Research Article

Biochemical Evidence for a Putative Inositol 1,3,4,5-Tetrakisphosphate Receptor in the Olfactory System of Atlantic Salmon (*Salmo salar*)

Jiongdong Pang[1,2] and Dennis E. Rhoads[2,3]

[1] *Chemistry Department, Southern Connecticut State University, New Haven, CT 06515, USA*

[2] *Department of Biochemistry, Microbiology and Molecular Genetics, University of Rhode Island, Kingston, RI 02881, USA*

[3] *Department of Biology, Monmouth University, West Long Branch, NJ 07764-1898, USA*

Correspondence should be addressed to Dennis E. Rhoads; drhoads@monmouth.edu

Olfactory receptor neurons in Atlantic salmon (*Salmo salar*) appear to use a phosphoinositide-directed phospholipase C (PLC) in odorant signal transduction. The consequences of odor-activated PLC depend on its product, inositol 1,4,5-trisphosphate (IP_3). Therefore, a plasma membrane rich (PMR) fraction, previously characterized from salmon olfactory rosettes, was used to study binding sites for IP_3 and its phosphorylation product, inositol 1,3,4,5-tetrakisphosphate (IP_4). Binding sites for IP_3 were present at the lower limit for detection in the PMR fraction but were abundant in a microsomal fraction. Binding sites for IP_4 were abundant in the PMR fraction and thus colocalized in the same subcellular fraction with odorant receptors for amino acids and bile acids. Binding of IP_4 was saturable and high affinity ($K_d = 83$ nM). The rank order for potency of inhibition of IP_4 by other inositol polyphosphates ($InsP_x$) followed the phosphorylation number with $InsP_6 > InsP_5 >$ other $InsP_4$ isomers $> InsP_3$ isomers $> InsP_2$ isomers, with the latter showing no activity. The consequences of PLC activity in this system may be dictated in part by a putative receptor for IP_4.

1. Introduction

Adenylyl cyclase and cAMP appear to dominate odor signal transduction in mammals (for reviews, see [1–3]). Phosphoinositides may play a divergent role in olfaction, mediating inhibitory signaling through phosphoinositide-3-kinase [4] or excitatory signaling through phospholipase C [1, 5]. For fish, components of a phospholipase C-based olfactory signal transduction system have been characterized in catfish [6–13] and are seen in carp [14, 15], zebrafish [16], and Atlantic salmon [17, 18].

As potent olfactory stimuli for Atlantic salmon, amino acids and bile acids interact with distinct subclasses of olfactory receptors to begin the process of olfactory reception [18, 19]. The amino acid and bile acid receptors appear to be coupled through G proteins to the activation of phospholipase C (PLC) and the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) [17, 18]. Early biochemical data characterizing these as G protein-coupled receptors is now supported by molecular studies characterizing olfactory receptor gene sequences from Atlantic salmon [20–23]. Underscoring the importance of these receptors in salmon physiology, odorant receptor expression has been shown to change during the parr-smolt transformation, a period characterized by increased olfactory sensitivity and olfactory-based learning [24].

The significance of olfactory PLC activity resides in part with the location and characteristics of receptors for IP_3. In most cells, IP_3 receptors mediate the release of Ca^{2+} from internal stores in the endoplasmic reticulum (for review, see [25]). However, in association with PLC-based olfactory signal transduction, IP_3 receptors have been found in olfactory cilia of catfish [6], carp [14], and lobster [26, 27]. From this position, IP_3 may gate Ca^{2+} influx through the plasma

membrane rather than the release from intracellular stores. Another important part of IP_3 signaling in other systems has been its metabolism, including phosphorylation by a 3-kinase to generate the biologically active inositol 1,3,4,5-tetrakisphosphate (IP_4) [28–30]. While IP_4 continues to be studied in mammalian systems for roles as diverse as regulating nuclear calcium signaling [31], tyrosine kinase [32], and mitochondrial permeability and apoptosis [33, 34], Fadool and Ache [26] showed that olfactory receptor neurons of lobster express an IP_4 receptor acting as a functional channel in the plasma membrane. In lobster, plasma membrane IP_3 and IP_4 receptors may interact reciprocally to regulate Ca^{2+} entry in olfactory neurons.

The goal of the present study was to characterize further the PLC-based olfactory signal transduction system of Atlantic salmon, beginning with the hypothesis that IP_3 binding sites would colocalize with odor receptor binding sites in a plasma membrane rich fraction (PMR) that we characterized previously [17–20, 35]. Finding that binding of IP_3 was marginal in this fraction, we proceeded to detect and characterize PMR binding sites for IP_4 which may play a critical role in salmon olfactory transduction. Binding sites for IP_3 were subsequently detected in the endoplasmic reticulum-rich microsomal fraction.

2. Materials and Methods

2.1. Isolation of the Plasma Membrane Rich (PMR) and Microsomal Fractions. Atlantic salmon (*Salmo salar*) were raised under conditions of simulated natural photoperiod and temperature in the aquaculture facility of University of Rhode Island. Using a modification of a method devised originally for rainbow trout by Cagan and Zeiger [36], a plasma membrane rich (PMR) fraction was obtained from the olfactory rosettes as described previously [19]. Rosettes were pooled from ten salmon for each analysis. The microsomal fraction was isolated from the olfactory rosettes using the method of Kalinoski et al. [6]. For comparative purposes, PMR fractions and microsomal fractions were also prepared from salmon brain and rat brain. Concentrations of proteins were determined by the method of Bradford (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as a standard.

2.2. IP_3 Binding. Binding of [^{3}H]IP_3 ([inositol-1-^{3}H]; 21.0 Ci/mmol; New England Nuclear, Boston, MA) was measured using conditions described by Kalinoski et al. [6] except that microsomal fractions (100 μg protein per assay) or PMR fractions (100–300 μg protein per assay) were from salmon olfactory rosettes or from salmon or rat brain. Digitonin (50 μg/mL) was added to permeabilize any membrane vesicles and insure that all binding sites are accessible [6]. The incubation buffer consisted of 110 mM KCl, 1 mM EGTA/0.2 mM $CaCl_2$ (free Ca^{2+} concentration = 20 nM), and 10 mM HEPES, pH 7.4. Incubations were carried out for 30 min at 4°C. Separation of bound and free [^{3}H]IP_3 was achieved by rapidly filtering through Whatman GF/C filters and washing 3 times with assay buffer. Filters were

extracted in scintillation cocktail for 4 hr, and the amount of associated radioactivity was determined by scintillation spectrometry. The amount of binding was determined in the absence (total binding) and presence (nonspecific binding) of excess (120 μM) unlabeled InsP$_3$. Two concentrations of [^{3}H]IP_3 (7 and 14 nM) were tested. The calculated difference between total and nonspecific binding was operationally defined as a specific binding.

2.3. IP_4 Binding. The binding assay for [^{3}H]IP_4 ([Inositol-1-^{3}H]; 21.0 Ci/mmol; New England Nuclear, Boston, MA) was performed under conditions identical to those described by Challiss et al. [37]. The assay buffer consisted of 25 mM CH_3COONa, 25 mM KH_2PO_4, 5 mM $NaHCO_3$, 1 mM EDTA, pH 5.0, and the indicated concentrations of [^{3}H]IP_4. Nonspecific binding was defined by the inclusion of 120 μM unlabeled IP_4. To characterize the binding specificity, competition assays were conducted with a minimum of three concentrations of other inositol polyphosphates (InsP$_x$): InsP$_6$, Ins(1,3,4,5,6)P$_5$, Ins(3,4,5,6)P$_4$, Ins(1,4,5,6)P$_4$, Ins(1,3,4)P$_3$, Ins(1,4,5)P$_3$, Ins(1,4)P$_2$, and Ins(4,5)P$_2$ (all generously provided by Dr. Ching-Shih Chen, School of Pharmacy, University of Rhode Island). Reactions were initiated by the addition of PMR fraction (100 μg protein), and samples were maintained at 4°C for 30 min with gentle rocking. Separation of bound and free [^{3}H]IP_4 was achieved by rapidly filtering through Whatman GF/C filters and washing 3 times with assay buffer. Filters were extracted in scintillation cocktail for 4 hr, and radioactivities were determined.

Binding assays for both [^{3}H]IP_3 and [^{3}H]IP_4 were based on conditions optimized by others ([6, 36], resp.). To rule out any effect of the different incubation conditions (most notably pH) on conclusions regarding binding of [^{3}H]IP_3 or [^{3}H]IP_4, each was tested at the conditions that had been optimized for the other. As expected, binding was negligible when measured at nonoptimal conditions.

3. Results

3.1. IP_3 Binding. At a radioligand concentration of 7 nM, no specific binding of IP_3 was detectable with the olfactory PMR fraction. At 14 nM radioligand, IP_3 binding to the olfactory PMR fraction was at the lower limit of detection in the assay (see data labeled IP_3-PMR in Figure 1). Nonspecific binding accounted for almost 90% of the small amount of total binding of [^{3}H]IP_3 to the PMR fraction. Similar results were obtained with a salmon brain PMR fraction, analyzed as a negative control. The specific binding of [^{3}H]IP_3 corresponded to a maximum of 16 fmol bound per mg olfactory PMR protein and 10 fmol per mg salmon brain fraction.

In contrast, specific binding sites for [^{3}H]IP_3 were readily detected in a microsomal (MS) preparation from salmon olfactory rosettes (see data labeled IP_3-MS in Figure 1). In this preparation, specific binding accounted for at least 75% of the total binding of [^{3}H]IP_3 and corresponded to 1.2 pmol IP_3 bound per mg MS protein, a level nearly 100 times higher than the PMR fraction. This compares favorably to the level
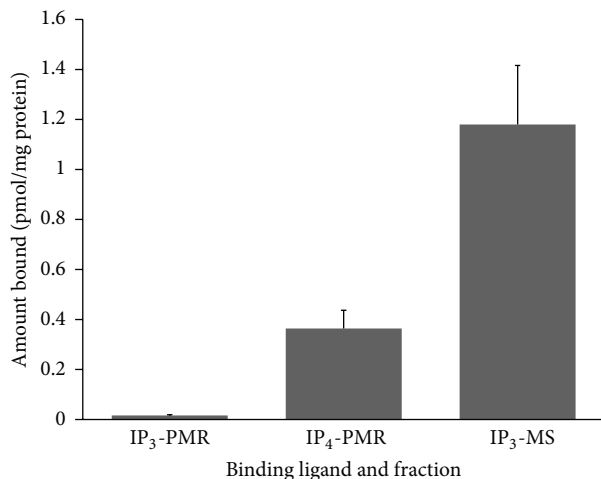
FIGURE 1: Initial screening of IP_3 and IP_4 binding sites in membrane fractions from salmon olfactory rosettes. The plasma membrane rich (PMR) fraction and a microsomal (MS) fraction were prepared from olfactory rosettes of Atlantic salmon. Specific binding of $[^3H]IP_3$ was determined in incubations with 14 nM $[^3H]IP_3$ in the presence and absence of excess unlabeled $InsP_3$. Specific binding of IP_4 to the PMR fraction is shown for comparison to IP_3. The concentration of $[^3H]IP_4$ in the reaction mixture was also 14 nM.

FIGURE 2: Saturation binding of $[^3H]IP_4$ to a plasma membrane rich fraction from salmon olfactory rosettes. Specific binding of IP_4 was determined at each of the IP_4 concentrations shown. Results are averaged from a single experiment performed in duplicate using olfactory rosettes from 10 Atlantic salmon and are representative of the results of three independent experiments. The inset shows a Scatchard analysis of the binding of IP4 to the plasma membrane rich fraction. Binding data as in Figure 2 was transformed to estimate the K_d and B_{max}. The data fit a straight line ($R^2 = 0.93$) indicative of a single class of binding sites.

of IP_3 binding measured in a rat brain microsomal fraction that was analyzed as a positive control.

3.2. IP_4 Binding. While IP_3 binding to the salmon olfactory PMR fraction was at the lower limit for detection in our assay, binding sites for IP_4 were readily detected and were present at high density (see data labeled IP_4-PMR in Figure 1). At comparable ligand concentration (14 nM), the olfactory PMR fraction supported binding of 364 fmol IP_4 per mg protein (contrasted with 16 fmol IP_3 per mg protein). Nonspecific binding represented less than 20% of total binding. In a single trial with the microsomal preparation from salmon olfactory rosettes, specific binding of $[^3H]IP_4$ was at the lower limit of detection (not shown). Thus, IP_4 sites were readily detected in the PMR but not the microsomal fraction, a result opposite of that for IP_3 binding.

Experiments performed with increasing concentrations of $[^3H]IP_4$ demonstrated that specific binding was saturable (Figure 2). Scatchard analysis of the binding data (Figure 2, inset) yielded 83 nM for the K_d and 3811 fmol/mg protein for the B_{max} for IP_4 binding to the olfactory PMR fraction.

To further characterize the specificity of IP_4 binding to the olfactory PMR fraction, competition experiments were performed using 14 nM $[^3H]IP_4$ and various other inositol polyphosphates ($InsP_x$) differing in degree and position of phosphorylation (Figure 3). If an analog competes with IP_4, then binding will decrease as the concentration of the analog increased (Figure 3).

$InsP_5$ and $InsP_6$ showed reasonably potent inhibition of $[^3H]IP_4$ binding. Other IP_4 analogs ($Ins(3,4,5,6)P_4$ and $Ins(1,4,5,6)P_4$) were intermediate in potency as inhibitors,

while the IP_3 analogs ($Ins(1,3,4)P_3$ and $Ins(1,4,5)P_3$) showed little or no activity. Similarly, $Ins(1,4)P_2$ and $Ins(4,5)P_2$, the dephosphorylation products formed from the inactivation of $Ins(1,4,5)P_3$, had no inhibitory effect on $[^3H]IP_4$ binding when incubated at 10 μM (data not included in Figure 3). From these competition assays, the effective concentration of analog giving 50% inhibition of $[^3H]IP_4$ binding (EC_{50}) was estimated (Table 1). From this analysis, the rank order for potency of inhibition to $[^3H]IP_4$ binding was $InsP_6$ > $Ins(1,3,4,5,6)P_5$ > $Ins(3,4,5,6)P_4$ > $Ins(1,4,5,6)P_4$ > $Ins(1,3,4)P_3$ = $Ins(1,4,5)P_3$.

4. Discussion

Previous characterization of the PMR fraction showed high levels of the plasma membrane marker Na, K-ATPase and binding sites for amino acid [19, 20] and bile acid [18] odors. This fraction had minimal contamination with endoplasmic reticulum as suggested by the absence of thapsigargin-sensitive Ca^{2+}-ATPase [35]. The low level of observed IP_3 binding can also be considered as evidence of the lack of ER especially when compared to the microsomal fraction which is traditionally used as a source of endoplasmic reticulum and IP_3 receptors [25]. A comparison of IP_3 binding to the two subcellular fractions is consistent with the presence of IP_3 receptors in endoplasmic reticulum rather than plasma membranes. This does not rule out the possibility that IP_3 receptors would be detected at a higher level in isolated cilia [6] rather than the PMR fraction, but the low level of IP_3 binding to the olfactory PMR fraction contrasts sharply with the high density of binding sites corresponding to odorant amino acid receptors [19, 20]. Clearly, IP_3 receptors do not

Acknowledgments

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