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# A subtype of the metabotropic glutamate receptor family in the olfactory system of Atlantic salmon

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**Abstract** A plasma membrane rich fraction was prepared from olfactory rosettes of Atlantic salmon and used to study binding of L-glutamic acid and activation of phospholipase C (PLC). Glutamate binding was saturable, high affinity, and inhibited by aspartic acid and taurocholate but not by alanine and lysine. Binding of glutamate was potently inhibited by various ligands for rat brain metabotropic glutamate receptors (mGluR) and also by kainate and *N*-methyl-D-aspartate. Glutamate stimulated phosphatidylinositol 4,5-bisphosphate breakdown consistent with G protein-dependent activation of PLC. Northern blot analyses demonstrated the presence of olfactory rosette RNA that hybridizes with cDNA probes for mGluR1 and mGluR4 under low stringency conditions. The results indicate the salmon olfactory system includes a subtype of the metabotropic glutamate receptor family.

*Key words:* Glutamate; Olfactory reception; Glutamate receptor; Phospholipase C; Atlantic salmon

## 1. Introduction

Amino acids [1], bile acids [2] and steroid hormones [3] are potent olfactory stimuli for Atlantic salmon. The water solubility of these odors makes the olfactory system of Atlantic salmon and other fish readily amenable to direct receptor binding studies that are unapproachable with the volatile odors of terrestrial organisms. Binding studies with odorant amino acids and olfactory membrane preparations from a number of different fish species have led to a consensus that olfactory discrimination of amino acids occurs at least in part through the interaction of amino acids with receptors having distinct but not necessarily exclusive binding specificities [4–10]. For example, a combination of physiological [11] and biochemical approaches [9] has indicated that there are four subclasses of amino acid receptors, recognizing acidic, basic, long chain neutral and short chain neutral amino acids, respectively, in catfish olfactory receptor cells. In both rainbow trout [12] and Atlantic salmon [10], there was relatively little enrichment of amino acid binding sites in preparations of olfactory cilia. Based on these observations and the presence of both ciliated and microvillous olfactory receptor cells in fish olfactory epithelium [13], it has been suggested that microvillous receptor cells play a major role in olfactory reception of amino acids [10].

The present study has focused on the binding specificity of a putative olfactory glutamate receptor (oGluR) in Atlantic salmon using an olfactory plasma membrane rich fraction that would include contributions from both ciliated and microvillous receptor cells. Included in the analyses are glutamate analogs that have led to pharmacological characterization of distinct glutamate receptor subtypes in mammalian brain (reviewed in [14]). Because these latter glutamate receptors are also distinguished on the basis of the associated signal transduction mechanism as ionotropic (coupled to an ion channel) or metabotropic (coupled through a G protein to phospholipase C or adenylyl cyclase), we have also examined effects of glutamate on phospholipase C (PLC) activity.

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## 2. Materials and methods

### 2.1. Isolation of olfactory membranes

Atlantic salmon (*Salmo salar*) were raised under conditions of simulated natural photoperiod and temperature. Using a modification [10] of a method originated for rainbow trout by Cagan and Zeiger [4], a plasma membrane rich (PMR) fraction was obtained from the olfactory rosettes. Rosettes were pooled from six salmon for each experiment. For comparative purposes, PMR fractions were also prepared from salmon brain and rat brain.

### 2.2. Glutamate binding

Specific binding of glutamate to the PMR fraction was measured as described previously for alanine and serine [10]. The PMR fraction (80–100 $\mu$g protein) was incubated for 60 min on ice in 0.5 ml of 50 mM MOPS buffer, pH 7.2, containing 2.5 mM $CaCl_2$, 50 nM L-[G-$^3$H]glutamic acid ([$^3$H]Glu; 62 Ci/mmol, Amersham Corp., Arlington Heights, IL) and potential inhibitors (all from Sigma Chemical, St. Louis, MO) as indicated in the results and figure legends. As described previously [10], the basic operational definition of specific Glu binding was based on the difference between the total binding of [$^3$H]Glu to the PMR fraction and the nonspecific binding of [$^3$H]Glu in the presence of a 1000-fold excess of unlabeled Glu.

### 2.3. Enzyme assays

PLC activity was measured in the PMR fraction using hydrolysis of exogenous [$^3$H]phosphatidylinositol-4,5-bisphosphate ($PIP_2$) into water-soluble [$^3$H]inositol phosphate(s). Complete characterization of the stimulation of PLC by an amino acid cocktail has been presented in detail elsewhere [15]. The reaction mixture contained (final concentrations): 50 mM HEPES, pH 6.9, 10 $\mu$M GTP$\gamma$S, 2.5 mM $MgCl_2$ and 1.0–1.2 $\mu$M [inositol-2-$^3$H]$PIP_2$ (from Amersham Corp.). The free $Ca^{2+}$ concentration was maintained at $10^{-9}$ M with a $Ca^{2+}$/1 mM EGTA buffer as described elsewhere [15]. Reactions were started with the addition of 20–35 $\mu$g PMR protein. Reactions were terminated after a 10 min incubation at room temperature by the addition of 200 $\mu$l 10% $HClO_3$, placed on ice for 10 min, and centrifuged for 2 min with a benchtop microcentrifuge. Fifty microliters of 10 mM EDTA, pH 7.0, were added to a 200 $\mu$l aliquot of the supernatant along with 200 $\mu$l of a 1:1 (v/v) mixture of 1,1,2-trichlorotrifluoroethane and tri-*n*-octylamine to yield three phases. The upper aqueous phase contained the water soluble [$^3$H]inositol-containing products and was used for determination of radioactivity associated with $PIP_2$ breakdown.

### 2.4. RNA extraction and analyses

Total RNA was obtained from 10 salmon olfactory rosettes (1 g wet weight), 3 salmon brains (1.2 g wet weight), or 1 rat brain (1 g wet weight) using the single step acid guanidium thiocyanate–phenol–


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chloroform extraction of Chomczynski and Sacchi [22]. The RNA was resolved by agarose gel electrophoresis [23] and transferred to GeneScreen membranes (DuPont Corp., Wilmington, DE), using the protocol provided by the manufacturer. The full-length cDNAs for mGluR1 and mGluR4 were provided by E. Mulvihill (ZymoGenetics, Seattle, WA). These were prepared for use as RNA probes by enzymic radiolabeling (Pharmacia) with the Klenow fragment of DNA polymerase and [α-$^{32}$P]dCTP. For hybridization, membranes were incubated with the probe for 24 to 48 h at 42°C in 5 × SSPE (0.75 M NaCl, 0.05 M $NaH_2PO_4 \cdot H_2O$, 0.005 M EDTA·$Na_2$), 35% (w/v) formamide, 5 × Denhardt's solution, 1% sodium dodecyl sulfate (SDS), 10% Dextran sulfate and 100 μg/ml calf thymus DNA. Membranes were washed and subjected to autoradiography (Kodak X-Ray film) overnight at −70°C. The wash conditions were: twice for 15 min with 2 × SSPE at room temperature, once for 45 min with 2 × SSPE containing 2% SDS at 42°C, and twice for 10 min with 0.1 × SSPE at room temperature. Higher stringency hybridization and wash conditions were not tested.

## 3. Results and discussion

Binding of [$^3$H]Glu to the olfactory PMR fraction was inhibited by up to 90% in the presence of excess unlabeled Glu. This specific binding of Glu was saturable (Fig. 1). Scatchard analysis of the binding data yielded 1.5 μM for the $K_d$ and 259 pmol/mg protein for the $B_{max}$ of Glu binding to the PMR fraction (Fig. 1, inset). This affinity correlates well with physiological responses of the salmon olfactory system to amino acids [1]. A comparable analysis with a PMR fraction from salmon brain yielded 2.2 μM for the $K_d$ and 169 pmol/mg protein for the $B_{max}$ of Glu binding. Thus, Glu binding sites are even more abundant in the olfactory system than in the fish central nervous system and they are of comparable affinity in the two sites.

To further characterize the specificity of [$^3$H]Glu binding to the olfactory PMR fraction, a single high concentration (500 μM) of other known salmon odors and of Glu analogs was used to identify inhibitors of [$^3$H]Glu binding (Fig. 2). While limited, the single high concentration allowed for an initial screening of selected odors and Glu analogs without the large numbers of salmon that would be required to obtain sufficient olfactory membranes for a more detailed binding study. Alanine and lysine which represent ligands for putative neutral and basic amino acid receptor subtypes, respectively, had no effect on

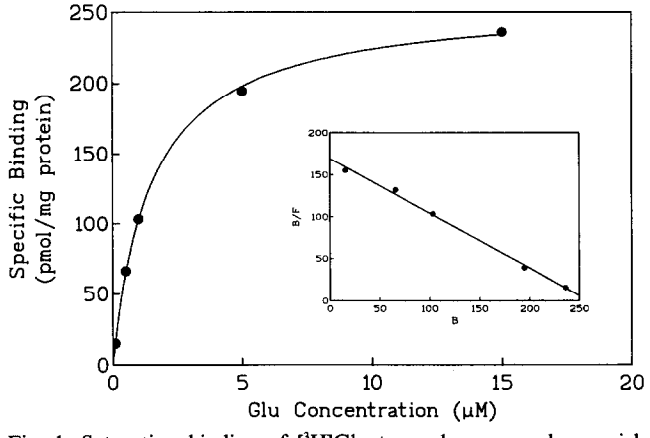


Fig. 1. Saturation binding of [$^3$H]Glu to a plasma membrane rich fraction from salmon olfactory rosettes. Specific binding of Glu was determined at each of the Glu concentrations shown. Results are from a single experiment, are the averages of incubations performed in duplicate, and are representative of the results of three independent experiments. Inset, Scatchard analysis of the binding data.

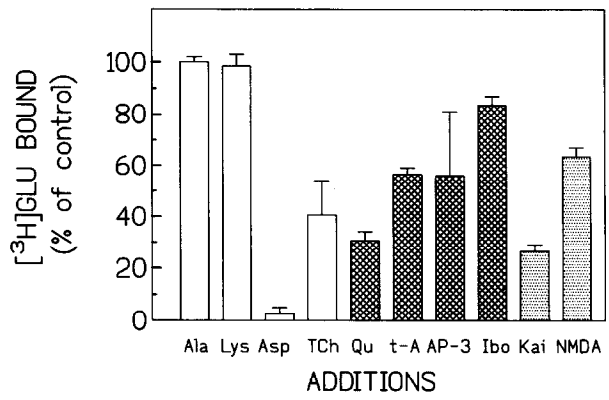


Fig. 2. Inhibition of [$^3$H]Glu binding to a plasma membrane rich preparation from salmon olfactory rosettes. Specific binding of [$^3$H]Glu was determined in the presence of known odors: alanine (ala), lysine (lys), aspartate (asp), and taurocholate (TCh); (2) metabotropic Glu receptor analogs: quisqualate (Qu), *trans*-1-aminocyclopentenyl-1,3-dicarboxylate (t-A), 2-amino-3-phosphonoproprionate (AP-3), and ibotenate (Ibo); or (3) ionotropic Glu receptor ligands: kainate (Kai) and *N*- methyl-D-aspartate (NMDA). All potential inhibitors were tested at a concentration of 500 μM except TCh (20 μM). Results are mean ± S.E.M. ($n$ = 3) and are expressed as a % of the control specific binding of 50 nM [$^3$H]Glu. Nonspecific binding was subtracted from all values.

[$^3$H]Glu binding. In contrast, the acidic amino acid, aspartate, was a potent inhibitor, effectively eliminating all specific Glu binding at this high concentration. In this respect, similar results were obtained with the salmon brain PMR fraction (Fig. 3) and Glu binding in each case is consistent with binding to an acidic amino acid receptor. Because of the high concentration of aspartate used, this analysis does not distinguish between possible differences in the respective affinities of the binding site for Glu and Asp. The odorant bile acid, taurocholate, inhibited [$^3$H]Glu binding by more than 50%. A lower concentration (20 μM) of taurocholate (TCh) was used in this analysis because of potential detergent effects at high concentrations. This inhibition by TCh was greater for olfactory Glu binding than for Glu binding to the salmon brain PMR fraction (Fig. 3). This inhibition of Glu binding by TCh was observed with the two salmon membrane preparations but was not observed with a PMR fraction from rat brain (data not shown). This latter finding is significant because it argues against a nonspecific reduction in Glu binding due to a detergent effect of TCh on the membranes at the concentration of TCh used. Such a detergent effect would not be expected to distinguish between rat and fish membranes. Thus, while olfactory discrimination among amino acids may occur at least in part through the interaction of amino acids with distinct receptor binding sites in Atlantic salmon, there may be some interaction between bile acids and amino acids at the receptor level. Further analysis is needed to address the possible subtypes within this category of receptors that might lead to olfactory discrimination of these two classes of organic acids. In addition, the potential significance of an apparent Glu/taurocholate interaction in salmon brain needs additional investigation.

Ligands that have been well characterized as agonists (quisqualate, *trans*-1-aminocyclopentenyl-1,3-dicarboxylate, *trans*-A, and ibotenate) or an antagonist (2-amino-3-phosphonoproprionate, AP-3) for metabotropic Glu receptors (mGluR) in

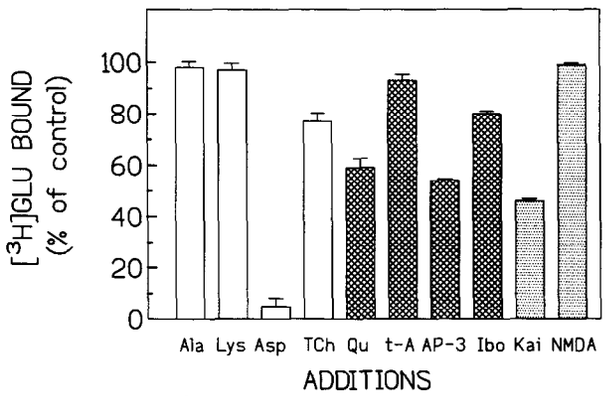


Fig. 3. Inhibition of [$^3$H]Glu binding to a plasma membrane rich preparation from salmon brain. Specific binding of [$^3$H]Glu was determined in the presence of known odors: alanine (ala), lysine (lys), aspartate (asp), and taurocholate (TCh); (2) metabotropic Glu receptor analogs: quisqualate (Qu), *trans*-1-aminocyclopentenyl-1,3-dicarboxylate (t-A), 2-amino-3-phosphonoproprionate (AP-3), and ibotenate (Ibo); or (3) ionotropic Glu receptor ligands: kainate (Kai) and *N*-methyl-D-aspartate (NMDA). All potential inhibitors were tested at a concentration of 500 $\mu$M except TCh (20 $\mu$M). Results are mean ± S.E.M. ($n$ = 3) and are expressed as a % of the control specific binding of 50 nM [$^3$H]Glu. Nonspecific binding was subtracted from all values.

mammalian brain (see [14]), all inhibited Glu binding to the olfactory PMR preparation. The rank order of potency for inhibition was quisqualate > *trans*-A = AP-3 > ibotenate (Fig. 2). Interestingly, *N*-methyl-D-aspartate (NMDA) and kainate, ligands for distinct ionotropic Glu receptors [14], also inhibited Glu binding to the olfactory PMR preparation (Fig. 2). Kainate was as potent as quisqualate while NMDA was comparable to *trans*-A and AP-3 in inhibiting Glu binding. The rank order of potency for these Glu analogs was different for inhibition of [$^3$H]Glu binding to the salmon brain PMR fraction: kainate > AP-3 = quisqualate > ibotenate > *trans*-A (Fig. 3). NMDA had no effect on Glu binding to the brain PMR fraction.

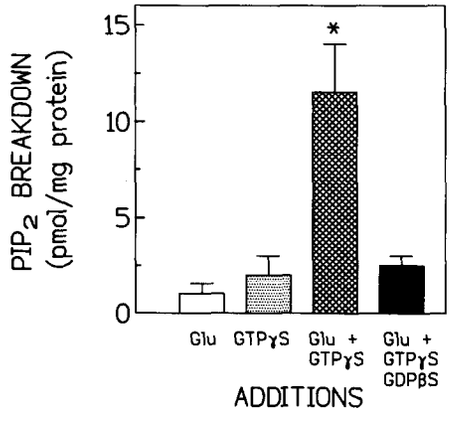


Fig. 4. Stimulation of olfactory PLC activity by Glu. Breakdown of [$^3$H]$PIP_2$ by the olfactory plasma membrane rich fraction was measured in the presence of: 10 $\mu$M Glu, 10 $\mu$M GTP$\gamma$S, 10 $\mu$M Glu + GTP$\gamma$S or 10 $\mu$M Glu + GTP$\gamma$S following preincubation of the membranes for 10 min with 1 mM GDP$\beta$S. Values for basal (no stimuli) $PIP_2$ breakdown were subtracted from all values so only stimulus-dependent phospholipase C activity is presented. Results are means ± S.E.M. ($n$ = 4). *Only this value for PLC activity was significantly greater than basal (no stimuli) activity ($P < 0.01$) when analyzed by Student's $t$-test.

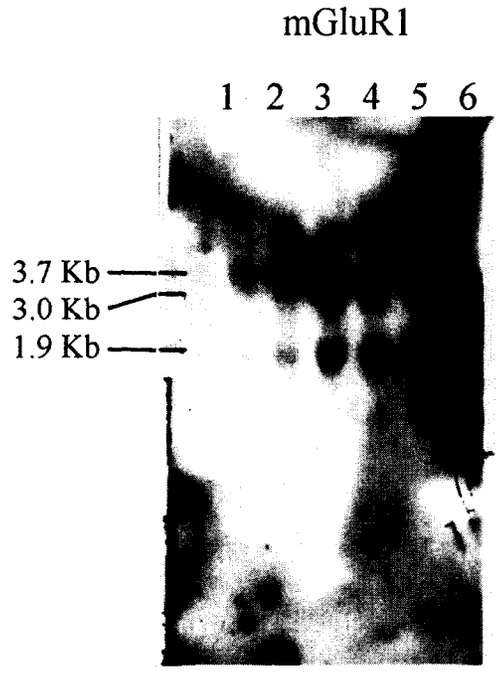


Fig. 5. Northern analysis of total RNA from rat brain, salmon brain and salmon olfactory rosettes. Total RNA was extracted from each of the tissues indicated, resolved by agarose gel electrophoresis and hybridized with a cDNA probe for mGluR1 (see section 2 for details). Lanes 1 and 2, rat brain (positive control); lanes 3 and 4, salmon brain; lanes 5 and 6, salmon olfactory rosette. Each lane was loaded with 20 $\mu$g total RNA.

In addition to ligand binding specificity, mammalian brain Glu receptors also differ in characteristic signal transduction mechanisms [14]. In particular the various mGluR subtypes are known to either stimulate PLC or inhibit adenylyl cyclase via one or more specific G proteins [14]. In the olfactory PMR fraction, Glu stimulated $PIP_2$ breakdown in a manner consistent with G protein mediated activation of PLC (Fig. 4). A concentration of Glu (10 $\mu$M) was chosen because it was approximately 10-times the $K_d$ and equivalent to that shown to give maximal stimulation of salmon olfactory PLC activity by an amino acid cocktail that contained representative amino acids from different putative receptor subtypes [15]. Stimulation of PLC by Glu did not occur in the absence of GTP$\gamma$S (Fig. 4). GTP$\gamma$S by itself had no effect on olfactory PLC activity, suggesting there was negligible guanine nucleotide exchange on the putative G protein in the absence of Glu. Dependence on a G protein was strongly suggested by previous work showing that activation of salmon olfactory PLC by a cocktail of amino acids in the presence of GTP$\gamma$S was prevented by preincubation of the PMR fraction with GDPbS to block the G protein [15]. A requirement for a G protein was similarly demonstrated for activation of salmon olfactory PLC by the bile salt taurocholate [24]. Several components of an olfactory PLC-based signal transduction system, including PLC [16] and a $Ca^{2+}$-mobilizing inositol 1,4,5-trisphosphate receptor [17–19], have been recently described for odorant amino acids in catfish. In both catfish and rat, olfactory receptors are thought to belong to the superfamily of G protein-coupled receptors [20,21].

Under low stringency conditions, both the mGluR1 cDNA

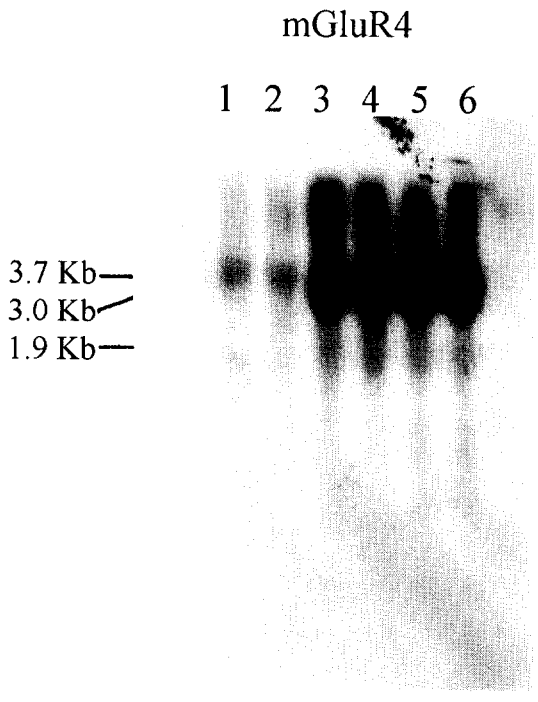


Fig. 6. Northern analysis of total RNA from rat brain, salmon brain and salmon olfactory rosettes. Total RNA was extracted from each of the tissues indicated, resolved by agarose gel electrophoresis and hybridized with a cDNA probe for mGluR4 (see section 2 for details). Lanes 1 and 2, rat brain (positive control); lanes 3 and 4, salmon brain; lanes 5 and 6, salmon olfactory rosette. Each lane was loaded with 20 $\mu$g total RNA.

(Fig. 5) and the mGluR4 cDNA (Fig. 6) hybridized with olfactory RNA. Hybridization of the two cDNA probes with rat brain RNA as a positive control and with salmon brain RNA are shown for comparison. With the mGluR1 probe (Fig. 5), two bands were observed in each of the RNA analyses: 3.7 and 1.9 kb for the rat brain RNA and 3.0 and 1.9 kb for both the salmon olfactory rosette and salmon brain RNA preparations. With the mGluR4 probe, there was a prominent band at 3.7 kb for the rat brain RNA and at 3.0 kb for the salmon brain and olfactory rosette preparations.

In summary, the evidence suggests that a Glu receptor is present in the salmon olfactory system with properties similar to metabotropic Glu receptors found in mammalian brain. These properties include inhibition of olfactory Glu binding by mGluR ligands and apparent coupling to PLC. Inhibition of olfactory Glu binding both by mGluR ligands and by kainate and NMDA suggests that multiple Glu receptors may be present in the olfactory PMR fraction. Alternatively, a single oGluR exists with a much less restrictive ligand binding profile for Glu analogs than is observed in mammalian brain. In either case, this work raises the possibility that olfactory reception of acidic amino acids in Atlantic salmon is mediated in part by an oGluR that is a subtype of the mGluR family. The presence of RNA that hybridizes with cDNA probes for mGluRs lead us to propose that the salmon olfactory system includes a subtype of the metabotropic glutamate receptor family.

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