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High-affinity Ca^{2+},Mg^{2+}-ATPase in plasma membrane-rich preparations from olfactory epithelium of Atlantic salmon

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Abstract

High-affinity Ca^{2+},Mg^{2+}-ATPase was identified in a plasma membrane-rich fraction of olfactory epithelium from Atlantic salmon (*Salmo salar*). The enzyme required both Ca^{2+} and Mg^{2+} for activation. The apparent K_m for Ca^{2+} was 9.5 nM and V_{max} was 0.85 μmol P_i/mg of protein per min. Stimulation by Ca^{2+} was optimal at 5–100 μM $MgCl_2$. Bovine brain calmodulin had no effect on Ca^{2+},Mg^{2+}-ATPase, even after multiple washes of the membrane preparation with EDTA or EGTA. Endogenous calmodulin was somewhat resistant to removal and could be detected with immunobloting after multiple washes of the membrane preparation with EDTA or EGTA. This endogenous calmodulin may regulate Ca^{2+},Mg^{2+}-ATPase activity because the activity was inhibited by calmidazolium. Vanadate inhibited Ca^{2+},Mg^{2}-ATPase activity and thapsigargin, a specific inhibitor for Ca^{2+},Mg^{2+}-ATPase of endoplasmic reticulum, had no effect on the enzyme activity. High affinity Ca^{2+},Mg^{2+}-ATPase exists in both ciliary and nonciliary membranes with a similar K_m for Ca^{2+}. Ca^{2+},Mg^{2+}-ATPase activity is greater in cilia preparations than in membranes from the deciliated olfactory epithelium. As a putative plasma membrane Ca^{2+} pump, this high-affinity Ca^{2+},Mg^{2+}-ATPase may play an important role in the regulation of intracellular Ca^{2+} in olfactory epithelia. In particular, the ciliary membrane may play a prominent role in the removal of Ca^{2+} from ciliated olfactory receptor cells after odorant stimulation.

Key words: Chemoreception; Calmodulin; Cilia; Calcium; Olfactory reception; Olfactory signal transduction

1. Introduction

Recent electrophysiological and biochemical evidence has underscored the variety of potential roles Ca^{2+} may play in olfactory reception (for recent review, see Refs. [1,2]). In odor-responsive olfactory neurons of catfish, the resting intracellular Ca^{2+} concentration was 18 ± 12 nM, more than ten thousand times lower than the extracellular concentration [3].

The mechanisms responsible for the control of intracellular free Ca^{2+} in olfactory sensory cells are unknown. In neurons, plasma membrane Na^+/Ca^{2+} exchange [4] and Ca^{2+},Mg^{2+}-ATPase (Ca^{2+} pump) activities are the two major mechanisms for the removal of cytosolic Ca^{2+}. In a variety of cell types, Ca^{2+}, Mg^{2+}-ATPase is located in the plasma membrane where it is responsible for extrusion of Ca^{2+} from the cell [5] and/or in the membrane of an internal organelle such as the endoplasmic reticulum where it is responsible for sequestering Ca^{2+} within the organelle [6].

Cilia and other plasma membrane rich fractions from the olfactory rosettes of Atlantic salmon were previously shown to be rich in binding sites for amino acids that are potent olfactory stimuli [7]. These putative olfactory receptors appear to be coupled to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) in a G protein-dependent and Ca^{2+}-sensitive manner [8]. One or more components of this well-known Ca^{2+}-mobilizing signal transduction system [9] have now been identified in several olfactory systems [2,3,10–14].

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The present study continues analysis of olfactory membrane preparations from Atlantic salmon (*Salmo salar*) and focuses on Ca^{2+},Mg^{2+}-ATPase as a potential mechanism for regulating intracellular Ca^{2+} levels.

2. Materials and methods

2.1. Tissue fractionation

Atlantic salmon (1–3 years old) were sacrificed and the olfactory rosettes were removed from the nares. As described and characterized previously [7,8], the rosettes were minced, homogenized and subjected to differential centrifugation to obtain a plasma membrane-enriched fraction (P2), a modification of procedures developed with olfactory rosettes from rainbow trout by Cagan and Zeiger [15]. To prepare a cilia-enriched fraction, rosettes were subjected to calcium shock by a procedure modified from that of Rhein and Cagan [16] as described previously [7]. A P2 fraction was then prepared from the deciliated rosette [7]. Protein was determined by the Bradford method [17].

2.2. ATPase assays

ATPase activities were measured by the release of P_i using a previously described spectrophotometric assay [18] with slight modification. Membrane fractions (3–10 μg protein) were incubated at room temperature in 0.25 ml of 2.5 mM Hepes-Pipes buffer (pH 7.5), containing 1 mM ouabain, 2.5 mM ATP, 5 μM Mg^{2+} and various concentrations of Ca^{2+} for 15 min. Ca^{2+}-ATPase was defined by the difference in ATPase activity in the absence and presence of calcium. A low ionic strength buffer was used to help maintain membrane fragments rather than resealed vesicles. Any non-specific ATP hydrolysis was determined in the presence of 1 mM EDTA with no added divalent cations. Mg^{2+}-ATPase was determined by the difference in ATPase activity in the presence and absence of Mg^{2+} with no Ca^{2+}. 1 mM EGTA was used to buffer the free Ca^{2+} concentration [19]. Free Ca^{2+} was calculated using the following binding constants: CaEGTA: $10^{7.8}$, CaATP: $10^{3.93}$, MgEGTA: $10^{2.04}$, MgATP: $10^{4.61}$.

2.3. Immunological characterization of calmodulin

Plasma membrane enriched fractions (P2) which had been subjected to four cycles of centrifugation and resuspension in 1 mM EDTA were analyzed by SDS-PAGE (12% acrylamide) [20] and transferred to nitrocellulose membranes for immunoblotting [21]. Immunoblot analysis was performed with monoclonal antibody against bovine brain calmodulin (Upstate Biotech., Lake Placid, NY).

3. Results

In a concentration-dependent manner, Ca^{2+} activated ATPase activity in the presence of 5 μM $MgCl_2$ (Fig. 1A). Eadie-Hofstee analysis of the Ca^{2+} activation curve (Fig. 1B) was consistent with the presence of

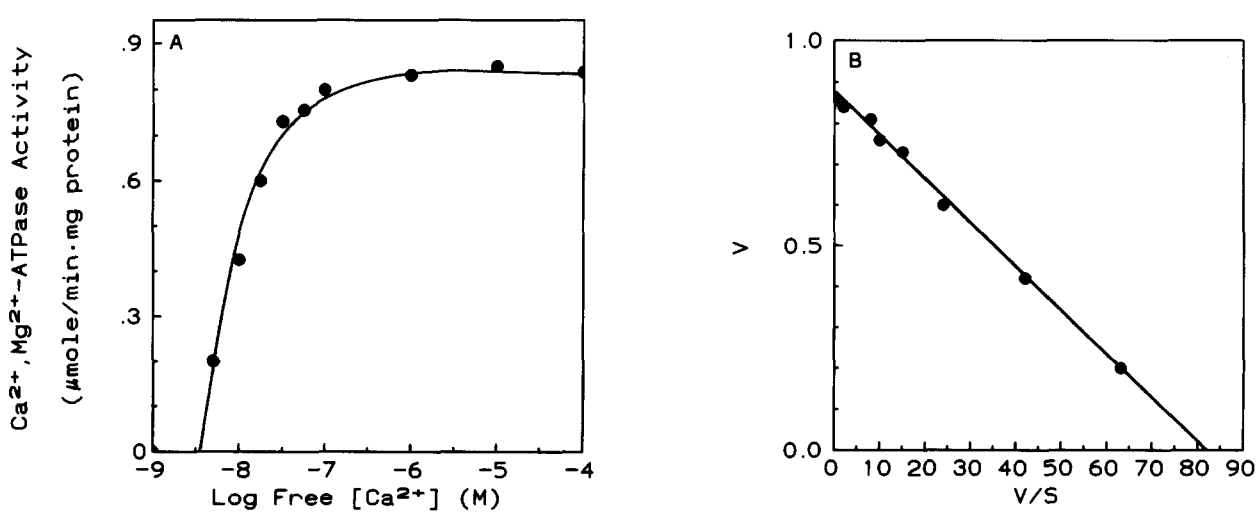

Fig. 1. Ca^{2+} concentration dependence of Ca^{2+},Mg^{2+}-ATPase activity in plasma membrane rich preparation from olfactory rosettes of Atlantic salmon. (A) Production of inorganic phosphate from ATP hydrolysis was determined in the presence of indicated Ca^{2+} concentration and absence of Ca^{2+} with the difference defined as Ca^{2+},Mg^{2+}-ATPase activity. The incubating solution contained 2.5 mM Hepes-Pipes (pH 7.5), 2.5 mM ATP, 5 μM $MgCl_2$ and 1 mM ouabain. Free Ca^{2+} concentration was buffered with 1 mM EGTA. (B) Eadie-Hofstee plot of the same data. Results are representative of three separate experiments each performed in triplicate.

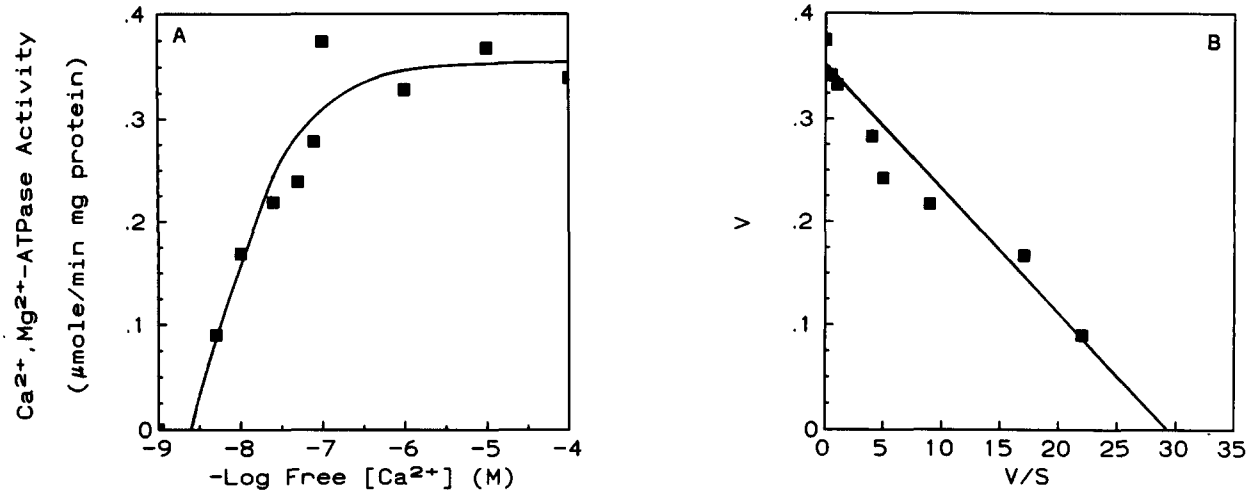

Fig. 2. Ca^{2+} dependence of Ca^{2+}, Mg^{2+}-ATPase activity in plasma membrane rich preparations at 0.5 mM Mg^{2+}. (A) Ca^{2+},Mg^{2+}-ATPase activity was determined as described in Fig. 1 except 0.5 mM Mg^{2+} was added to the incubating solution. (B) Eadie-Hofstee plot of the same data. Results are those of a single representative experiment performed in triplicate.

a single class of Ca^{2+} binding sites in this concentration range of Ca^{2+} and yielded an apparent K_m of 10 nM and a V_{max} of 0.84 μmol/mg protein per min. Initial data had shown that Ca^{2+},Mg^{2+}-ATPase activity was optimal between 5 and 100 μM $MgCl_2$ and that higher concentrations of Mg^{2+} (> 100 μM) decreased the Ca^{2+}-dependent ATPase activity. When Mg^{2+} was increased to 500 μM, V_{max} was reduced maximally to 0.34 μmol/mg protein per min while the apparent K_m for Ca^{2+} remained unchanged (Fig. 2). No Ca^{2+}-ATPase activity was detected when the free Ca^{2+} concentration was buffered with EDTA to eliminate all Mg^{2+}. The dependence of Ca^{2+}, Mg^{2+}-ATPase on ATP concentration was also investigated. Ca^{2+}-ATPase activity was maximal at 1 mM ATP (Fig. 3).

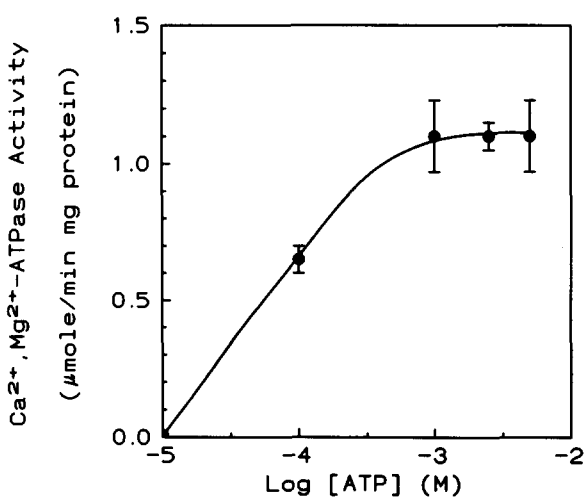

Fig. 3. ATP concentration dependence of Ca^{2+}, Mg^{2+}-ATPase in plasma membrane rich preparation from olfactory rosette of Atlantic salmon. Ca^{2+}, Mg^{2+}-ATPase activity was determined as described in Fig. 1. Free Ca^{2+} concentration was buffered at 100 nM and free Mg^{2+} was within the range (5–100 μM) for optimal activity. Results are representative of two separate experiments each performed in triplicate.

Thapsigargin (200 nM and 2 mM), a specific inhibitor for Ca^{2+},Mg^{2+}-ATPase of endoplasmic reticulum, had no effect on Ca^{2+},Mg^{2+}-ATPase activity in this preparation (Table 1). Vanadate (40 μM), a transitional analogue of phosphate, inhibited the Ca^{2+}, Mg^{2+}-ATPase (Table 1). Bovine brain calmodulin had no effect on the enzyme from either cilia preparations or a plasma membrane rich fraction from deciliated olfactory rosettes (Fig. 4). Concentrations of calmodulin up to 60 μg/ml were tested without effect. Proteins from the plasma membrane rich fraction were resolved by SDS-PAGE and analyzed for the presence of immunoreactivity toward calmodulin. Immunoreactivity consistent with the presence of calmodulin was observed (Fig. 5). The latter was observed even after multiple (4 ×) washes of the membrane preparation with buffer containing 1 mM EDTA prior to enzyme assay. Similar results were obtained with EGTA (data not shown). If endogenous calmodulin were acti-

Table 1
Effect of thapsigargin and vandadate on Ca^{2+},Mg^{2+}-ATPase activity

Addition to the incubation	Ca^{2+},Mg^{2+}-ATPase activity (% of Control)
None	100
Thapsigargin (200 nM)	101 ± 10
Thapsigargin (2 mM)	113 ± 10
Vanadate (40 μM)	15 ± 3

Ca^{2+},Mg^{2+}-ATPase was determined as described in Fig. 1. Free Ca^{2+} concentration was buffered at 100 nM with 1 mM EGTA. Ca^{2+},Mg^{2+}-ATPase activity when no modulator was added was used as the control. Results are means ± S.E. of four determinations.

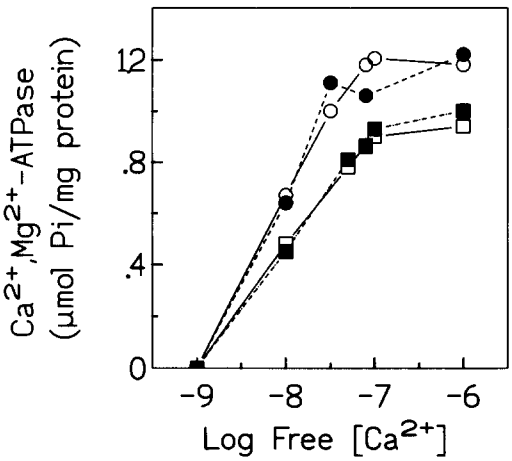

Fig. 4. Lack of effect of calmodulin on Ca^{2+},Mg^{2+}-ATPase activities in cilia and deciliated rosettes. Ca^{2+},Mg^{2+}-ATPase activity was measured as described in the legend to Fig. 1 using: (i) cilia preparations in the absence (open circles) or presence (closed circles) of calmodulin, or (ii) P2 preparations from the deciliated olfactory rosette in the absence (open squares) or presence (closed squares) of calmodulin. The calmodulin concentration was 1 μM. Results are each from a single preparation assayed in duplicate.

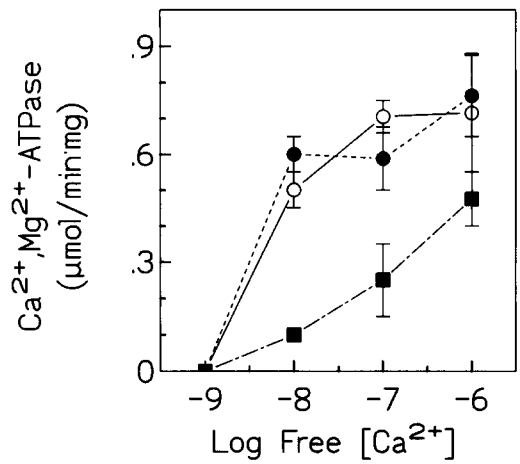

Fig. 6. Lack of effect of calmodulin and inhibition of olfactory Ca^{2+},Mg^{2+}-ATPase activity by calmidazolium. Control (open circles) Ca^{2+},Mg^{2+}-ATPase activity was measured as described in the legend to Fig. 1. Calmodulin (closed circles) was tested at 1 μM and calmidazolium (closed squares) was tested at 1 μM. Results are means ± S.E. for two separate experiments, performed in duplicate.

vating the enzyme and masking effects of added calmodulin, the enzyme activity would be sensitive to a calmodulin antagonist such as calmidazolium. This was tested using a P2 preparation from the whole rosette, again washed four times with buffer containing 1 mM EDTA. Under conditions where calmodulin had no effect, calmidazolium inhibited Ca^{2+},Mg^{2+}-ATPase activity (Fig. 6). Inhibition was greatest at low Ca^{2+} concentrations. In the presence of calmidazolium, the apparent K_m for Ca^{2+} was increased to approximtely 300 nM.

Ca^{2+},Mg^{2+}ATP-ase activity was highest in cilia preparations and was significantly greater than that of plasma membrane-rich (P2) fraction from deciliated olfactory rosettes. The V_{max} for Ca^{2+},Mg^{2+}-ATPase was about 1.5-fold higher in the cilia than the deciliated membrane preparation (Table 2). The Ca^{2+}, Mg^{2+}-ATPase in both the cilia preparation and the plasma membrane-rich fraction of the deciliated rosette had a similar low K_m for Ca^{2+}.

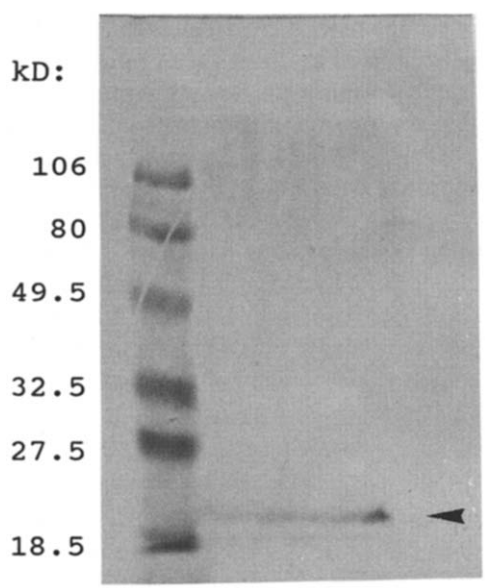

Fig. 5. Identification of calmodulin by immunoblotting. Membranes were solubilized and separated by SDS/PAGE (12% gel) and transferred to nitrocellulose membrane. Immunoblot analysis was performed with a monoclonal antibody against bovine brain calmodulin. Left lane: prestained molecular standards (1, phosphorylase B (106 kDa); 2, BSA 80 kDa); 3, ovalbumin (49.5 kDa); 4, carbonic anhydrase (32.5 kDa); 5, soybean trypsin inhibitor (27.5 kDa); 6, lysozyme (18.5 kDa)). The arrow indicates the presence of calmodulin immunoreactivity.

Table 2
Ca^{2+},Mg^{2+}-ATPase activity in membrane fractions from atlantic salmon olfactory rosettes

Fraction	K_m (nM)	V_{max} (μmol P_i /mg per min)
Whole rosette (P_2)	10 ± 1	0.85 ± 0.04
Deciliated rosette (P_2)	12 ± 2	0.66 ± 0.03 *
Cilia	10 ± 1	1.05 ± 0.18

Apparent K_m and V_{max} were derived from Eadie-Hofstee plots of Ca^{2+} dose-response curves of Ca^{2+},Mg^{2+}-ATPase using cilia preparations and plasma membrane rich preparations (P2) from whole rosette and deciliated rosette. Result for whole rosette is mean ± SEM from three experiments each performed in triplicate. Results for cilia and deciliated rosette are means ± S.E. from four determinations. * By Student's *t*-test, the V_{max} for the deciliated rosette P2 was significantly less than that of the whole rosette ($P < 0.05$). It was also significantly less than that of the cilia fraction ($P < 0.05$).

4. Discussion

It has been shown that odor stimulation triggers influx of Ca^{2+} into olfactory neurons of the catfish [3,11]. The influx may be mediated by a plasma membrane inositol 1,4,5-trisphosphate IP_3 receptor [3,12]. Inositol 1,4,5-trisphosphate is produced by a signal transduction cascade involving activation of phospholipase C (PLC) and PIP_2 hydrolysis as has been observed in several olfactory systems (for recent review, see Ref. [9]), including that of Atlantic salmon [8]. In Atlantic salmon, low intracellular free Ca^{2+} sensitizes the olfactory system to odorant stimulation of PLC activity. Stimulation by odorant amino acids was not observed above 100 nM Ca^{2+}. The optimal Ca^{2+} concentration for activation of PLC by odorants was below 10 nM. Above 10 nM, Ca^{2+} directly activated PLC, a characteristic we have suggested may provide amplification in olfactory signal transduction through 'feedback activation' of PLC by Ca^{2+} initially entering the olfactory receptor cell in response to odors [8]. This mechanism, however, presumes an efficient system for maintenance of very low Ca^{2+} ($\sim$ 10 nM) in olfactory receptor cells. Catfish olfactory receptor cells, which have resting free Ca^{2+} concentrations in this range [3,11] must possess a system of similar efficiency. Further, the increased intracellular free Ca^{2+} concentration has to be restored to and maintained at its low resting level after each stimulation. A high affinity Ca^{2+},Mg^{2+}-ATPase activity has been identified in the olfactory epithelium from Atlantic salmon. This enzyme has an extremely low K_m for Ca^{2+} (10 nM). Such a low K_m suggests that this enzyme could be capable of reducing Ca^{2+} to the level required for sensitization to olfactory stimuli. However, definitive evidence that the Ca^{2+}-ATPase is associated with ATP-dependent Ca^{2+} transport (Ca^{2+} pump) is needed. With a similar K_m for Ca^{2+}, the Ca^{2+},Mg^{2+}-ATPases from *Leishmania donovani* promastigote [22] and from gypsy moth larval midgut [23] were implicated in high-affinity Ca^{2+} transport.

The olfactory enzyme required both Ca^{2+} and Mg^{2+} for activation. However, Mg^{2+} at a higher concentration was found to decrease the specific activity of the enzyme versus a given Ca^{2+} concentration. There was no effect of Mg^{2+} on the high affinity of the olfactory enzyme for Ca^{2+} and the significance of this noncompetitive inhibition by Mg^{2+} is not clear. Apparent inhibition of Ca^{2+}-ATPase by Mg^{2+} has been reported previously [22,24]. Because the assay was performed under hypoosmotic conditions to favor maintenance of open membrane fragments it is feasible that Mg^{2+} may simply promote vesicle formation at higher concentrations. Right side-out vesicles (ATPase site inside) may show significantly reduced ATPase activity in this assay. This could explain reduced V_{max}.

Thapsigargin, a specific inhibitor of endoplasmic reticulum Ca^{2+},Mg^{2+}-ATPase [25] had no effect on Ca^{2+},Mg^{2+}-ATPase activity in our preparation. This may indicate that there is little contamination of the plasma membrane-rich fraction with Ca^{2+},Mg^{2+}-ATPase from endoplasmic reticulum or may reflect a general insensitivity of tissue from this species to thapsigargin. The Ca^{2+}, Mg^{2+}-ATPase identified in the olfactory epithelium was sensitive to inhibition by vanadate, a transitional analogue of phosphate which had been shown to inhibit the E_1/E_2 transition of the aspartyl phosphate class of ATPase [26].

The enzyme could not be stimulated by a heterologous source of calmodulin (bovine). Although calmodulin usually can be easily removed by a chelating agent such as EDTA or EGTA, the complete removal of calmodulin is difficult in some systems [27,28]. Despite the fact that we have washed our membrane preparation extensively with EDTA or EGTA, calmodulin was detected by immunoblotting. Calmodulin mRNA is expressed at high levels in both mature and immature olfactory neurons of other species [29]. The presence of endogenous calmodulin could explain the lack of stimulation by exogenous calmodulin and could contribute to the high affinity of the enzyme for calcium. However, the enzyme should still show sensitivity to a calmodulin antagonist such as calmidazolium [30]. This was the case and the results indicate that olfactory Ca^{2+}, Mg^{2+}-ATPase may be regulated by calmodulin in this species. However, the results are indirect and additional experiments, perhaps with salmon calmodulin, or detailed analysis of the structure of olfactory Ca^{2+},Mg^{2+}-ATPase are needed to confirm regulation by calmodulin. Two laboratories have reported anomalous effects of CaEGTA on Ca^{2+}, Mg^{2+}-ATPase [31,32]. In each of these reports stimulation by calmodulin was masked by the presence of CaEGTA at concentrations equivalent to those used in the present study.

High-affinity Ca^{2+},Mg^{2+}-ATPase exists in both ciliary membrane and membranes from the deciliated rosettes with a similar high affinity for Ca^{2+} (Table 2). Ca^{2+},Mg^{2+}-ATPase activity was 1.5-fold higher in the cilia than in the plasma membrane-rich fraction of the deciliated rosettes. However, in preliminary studies [33], we observed up to 8-fold enrichment of cilia for Ca^{2+}, Mg^{2+}-ATPase. The differences in the degree of enrichment were due to the fluctuation of Ca^{2+}, Mg^{2+}-ATPase activity in the deciliated preparation. The ciliary activity was consistently high. Differences in age, developmental stage or season when the fish are sampled could contribute to these variations in nonciliary Ca^{2+}, Mg^{2+}-ATPase. For example, it has been shown that branchial Na^+,K^+-ATPase activity changes during salmonid smoltification [34]. Thorough investigation of the effects of these factors on Ca^{2+},Mg^{2+}-

ATPase activity is needed to address the significance of the observed variability. Because cilia lack organelles like the endoplasmic reticulum to help sequester elevated cytosolic Ca^{2+}, the high Ca^{2+},Mg^{2+}-ATPase activity in ciliary membrane may be required for the rapid removal of Ca^{2+} from cilia after odorant stimulation. Olfactory cilia preparations from trout [16], catfish [12,35], and Atlantic salmon [7] are rich in odorant binding sites. Together with our previous analysis of odorant binding and Na^+,K^+-ATPase [7], the present studies indicate that olfactory cilia provide increased plasma membrane surface area for both olfactory reception and active formation and maintenance of critical ion concentration gradients.

The existence of L-alanine binding sites in both ciliated and extraciliary plasma membranes has been demonstrated in Atlantic salmon olfactory rosettes [7]. The latter membrane preparations, where most ($\geq$ 85%) of high-affinity alanine binding was recovered, may indicate substantial contribution of microvillar (nonciliated) olfactory receptor cells to alanine reception in Atlantic salmon. The high affinity for calcium demonstrated by the Ca^{2+}, Mg^{2+}-ATPase in both ciliary and nonciliary plasma membrane rich preparations establishes this enzyme as a strong candidate for the regulation of free Ca^{2+} concentration in both types of receptor cells.

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