Stimulation of Ca^{2+}-Regulated Olfactory Phospholipase C by Amino Acids†

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ABSTRACT: L-Amino acids are potent olfactory stimuli for Atlantic salmon. A plasma membrane fraction, previously shown to be rich in amino acid binding sites, was prepared from olfactory rosettes of Atlantic salmon (*Salmo salar*) and utilized to investigate the role of phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis in olfactory signal transduction. A cocktail of L-amino acids (Ser, Glu, Lys, and Gly) stimulated PIP_2 hydrolysis by phospholipase C (PLC) in a dose-dependent manner with half-maximal stimulation when all amino acids were present at approximately 1 μM. Stimulation of PIP_2 hydrolysis by amino acids required GTPγS, which alone had no effect on PLC activity. Unlike GTPγS, AlF_4^- and Ca^{2+} stimulated PIP_2 breakdown. Preincubation with 1 mM GDPβS eliminated the effect of amino acids and AlF_4^- on PIP_2 hydrolysis, suggesting the involvement of G protein regulation. The lack of stimulation by GTPγS alone suggested that there was negligible exchange of GTPγS for GDP in the absence of odorant. There were no significant effects of amino acids on either adenylate cyclase or guanylate cyclase activities in the membrane preparation under these conditions. The effect of the amino acid cocktail was maximal at 1–10 nM free Ca^{2+}. At or above 100 nM free Ca^{2+}, no effect of amino acids on PIP_2 hydrolysis was found. However, between 100 nM and 100 μM, Ca^{2+} directly stimulated PLC activity in a dose-dependent manner. This stimulation by Ca^{2+} appeared to be G protein independent because it did not require GTPγS and was not inhibited by GDPβS. Thus, low Ca^{2+} appears to sensitize olfactory PLC to G protein dependent stimulation by amino acids, whereas direct activation of PLC by elevated Ca^{2+} may contribute to amplification in olfactory signal transduction.

The initial step of olfactory reception is believed to be the interaction between odorant and receptor on the olfactory sensory membrane which leads to the production of an action potential (Getchell, 1986; Lancet, 1986). Cumulative evidence has suggested that participation of adenylate cyclase in olfactory signal transduction (Pace et al., 1985; Sklar et al., 1986; Jones & Reed, 1989; Bakalyar & Reed, 1990; Dhallan et al., 1990; Boekhoff et al., 1990). However, not all odorants activate adenylate cyclase, and in several systems, odorants which are poor stimulants for adenylate cyclase appear to cause activation of phospholipase C (PLC), producing inositol 1,4,5-trisphosphate (IP_3), a possible alternative mechanism for olfactory signal transduction (Huque & Bruch, 1986; Boekhoff et al., 1990; Breer et al., 1990; Breer & Boekhoff, 1991; Fadool & Ache, 1992). In olfactory cells of the catfish, a plasma membrane Ca^{2+} channel is activated by IP_3 and may participate in Ca^{2+} mobilization and membrane depolarization in response to odorant amino acids (Restrepo et al., 1990; Restrepo & Boyle, 1991; Kalinoski et al., 1992). IP_3 activates two types of plasma membrane channels and evokes an inward current in cultured lobster olfactory receptor neurons (Fadool & Ache, 1992).

Many cell surface receptors are coupled to PLC (Berridge, 1987). Upon the binding of an appropriate agonist, phosphatidylinositol 4,5-bisphosphate (PIP_2) is hydrolyzed into IP_3 and diacylglycerol. The mechanisms of receptor activation of this enzyme are beginning to be understood and G proteins are involved in coupling of receptors to at least some isozyme(s) of PLC (Rhee et al., 1989; Harden, 1990; Gutowski et al., 1991; Smrcka et al., 1991; Taylor et al., 1991; Lee et al., 1992).

Alanine and other amino acids are potent olfactory stimuli for Atlantic salmon (*Salmo salar*) (Sutterlin & Sutterlin, 1971). Specific membrane binding sites have been partially characterized and presumably represent the site of initial interaction between amino acids and olfactory receptors (Lo et al., 1991). Radioligand binding studies in Atlantic salmon were consistent with the presence of a neutral amino acid receptor that discriminated between neutral and acidic or basic amino acids (Lo et al., 1991). The present study was designed to examine whether amino acids stimulate PLC activity and/or other signal transduction pathways in the olfactory epithelium of Atlantic salmon. We have also examined the possible involvement of G protein and Ca^{2+} in this transduction process.

MATERIALS AND METHODS

Tissue Fractionation. Two to three year old Atlantic salmon, reared under conditions of simulated natural photoperiod and natural water temperature, were killed by decapitation and the olfactory rosettes were removed from the nares. To prepare a plasma membrane-enriched fraction, the rosettes were minced, homogenized, and subjected to differential centrifugation to obtain fraction P_2 (Cagan & Zeiger, 1978) as described previously (Lo et al., 1991). A conventional microsomal fraction was also prepared as described previously (Lo et al., 1991). Protein was determined by the method of Bradford (1976).

Enzyme Assays. PLC activity was measured in the membrane fractions using hydrolysis of exogenous ^{3}H-PIP_2 into water-soluble ^{3}H-inositol phosphates. The protocol for extraction of water-soluble inositol phosphates was from

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Boekhoff et al. (1990) with slight modification. PLC activity was assayed in a reaction volume of 0.1 mL containing (final concentrations) 50 mM HEPES, pH 6.9, 2.5 mM $MgCl_2$, and 1.0–1.2 μM [*inositol*-2-^{3}H(N)]phosphatidylinositol 4,5-bisphosphate (from Amersham Corp., Arlington Heights, IL.). Effects of amino acids on PLC were determined in the presence and absence of 10 μM GTPγS and 0.1–100 μM amino acid. Amino acids were presented either individually or in a mixture (cocktail). Amino acids were selected to represent proposed subclasses of odorant binding sites (neutral, alanine and glycine; acidic, glutamic acid; basic, lysine). All amino acids were used as the free acids (Sigma Chemical Co., St. Louis, MO). The effect of GTPγS was tested at concentrations of 1–100 μM and AlF_4^- was tested at 10 mM. To examine the effect of GDPβS, membranes were preincubated 10 min with 1 mM GDPβS prior to PLC measurement. Free Ca^{2+} concentrations were maintained with Ca^{2+}/EGTA buffers using 1 mM EGTA according to Pershadsingh and McDonald (1979). Reactions were started with the addition of 20–35 μg of membrane protein. After 10 min of incubation at room temperature, the reactions were terminated by the addition of 200 μL of 10% $HClO_3$, placed on ice for 10 min, and centrifuged for 2 min with a benchtop microcentrifuge. An aliquot of the supernatant was removed for measurement of inositol phosphates. Fifty microliters of 10 mM EDTA, pH 7.0, was added to a 200-μL aliquot of the supernatant, which was then neutralized with 200 μL of a 1:1 (v/v) mixture of 1,1,2-trichloro-1,2,2-trifluoroethane and tri-*n*-octylamine. After being mixed for 30 s, the samples were centrifuged for 2 min with a microcentrifuge to yield three phases. The upper aqueous phase contained the water-soluble ^{3}H-inositol phosphates and was used for determination of radioactivity.

Adenylate cyclase and guanylate cyclase activities were measured at room temperature in a final volume of 200 μL containing 50 mM HEPES, pH 7.5, 1 mM ATP, 0.5 mM methylisobutylxanthine, and 2.5 mM $MgCl_2$. Free Ca^{2+} concentrations were maintained with Ca^{2+}/EGTA buffers, as described above. In addition to the amino acid cocktail used in the PLC measurements, histidine and additional combinations of classes of amino acids: neutral (serine, alanine, and threonine), basic (lysine and arginine), and acidic (glutamic and aspartic acids) were tested in these assays with each amino acid present at a final concentration of 100 μM. Reactions were started with the addition of 20–40 μg of membrane protein. After 15 min of incubation, reactions were terminated by inserting the incubation tubes in boiling water for 5 min. The samples were then centrifuged with a benchtop microcentrifuge and the supernatant was collected for cAMP and cGMP assays. The quantities of cAMP and cGMP generated during the 15-min incubation period were determined using commercially available competition binding assays (Amersham Corp). The assays are based on the competition between unlabeled cAMP or cGMP and a fixed quantity of radiolabeled cAMP or cGMP, respectively, for binding to a protein with high specificity for binding the respective cyclic nucleotide. Sensitivity of these assays is in the range of 0.2–16 pmol for cAMP and 0.04–8 pmol for cGMP.

RESULTS

A cocktail of amino acids (Ser, Gly, Glu, and Lys) stimulated the hydrolysis of ^{3}H-PIP_2 by the olfactory membrane preparation (P_2). GTPγS, a nonhydrolyzable GTP analogue, had no effect on PLC activity but it was required for the activation of the enzyme by the amino acid cocktail (Figure 1A). In the absence of GTPγS, the amino acid cocktail had no effect on

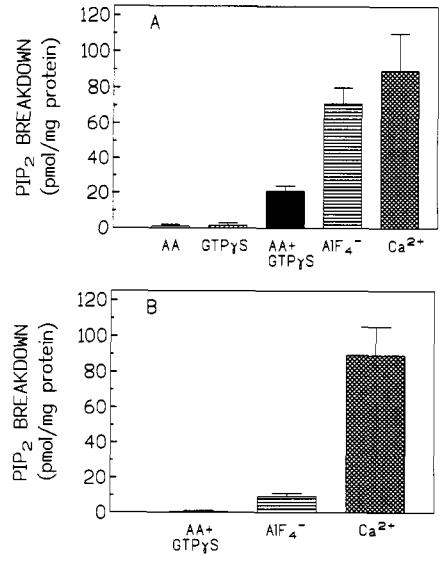

FIGURE 1: Effect of potential activators of PIP_2 hydrolysis. (A) PIP_2 hydrolysis was measured by the amount of water-soluble inositol phosphates released into the aqueous phase. Activity was measured in incubation solution containing 50 mM HEPES, pH 6.9, 1 μM ^{3}H-PIP_2, and 2.5 mM $MgCl_2$ in a total volume of 0.1 mL. Ca^{2+} concentration was buffered at 1 nM with 1 mM EGTA. Reactions were initiated by addition of the plasma membrane rich preparation (P_2). Basal activity was determined by PIP_2 hydrolysis caused by addition of sample in the absence of any potential stimulant and was substracted from all measurements. The potential stimulants were 100 μM amino acid cocktail (AA), 10 μM GTPγS, 100 μM amino acid cocktail plus 10 μM GTPγS (AA + GTPγS), 10 mM AlF_4^-, and 100 μM Ca^{2+}. The amino acid cocktail was made up of 100 μM each serine, glutamate, lysine, and glycine. Results are expressed as picomoles of PIP_2 per milligram of P_2 protein and represent means ● SEM from four independent determinations. (B) To examine the effect of GDPβS on stimulation of PIP_2 hydrolysis, samples were preincubated with 1 mM GDPβS at room temperature for 10 min before the assay. The other conditions for stimulation of PIP_2 hydrolysis and presentation of the results were as described for panel A.

the enzyme (Figure 1A). In the presence of GTPγS, the amino acid cocktail stimulated PLC activity. GTP, itself, was much less effective than GTPγS (data not shown). Although the enzyme could not be stimulated by GTP or GTPγS alone, both AlF_4^- and Ca^{2+} strongly stimulated PLC activity (Figure 1A). Stimulation of PLC by AlF_4^- and Ca^{2+} occurred without addition of GTP. After preincubation of the membrane preparation with 1 mM GDPβS, the stimulatory effects of amino acids and AlF_4^- on the enzyme were eliminated (Figure 1B). Stimulation of PLC by Ca^{2+} was unaffected by GDPβS (Figure 1B). This suggested that the activation of PLC by amino acids or AlF_4^-, but not by Ca^{2+}, required G protein.

The effect of the amino acid cocktail on PLC activity was dose-dependent and was maximal when the concentration of each amino acid was approximately 10 μM (Figure 2). Significant activation of PLC was observed at amino acid concentrations greater than 10^{-7} M with half-maximal stimulation at approximately 10^{-6} M amino acids.

Basal (no amino acids) PLC activity was stimulated by Ca^{2+} in a dose-dependent manner (Figure 3A). The amino acid cocktail caused significant enhancement of PLC activity only when free Ca^{2+} concentration was at or below 10 nM (Figure 3A). Thus, amino acid-dependent PLC activity was observed only at free Ca^{2+} concentrations below 100 nM (Figure 3B).

Adenylate cyclase activity was detected in the membrane preparation under basal conditions (no GTP, no amino acids).

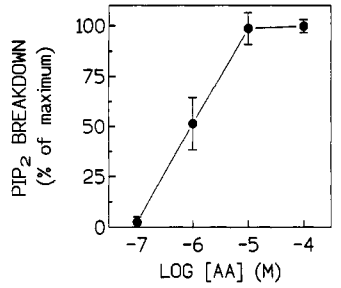

FIGURE 2: Dose response for stimulation of PIP_2 breakdown by the amino acid cocktail. Incubating solution used was as described in Figure 1 except 10 μM GTPγS and indicated concentration of amino acid cocktail were added. Results are means ± SEM from four independent determinations.

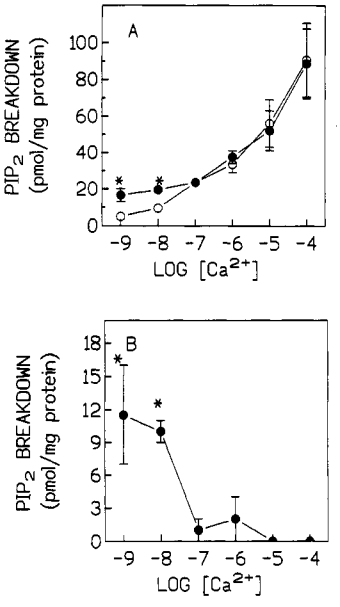

FIGURE 3: (A) Concentration dependence of PIP_2 hydrolysis on Ca^{2+} in the presence (●) and absence (○) of 100 μM amino acid cocktail. Basic incubation solution used was as described in Figure 1 except 10 μM GTPγS and indicated concentration of free Ca^{2+} buffered with 1 mM EGTA were added. Results are means ± SEM from four independent determinations. (B) The results presented in panel A were recalculated to represent amino acid-dependent PIP_2 breakdown vs Ca^{2+} concentration. For each Ca^{2+} concentration the value for PIP_2 breakdown in the absence of amino acid cocktail was subtracted from the value obtained in the presence of the cocktail.

Adenylate cyclase activity was substantially increased in the presence of GTPγS or AlF_4^- (Figure 4A). Concentrations of amino acids up to 10 μM had no effect on cAMP production in either the presence or absence of GTP (Figure 4A). Only a high concentration (100 μM) of amino acids stimulated cAMP production. This was seen with histidine (H) and groupings of neutral (alanine, serine and threonine; A,S,T), acidic (aspartic and glutamic acids; D,E) and basic (lysine and arginine; K,R) amino acids (Figure 4A).

A very low level of basal guanylate cyclase activity was detectable in the membrane preparation (Figure 5). There was no effect of amino acids on cGMP production.

Cyclic AMP measurements were sensitive to the free Ca^{2+} concentration. Ca^{2+} reduced cAMP levels in a dose-dependent manner (Figure 5). There was no effect of Ca^{2+} on cGMP levels.

DISCUSSION

Evidence from biochemical, electrophysiological, and behavioral studies has indicated that discrimination and reception

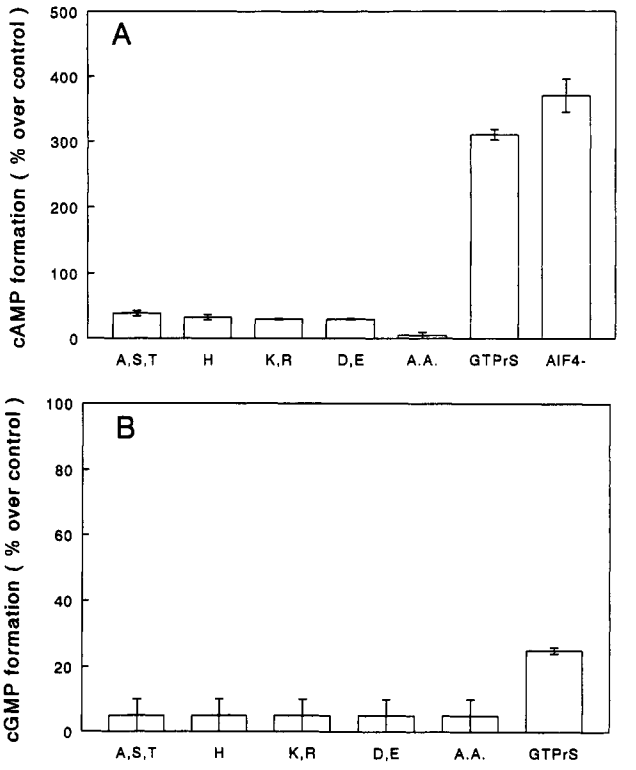

FIGURE 4: (A) cAMP formation in the presence of various potential stimulants. Incubating solution contained 50 mM HEPES, pH 7.5, 1 mM ATP, 10 μM GTP, 0.5 mM methylisobutylxanthine, and 2.5 mM $MgCl_2$. Free Ca^{2+} concentration was maintained at 1 nM with 1 mM EGTA. The concentration of each amino acid used in the assay was 100 μM (A, alanine; S, serine; T, threonine; H, histidine; K, lysine; R, arginine; D, glutamic acid; E, aspartic acid). The amino acid cocktail (AA) contained 10 μM each serine, glutamic acid, lysine, and glycine. Concentration of GTPγS was 10 μM. Formation of cAMP in the absence of any stimulant was used as the control. Results are means ± SEM from four independent determinations. (B) cGMP formation in the presence of various potential stimulants. Incubation solution and concentration of stimulants were as described for panel A. Formation of cGMP in the absence of any stimulant was used as the control. Results are means ± SEM from two independent determinations.

of amino acids in fish olfactory systems begin with binding to one or more distinct receptor subtypes for amino acids [reviewed in Lo et al. (1991)]. One of these proposed subtypes recognized L-Ala and L-Ser and, in Atlantic salmon, has a subcellular distribution that suggests it is abundant on nonciliary plasma membranes of receptor cells (Lo et al., 1991). The olfactory epithelium of teleost fish has both ciliated and microvillus olfactory receptor cells (Yamamoto, 1982). In the present study, plasma membrane fractions, previously shown to be rich in amino acid binding sites (Lo et al., 1991), were prepared from olfactory rosettes of Atlantic salmon and used to show that amino acids activated PLC, but not adenylate cyclase or guanylate cyclase, in a concentration range relevant to their roles as olfactory stimuli (Sutterlin & Sutterlin, 1971). Preliminary studies used a specific competition binding assay to measure IP_3 production by PLC in this membrane preparation. By the earliest time point possible under these conditions (approximately 10 s), IP_3 was produced and rapidly metabolized upon activation of the membrane preparation with amino acids (Y.H. Lo, unpublished results). Therefore, a batch assay of PIP_2 breakdown products was used in the present study to measure PLC activity. Work by Breer and co-workers has demonstrated production of IP_3 within 50 ms of stimulation of olfactory membranes with certain odors (Boekhoff et al., 1990; Breer et al., 1990, 1991). Also using

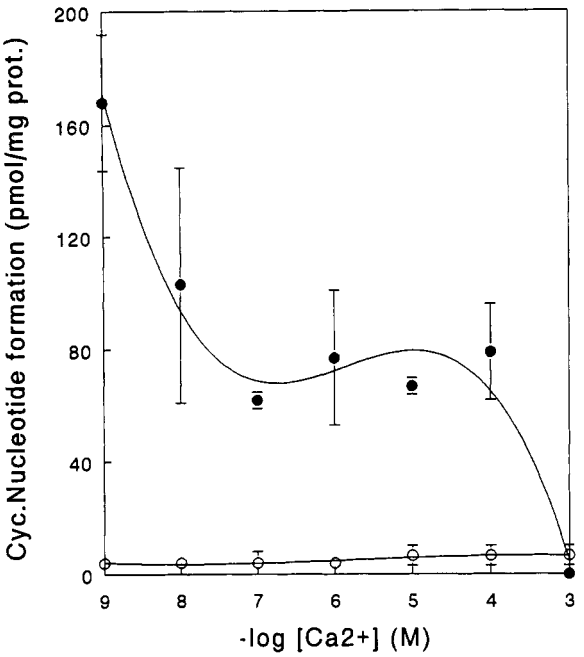

FIGURE 5: Effect of Ca^{2+} on cAMP (●) and cGMP (○) formation. Incubating solution was as described for Figure 4A with free Ca^{2+} concentrations buffered with 1 mM EGTA to the level indicated. Results are means ± SEM from three independent determinations.

rapid kinetics methodology, these workers have shown that other odors stimulate cAMP production with no effect on IP_3 production. While stimulation of PLC by odorant amino acids was observable with the batch assay used in the present work, the failure to observe stimulation of cAMP production except at a high (100 μM) concentration does not rule out the possibility of a small rapid increase in cAMP production that is stimulus-dependent but cannot be distinguished from the high basal activity under these batch assay conditions. A similar high basal activity is not observed with PLC (see Figure 3) and would indicate tight coupling between PLC activity and receptor activation.

Stimulation of PLC by amino acids was consistent with a requirement for a G protein. This was indicated by a requirement for GTPγS and by inhibition upon preincubation of membranes with GDPβS. Given the results with G protein regulation of adenylate cyclase, it was somewhat surprising that GTPγS by itself did not stimulate PLC. However, PLC activity was stimulated by AlF_4^-, which is also capable of activating G protein (Bigay et al., 1985). Instead of exchanging for bound GDP, AlF_4^- interacts with GDP and mimics the effect of GTP, causing activation and dissociation of α subunit from the $\beta\gamma$ complex (Gilman, 1987). Unlike GTPγS, AlF_4^- alone caused a large increase in the hydrolysis of PIP_2 in the olfactory membrane preparation. The effect of AlF_4^- could be blocked by preincubating the sample with 1 mM GDPβS. Bound GDPβS does not interact with AlF_4^- (Bigay et al., 1985; Cockroft & Taylor, 1987). This strongly suggested the participation of G protein in the activation of PLC from P_2 preparation. Generally, GTPγS stimulates G protein-regulated activity by displacing GDP from the α subunit and, because of its resistance to hydrolysis, promotes dissociation of this subunit from the $\beta\gamma$ complex and activation of the effector. The lack of effect of GTPγS alone could be due to tight binding of GDP to the α subunit. In the presence of amino acids, the amino acid–receptor complex could stimulate the exchange of tightly bound GDP for GTPγS and stimulate PLC activity. GDP has been shown to bind tightly to transducin, a specific G protein found in the rod outer segment (Stryer, 1986). Exchange of this tightly bound GDP for GTP or its analogue required the presence of light-activated rhodopsin (Fawzi & Northup, 1990). GDP has also been shown to bind tightly to G_q, a G protein which is postulated to be the pertussis toxin insensitive G protein regulating PLC (Smrcka et al., 1991; Gutowski et al., 1991). The G_q protein has been shown to regulate PLC-β1 activity (Taylor et al., 1991). This may be of further interest because initial immunoblotting analyses of olfactory membrane proteins from Atlantic salmon indicated positive immunoreactivity with an isozyme-specific monoclonal antibody to PLC-β1 (Cheng et al., 1992) and with antisera specific for the α_q family of G proteins (Rhoads et al., 1993). These results differ in several respects from those reported for channel catfish. First, GTPγS alone did stimulate PLC activity in olfactory ciliary membrane preparations from catfish rosettes (Huque & Bruch, 1986). This may be one indication of different G protein involvement in the two fish species. In catfish, odor-dependent PLC activity is pertussis toxin sensitive and the only pertussis toxin substrate identified cross-reacts with monoclonal antibody to $G_i\alpha$ (Bruch & Kalinoski, 1987; Abogadie & Bruch, 1992). We have not observed effects of pertussis toxin in salmon. Second, while a low level of Ca^{2+} was required for olfactory PLC activity in catfish, high concentrations of Ca^{2+} inhibited PLC activity (Boyle et al., 1987). This is in sharp contrast to the stimulation of PLC activity observed with salmon and may reflect involvement of different PLC isozymes as well as G protein, in these two systems. In both salmon (Figure 4A) and catfish (Bruch & Teeter, 1989), amino acids stimulated cAMP production only at concentrations well above the range where electrophysiological responses to amino acids were observed.

In the present investigation, PIP_2 hydrolysis could be stimulated by 1 μM amino acids with maximal stimulation at 10–100 μM amino acids. This is in the range expected from the reported K_D for amino acid binding (Lo et al., 1991). The magnitude of stimulation of amino acids was about one-third that of AlF_4^- and one-fourth that of 100 μM Ca^{2+}. Amino acids would be expected to stimulate PLC which is coupled to amino acid receptors and, thus, to stimulate only a subset of the G protein regulated PLC in the preparation.

Olfactory epithelial membrane preparations from a variety of fish have been shown to contain saturable binding sites for various amino acids. On the basis of both physiological and biochemical studies in catfish, these sites are broadly divided into the short-chain neutral (SCN), the long-chain neutral (LCN), the basic, and the acidic amino acid binding sites (Caprio & Byrd, 1984; Bruch & Rulli, 1988). These sites possess different but not necessarily exclusive specificities for various odorant amino acids. In olfactory membrane preparations from Atlantic salmon, there is mutual inhibition for binding between serine and alanine (Lo et al., 1991). In contrast, glutamate and lysine have no effect on serine or alanine binding. Thus, it is likely that separate neutral, acidic, and basic amino acid sites exist in Atlantic salmon olfactory receptor cells (Lo et al., 1991). For the purposes of the current study, the L-amino acid cocktail was composed of serine, lysine, glutamic acid, and glycine. Although glycine and serine are both neutral, glycine has been reported to be an attractant for salmonids while serine has been reported to be a repellent (Malmgren & Watson, 1987). Preliminary studies showed that each of the individual amino acids of the cocktail stimulated PLC activity (Y.H. Lo, unpublished results). An amino acid mixture (cocktail) was used in much of this study to maximize responses to odors by engaging more than one receptor subtype in the assay.

The effect of the amino acid cocktail on PLC activity was maximal when free Ca^{2+} was at or below 10 nM. There was no effect of amino acids when free Ca^{2+} was above 100 nM. Thus, the sensitivity of the receptor cells to amino acids depends upon the capability of these cells to maintain intracellular free Ca^{2+} below 100 nM. Although the intracellular free calcium ion concentration in olfactory neurons of Atlantic salmon is not known, very low intracellular free calcium (23 ± 19 nM) has been reported in olfactory neurons of channel catfish (Restrepo et al., 1990). Our related work has indicated the existence of a high-affnity, high-capacity Ca^{2+},Mg^{2+}-ATPase in salmon olfactory membrane preparations (Lo et al., 1993). With an extremely low K_m of 10 nM for Ca^{2+}, this Ca^{2+},Mg^{2+}-ATPase is a possible candidate for the tight control of free intracellular Ca^{2+} to the low level required to sensitize PLC to stimulation by amino acids.

PLC activity was directly stimulated by Ca^{2+} in a dose-dependent manner (Figure 3A). This stimulation required no GTP or GTPγS and was not affected by GDPβS. Direct effects of calcium on PLC activity have been reported in other systems (Cockroft & Taylor, 1987, 1986; Pnafoli et al., 1990; Fain, 1990). The phosphoinositide pathway is known to generate IP_3 and mobilize Ca^{2+} (Berridge, 1987). An IP_3 receptor (Kalinoski et al., 1992) and IP_3-gated Ca^{2+} channel activity (Restrepo et al., 1990; Restrepo & Boyle, 1991) have been identified in ciliary plasma membrane preparations and olfactory neurons, respectively, of channel catfish. If salmon have an IP_3 system with a plasma membrane orientation similar to that of catfish or lobster olfactory receptor cells (Fadool & Ache, 1992), activation of PLC by amino acids in salmon olfactory receptor cells is expected to cause a rise in intracellular Ca^{2+} by influx of extracellular Ca^{2+}. A rise in intracellular calcium could amplify the signal by further stimulation of PLC activity, a feedback activation of PLC by Ca^{2+} entering the receptor cell. However, in mammalian brain, elevated intracellular Ca^{2+} has numerous other effects including those on protein kinase activities and on the IP_3 receptor (Worley et al., 1987). The latter has been reported to be complex, involving sequential activation followed by inactivation (Finch et al., 1991). In rat olfactory cilia, rapid kinetics of IP_3 production are modified by protein kinase C activity (Boekhoff & Breer, 1992). Thus, in olfactory receptor mediated Ca^{2+} mobilization, there may be a variety of forms of positive and negative feedback regulation in which direct activation of PLC by Ca^{2+} may participate.

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