40\(\mu\)M. Next, we examined the effect of hippocalcin on AC activity at various free Ca\(^{2+}\) concentrations. Incubation with hippocalcin modulated ciliary AC activity in a Ca\(^{2+}\)-dependent manner. Specifically, hippocalcin increased AC activity at low free Ca\(^{2+}\) concentration and this activity decreased as free Ca\(^{2+}\) concentrations were increased (Fig. 4B). The normal intracellular Ca\(^{2+}\) concentration in the cilium is 40nM at rest and increases up to 300nM upon odorant stimulation [50]. Hippocalcin increased AC activity significantly at concentrations as low as 1nM (Fig. 4B). When free Ca\(^{2+}\) concentrations were raised above 300nM, which mimics the intracellular Ca\(^{2+}\) concentration upon odorant stimulation, hippocalcin no longer had an effect on AC activity. Thus, hippocalcin mediated activity of ACs at physiological Ca\(^{2+}\) concentrations. An opposite effect has been reported for VILIP-1; in vitro recombinant VILIP-1 attenuated odorant-induced AC activation in a Ca\(^{2+}\)-dependent manner [19]. Thus, VILIP-1 and hippocalcin may fine-tune the AC activity in the adaptation of ORNs to odorant stimulation.

Numerous reports have demonstrated that basal levels of phosphorylation are essential to maintain protein function [51–53]. Thus, we determined whether the phosphorylation level of ciliary proteins affected the modulation of AC by hippocalcin. At low free Ca\(^{2+}\) levels, protein kinase treatment inhibited the effect of hippocalcin on AC activity, whereas either native cilia or protein phosphatase-treated cilia showed increases in hippocalcin-mediated AC activity (Fig. 4C, left panel). At the high free Ca\(^{2+}\) conditions, only protein phosphatase-treated cilia showed significant increases in hippocalcin-mediated AC activity (Fig. 4C, left panel). Compared to native cilia, protein phosphatase treatment also increased the basal level of AC activity significantly, whereas protein kinase treatment significantly decreased it (Fig. 4C, left panel). Our data suggest that the dynamic status of phosphorylation of ciliary ACs may play a critical role in hippocalcin dependency; i.e.,
hyper-phosphorylation appears to desensitize the hippocalcin-mediated AC activity. Specifically, Ca\(^{2+}\) influx by odorant stimulation induces phosphorylation of AC3, which in turn causes AC3 desensitization [54,55]. Our data also showed that there is some degree of basal phosphorylation of AC3 [55]. AC3 is the most prominent isofrom of AC found in olfactory cilia [56–60], and phosphorylation-dependent regulation of AC by hippocalcin can be one of the mechanisms for odor adaptation. Other ACs, such as AC2 or AC4, are also present in the OE [61], and thus represent other potential targets for regulation by hippocalcin.

VILIPs are reported to modulate intracellular cGMP level [62,63]. We tested whether hippocalcin affected the activity of GCs in cilia. First, we determined the dose–response of GC to various concentrations of hippocalcin at a free Ca\(^{2+}\) concentration of 10nM (Fig. 5A). GC activity was significantly reduced as the concentration of hippocalcin protein increased. Next, we examined the effect of hippocalcin on GC activity at various free Ca\(^{2+}\) concentrations. Hippocalcin significantly inhibited the activity of particulate GCs over a limited range of free Ca\(^{2+}\) concentrations, specifically in the range of 1–10nM (Fig. 4C). The inhibitory effect of hippocalcin on GC activity decreased as intracellular Ca\(^{2+}\) concentrations increased. These results suggest that in the olfactory system, hippocalcin may play a role in keeping the level of cGMP low in ORNs at rest, lowering the activity of cGMP-dependent protein kinases (PKGs). PKGs are reported to inhibit AC activity in olfactory cilia [13]. Hippocalcin did not affect the activity of GCs at higher Ca\(^{2+}\) concentrations, suggesting that it is not involved in regulation of particulate GCs in the cilia upon odorant stimulation and subsequent increases in intracellular Ca\(^{2+}\). GCAP1 has been proposed to function in that role [13]. VILIP-1 can also stimulate a type of particulate GC, GC-B [63], which is abundantly expressed in the OE [64].

Hippocalcin has been also reported to perform other functions, such as inhibiting G-protein-linked receptor kinase 1 (GRK1) in a Ca\(^{2+}\)-dependent manner [65] and protecting neurons against Ca\(^{2+}\)-induced cell death.
by interacting with neuronal apoptosis inhibitory protein [66]. In cerebellar Purkinje cells in the brain, VI-LIP-3, which is the Ca\(^{2+}\) sensor displaying the highest sequence homology to hippocalcin, has been implicated in complex brain functions such as synaptic plasticity [67]. Thus, hippocalcin may play multiple roles in ORNs in addition to modulating second messenger signaling.

In summary, multiple Ca\(^{2+}\)-binding proteins are expressed in ORNs. One of their roles may be the fine-tuning of the response to odorant stimulation and adaptation as changes in intracellular Ca\(^{2+}\) level occur. Here, we have identified the Ca\(^{2+}\)-binding protein hippocalcin in cilia, and have demonstrated its ability to modulate ACs and GCs, which are involved in stimulus detection and adaptation, respectively. Hippocalcin may play a similar dual role in other systems, modulating signal transduction in response to fluctuations in intracellular Ca\(^{2+}\) concentration.

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Fig. 5. Effects of hippocalcin on GC activity. (A) Dose–response of hippocalcin on GC activities in rat olfactory cilia. Apparent GC activities are determined at the free Ca\(^{2+}\) concentration of 10nM. (B) Effects of hippocalcin on GC activity in rat olfactory cilia. Apparent GC activities were determined under various Ca\(^{2+}\) concentrations with and without hippocalcin. Statistical significances are indicated (one-way ANOVA, \(*p < 0.05\), \(**p < 0.01\), and \(***p < 0.001\)).


