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By
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<th>Full Form</th>
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<tbody>
<tr>
<td>7TMD</td>
<td>seven transmembrane domain</td>
</tr>
<tr>
<td>AP</td>
<td>adapter protein</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP-ribosylation factor</td>
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<tr>
<td>βAR</td>
<td>β-adrenergic receptor</td>
</tr>
<tr>
<td>βARK</td>
<td>β-adrenergic receptor kinase</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CamKII</td>
<td>calcium/calmodulin kinase II</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3’,5’-monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CNGC</td>
<td>cyclic nucleotide-gated channels</td>
</tr>
<tr>
<td>CRE</td>
<td>cAMP response element</td>
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<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>CTA</td>
<td>conditioned taste aversion</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DIGS</td>
<td>detergent-insoluble glycolipids</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EOG</td>
<td>electro-olfactogram</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
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<td>Abbreviation</td>
<td>Full Name</td>
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<td>--------------</td>
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</tr>
<tr>
<td>FOE</td>
<td>fish olfactory epithelium</td>
</tr>
<tr>
<td>GAPs</td>
<td>GTPase activating proteins</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GEM</td>
<td>glycolipid-enriched membrane</td>
</tr>
<tr>
<td>GIT</td>
<td>GRK interacting protein</td>
</tr>
<tr>
<td>GRK</td>
<td>G protein receptor kinase</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GSP</td>
<td>gene-specific primer</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>inositol 1,4,5 trisphosphate</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;R</td>
<td>inositol 1,4,5 trisphosphate receptor</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LCR</td>
<td>locus control region</td>
</tr>
<tr>
<td>LTP</td>
<td>long term potentiation</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase (ERK)</td>
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<tr>
<td>MAPKK</td>
<td>mitogen-activated protein kinase kinase (MEK)</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>mitogen-activated protein kinase kinase kinase (Raf)</td>
</tr>
<tr>
<td>MEK</td>
<td>MAP/ERK kinase</td>
</tr>
<tr>
<td>MKP</td>
<td>MAP kinase phosphatase</td>
</tr>
<tr>
<td>MOE</td>
<td>main olfactory epithelium</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-Morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>NHERF</td>
<td>Na&lt;sup&gt;+&lt;/sup&gt;/H&lt;sup&gt;+&lt;/sup&gt; exchanger regulatory factor</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NSF</td>
<td>N-ethylmaleimide-sensitive factor</td>
</tr>
<tr>
<td>OE</td>
<td>olfactory epithelium</td>
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<tr>
<td>OR</td>
<td>olfactory receptor</td>
</tr>
<tr>
<td>ORN</td>
<td>olfactory receptor neuron</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PIP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PKG</td>
<td>protein kinase G</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>RACE</td>
<td>rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>RAG</td>
<td>recombination activating proteins</td>
</tr>
<tr>
<td>RGS</td>
<td>regulators of G protein signaling</td>
</tr>
<tr>
<td>RKIP</td>
<td>Raf kinase inhibitor protein</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROE</td>
<td>rat olfactory epithelium</td>
</tr>
<tr>
<td>RSK</td>
<td>ribosomal S6 kinase</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>SAPK</td>
<td>stress-activated protein kinase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>SOS</td>
<td>son of sevenless</td>
</tr>
<tr>
<td>SSC</td>
<td>saline sodium citrate buffer</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris/acetic acid/EDTA</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline/Tween-20</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris[hydroxymethyl]aminomethane</td>
</tr>
<tr>
<td>TSA</td>
<td>tyramide signal amplification</td>
</tr>
<tr>
<td>V2R</td>
<td>vomeronasal 2 receptor</td>
</tr>
<tr>
<td>VNO</td>
<td>vomeronasal organ</td>
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ABSTRACT

Olfactory signal transduction is initiated by binding of odorous ligands to G protein-coupled odorant receptors, resulting in the activation of G protein and second messengers that culminates in the generation of action potentials to relay this signal to the brain for odor recognition. This signaling cascade comprises the primary olfactory response. Odorant receptors comprise the largest multigene family identified to date, enabling the olfactory system to respond to an enormous pool of potential odorant molecules. This work focuses on the study of odorant receptors beyond the initiation of the primary olfactory response.

The first set of experiments focuses on odorant receptor desensitization and internalization. Key components of the internalization pathway involve phosphorylation of the odorant receptor by GRKs, binding of arrestin to the receptor-ligand complex, and targeting of this moiety to clathrin-coated pits. Dynamin initiates the formation of an endocytic vesicle from the clathrin-coated pit. Data are presented that identify and localize dynamin and clathrin to the dendritic extension of olfactory receptors where olfactory signal transduction occurs. In addition, a functional internalization event was identified specifically upon odorant stimulation in isolated catfish ORNs.

The second set of experiments focuses on periphal signaling pathways mediated by odorant receptors. Activation of the ERK cascade in neurons has been shown to participate in neuronal plasticity and gene expression. Data presented here extend this observation to olfactory sensory neurons of the channel catfish. A mixture of L-methionine and L-lysine, potent olfactory stimuli for catfish, activates the Raf-MEK-ERK-RSK pathway in vivo, and the activation of this pathway is modulated by CamKII and PKC.
The last set of experiments presents data for the identification of a potential chemosensory receptor in channel catfish. RT-PCR and RACE performed on total RNA isolated from catfish olfactory epithelium produced a cDNA that is very similar to metabotropic glutamate receptors and putative pheromone receptors from multiple species. This clone was localized to neurons located in the sensory region of the olfactory epithelium by in situ hybridization. Northern analysis indicated that this protein is expressed largely in the olfactory epithelium.
CHAPTER 1: 
INTRODUCTION

The work presented here focuses on the study of odorant receptors in the channel catfish *Ictalurus punctatus*. The first chapter presents evidence for a functional internalization pathway in olfactory sensory neurons, the second focuses on peripheral signaling pathways, namely ERK, initiated upon odorant stimulation, and the last chapter presents evidence for the identification of a putative chemosensory receptor isolated from olfactory epithelium. The topic of each research chapter will be individually reviewed in this section; however, the topics will be discussed in a different order.

OLFACTORY SIGNAL TRANSDUCTION IN CATFISH

The olfactory system is remarkable in its ability to discriminate among an innumerable amount of potential odorants possessing a diverse array of functional groups. Odorant stimuli mediate a multitude of behaviors including feeding, predator avoidance, homing, and sexual behavior (reviewed in Sorenson and Caprio 1998), and for catfish are water-soluble. The ability of the olfactory system to detect such a diverse array of molecules is mediated through the organism’s repertoire of G protein-coupled odorant receptors. Initially, stimuli bind odorant receptors located in the cilia or microvilli of olfactory receptor neurons (ORNs). Upon agonist binding, the odorant receptors activate heterotrimeric G proteins that in turn initiate second messenger cascades that result in changes in membrane potential of the cell. The ORN extends a single axon that synapses with mitral cells in regions called glomeruli located in the olfactory bulb. This results in transduction of the detected odorant stimuli to higher brain centers and results in odor recognition.

In catfish, odorant signal transduction is mediated through the specific G proteins identified in this species. Agonist binding to odorant receptors induces activation of G
protein by promoting the exchange of GDP for GTP on the $G_\alpha$ subunit along with a concomitant dissociation of activated $G_\alpha$-GTP and the $\beta_\gamma$ dimer. $G_\alpha$ subunits include $G_s$, $G_{olf}$, $G_{i1}$, $G_{i2}$, and $G_q$ (Abogadie et al. 1995) while $\beta_\gamma$ subunits include $\beta 1$, $\beta 2$, $\gamma 2$, and $\gamma 3$ (Bruch et al. 1997a). $G_{olf}$ is the only G protein clearly restricted to the chemosensory area in catfish olfactory epithelium (Abogadie et al. 1995), suggesting it is the main candidate for activation of adenylate cyclase III (Bakalyar and Reed 1990) and signaling through the second messenger cAMP. Bruch and Teeter (1990) showed that cAMP increased upon amino acid stimulation in isolated cilia of catfish olfactory neurons; however, this response was only observed when the in vitro stimulation regime was performed over several minutes and the stimulus concentration was greater than 1 µM. Furthermore, Restrepo et al. (1993) observed that amino acid stimuli rapidly increased inositol 1,4,5-trisphosphate (IP$_3$) production with no increase in cAMP levels in the time frame necessary for signal transduction. This evidence suggests that IP$_3$ second messenger production is mediated through $G_i$ or $G_q$. $G_i/G_q$ binding to phospholipase C (PLC) induces conversion of phosphatidylinositol 4,5-bisphosphate (PIP$_2$) into the second messengers IP$_3$ and diacylglycerol (DAG). PLC isotype $\beta 1$ (Bruch, unpublished data) was identified by RT-PCR in catfish olfactory neurons and is likely to catalyze this reaction.

The olfactory signal transduction cascade is transduced through the action of the second messengers DAG and IP$_3$. DAG is believed to activate PKC$\beta$ even though PKC$\delta$ is also present in catfish because odorant induced phosphorylation was completely inhibited by Go6976 (Bruch et al. 1997b). Go6976 is a specific inhibitor of calcium-sensitive PKCs of which PKC$\beta$ is a member. PKC$\delta$ lacks the C2 calcium-binding region making it insensitive to calcium binding and Go6976. Both IP$_3$ and cAMP second messengers modulate the gating
of cation channels, permitting entry of Na\(^+\) and Ca\(^{2+}\) into ORNs resulting in depolarization of the cell. IP\(_3\) binds to a 107 kDa IP\(_3\) receptor (IP\(_3\)R) (Kalinoski et al. 1992) located in the plasma membrane, that is itself a cation channel, while cAMP binds to a cationic cyclic nucleotide-gated channel (CNGC) (Nakamura and Gold 1987). The odorant-elicited receptor current has two sequentially activated components: a primary cationic current carried by Ca\(^{2+}\) and an anionic current carried by Cl\(^-\) (Kurahashi and Yau 1993). Cl\(^-\) concentration in ORNs is unusually high, and when intracellular Ca\(^{2+}\) concentration becomes sufficiently high, Ca\(^{2+}\)-activated Cl\(^-\) channels open, enabling Cl\(^-\) efflux and amplifying the initial cation-mediated depolarization (reviewed by Menini 1999). Ca\(^{2+}\) can also activate a K\(^+\) channel located in the plasma membrane of ORNs (Morales et al. 1997). Activation of this K\(^+\) channel is correlated with the inhibitory response elicited by putrid odors in toad ORNs and may also be important in inhibitory responses in other species.

The second messenger cascades can be regulated by a class of proteins known as the regulators of G protein signaling (RGSs). RGSs function as GTPase activating proteins (GAPs) that dramatically accelerate the GTP hydrolysis activity of the G\(_{\alpha}\) subunits, switching G\(_{\alpha}\) from the active to inactive state (Berman and Gilman 1998). Interaction assays have shown that RGSs can interact with, and act as GAPs for, members of the G\(_i\) and G\(_q\) classes of G proteins (Berman and Gilman 1998)--both present in catfish olfactory epithelium (Abogadie et al. 1995). An RGS3 homolog was identified in catfish ORNs (Bruch and Medler 1996). Homologs for RGS2 and RGS4 have also been identified as well as what appears to be a novel RGS in single catfish ORNs (Rankin and Bruch, unpublished data). RGSs have also been observed in the mammalian olfactory and vomeronasal epithelium where they display zonal expression patterns (Norlin and Berghard 2001).
ODORANT RECEPTOR CHARACTERIZATION AND EXPRESSION

Odorant receptors were first identified in rat based on the observation that odorant stimulation induced an increase in cAMP and IP$_3$ second messengers, and these increases were GTP dependent. Receptor proteins coupled to second messenger stimulation belong to the G protein-coupled receptor (GPCR) superfamily (Buck and Axel 1991). This superfamily of proteins displays a characteristic seven transmembrane domain (7TMD) topology, and it was to this structural feature that degenerate PCR primers were designed that resulted in the initial identification of odorant receptors. A primer pair derived from transmembrane domains III and VII of the putative rat olfactory receptors were used by Ngai et al. (1993a) to generate an amplification product from catfish cDNA. This amplification product displayed significant homology to the rat receptors, and was used as a probe to screen a cDNA library generated from catfish olfactory epithelium for identification of odorant receptors in this species.

Screening of the catfish olfactory cDNA library revealed four families of odorant receptors that exhibited from 40-60% sequence similarity among one another and 30-40% similarity with the rat sequences (Ngai et al. 1993a). Maximal conservation in the olfactory receptors was observed in the second intracellular and extracellular loops as well as within transmembrane domains II, VI, and VII. In contrast to other members of the GPCR superfamily that exhibit maximal similarity within all seven transmembrane domains, great divergence was observed in transmembrane domains I, III, IV, and V of catfish odorant receptors. These transmembrane domains are believed to represent ligand interaction sites, their structural divergence reflecting the requirement for these receptors to interact with a diverse array of agonists (Ngai et al. 1993a; Dixon et al. 1987). Southern blots performed
using probes representing six of the most divergent receptors revealed that each receptor gene lacks introns and defines a distinct chromosomal locus. Three members of the catfish 32 group were the only odorant receptor genes found to co-localize within a 30 kb DNA chromosomal location (Ngai et al. 1993a). RNase protection assays showed that the identified receptors were specifically expressed in olfactory epithelium (receptor 202 was also found in testes). *In situ* hybridizations demonstrated that each receptor subtype was expressed in a small number of ciliated ORNs, and that neurons expressing a specific subtype were distributed randomly throughout the olfactory epithelium (Ngai et al. 1993a; Ngai et al. 1993b).

The identification of the ORs in several species (Buck and Axel 1991; Ngai et al. 1993a; Sengupta et al. 1996; Clyne et al. 1999) revealed that ORs comprise the largest gene family identified to date, and with very few exceptions, lack introns (for reviews see Mombaerts 1999 and Firestein 2001). Mammals were proposed to have about 1000 individual OR genes; however, with the release of the mouse and human genomes, data mining efforts revealed that humans have 70% pseudogenes with 347 full-length ORs (Zozulya et al. 2001) and mouse has 20% pseudogenes with about 900 full-length ORs (Zhang and Firestein 2002). Catfish are predicted to express about 100 different ORs (Ngai et al. 1993a).

Given the size of the odorant receptor gene family, the selective expression of a particular odorant receptor by an individual ORN is a remarkable process that remains unclear. Information available for fish derives from work performed on the zebrafish *Danio rerio*. Odorant receptor expression during development in this species was observed to be non-coordinate even for those receptor genes that are tightly linked in the genome (Barth et
al. 1997). This non-coordinate expression is not observed in mammals. Chess et al. (1994) observed in mice that odorant receptor gene expression is monoallelic and can be derived from either the maternal or paternal allele. The authors suggest that based on this evidence, each allele is governed by stochastic cis regulation (Chess 1998). Three models have been proposed to explain how an ORN might express a single odorant receptor from the multitude of available candidate genes (reviewed by Kratz et al. 2002). The short promoter model is based on the sequences just upstream from the coding region of the receptor gene. Activation of transcription of a particular gene is regulated by selective transcription factor expression. This model seems unlikely in that it would require an equally large repertoire of transcription factors that would have to co-evolve along with the receptor genes. A second model is based on the presence of a locus control region (LCR). In this model, transcription factors bound to proximal promoters are unavailable to initiate transcription until additional factors bind to a distal LCR inducing the region to become transcriptionally active. Such factors could be temporally or spatially regulated. The recombination model proposes that selection of a receptor gene occurs by translocation to a single active locus for expression, similar to V(D)J recombination used in the immune system. This recombination event requires the activities of recombination activating proteins RAG1 and RAG2, both of which have been identified in zebrafish (Jessen et al. 2001); however, mammals express only RAG1, making this an unlikely system in this species.

Abundant evidence exists to support the assumption that a single olfactory receptor gene is expressed in a small population of ORNs (Ngai et al. 1993b; Bettina et al. 1999; Buck and Axel 1991). These data are derived mainly from mammalian systems. In contrast, Medler and Bruch observed coexpression of more than one odorant receptor per ORN in
catfish (unpublished data). The catfish olfactory system differs from the mammalian system not only in the number of odorant receptor genes, but also in the composition of the olfactory epithelium. The mammalian main olfactory epithelium (MOE) is composed primarily of ciliated ORNs, while a separate vomeronasal organ (VNO) is composed largely of microvillar ORNs. The VNO plays a key role in detecting pheromonal cues in the environment that govern mating behavior; however, the VNO also participates in odorant detection (Eisthen 1992; Dulka 1993). In contrast, channel catfish possess a single olfactory organ composed of both ciliated and microvillar ORNs (Erickson and Caprio 1984; Morita and Finger 1998). Ngai et al. (1993b) performed in situ hybridization experiments in catfish olfactory tissue using probes representative of the four odorant receptor subtypes identified in catfish and showed that the hybridization patterns obtained for each probe did not overlap with any others tested. The entire family of odorant receptors has not yet been identified in catfish and it seems premature to extend the results obtained from experiments employing 10% of the estimated population of odorant receptor genes to those members that remain to be examined. Identification of additional odorant receptor genes will be necessary to determine the expression pattern of these gene products in both the olfactory epithelium by in situ hybridization and in single olfactory neurons by single cell RT-PCR techniques.

Speca et al. (1999) identified an odorant receptor in goldfish (receptor 5.24) that preferentially responds to basic amino acids, and this receptor shares significant structural similarity with the metabotropic glutamate receptors (mGluRs), calcium sensing receptors (CaSR), and putative pheromone receptors. The amino acid receptor contains an extended amino-terminal, extracellular domain (~500 amino acids) characteristic of mGluRs that differs from the truncated amino-terminal domain (~40 amino acids) present in the catfish
odorant receptors identified by Ngai et al. (1993a). *In situ* hybridization experiments performed in goldfish olfactory epithelium with probes generated from the amino acid receptor clone revealed that the majority of the labeling was observed in microvillar ORNs, however, a small population of ciliated ORNs was labeled (Speca et al. 1999). The identification of goldfish 5.24 receptor was surprising, since it had been previously thought that receptors exhibiting such a large extracellular amino-terminal domain would mediate pheromonal responses.

As mentioned above, the catfish olfactory epithelium is composed of a variety of neuronal morphologies, and the work presented by Ngai et al. (1993b) and Speca et al. (1999) indicate that these different cell types express different types of odorant receptors. Evidence also exists indicating that neurons with different cellular morphologies synapse at different locations within the olfactory bulb. Morita and Finger (1998) showed that deposition of 1,1-dilinoleyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) in the ventral region of the posterior catfish olfactory bulb preferentially stained tall, ciliated ORNs by retrograde labeling. Deposition of DiI into dorsal portions of the posterior olfactory bulb preferentially labeled short, microvillous ORNs. These data reveal that at least some of the synapses formed between ORNs in the olfactory epithelium and mitral cells in the glomeruli of the olfactory bulb correlates with the neuronal morphology present in the olfactory epithelium. Since different regions of the olfactory bulb display sensitivity to different types of odorants (Mombaerts et al. 1996), the correlation of ORN morphology in the epithelium to preferential sites of synapse formation within the olfactory bulb may represent one stage in the process of odor recognition.
Surprisingly, odorant receptors may participate in synaptic formation between ORNs in the epithelium to particular glomeruli within the olfactory bulb. In mouse, the odorant receptor P2 was replaced with odorant receptor M12 by targeted mutagenesis. The neurons that were now expressing M12 did not project to the usual P2 glomerular targets, but instead synapsed to regions near the P2 glomeruli. This work, together with the observation that odorant receptors are expressed in ORN axons, identifies a role for odorant receptors in axon guidance; however, additional cues are required for proper synapse formation (Mombaerts et al. 1996). This additional role of odorant receptors in axon guidance is exciting in that the receptor proteins could coordinate odorant discrimination with synaptic formation in the bulb—the two main functions of ORNs, yet odorant receptors have yet to be localized to growth cones.

The data in Chapter 4 present evidence for the identification of a putative chemosensory receptor in catfish olfactory epithelium. Presently, there are only five full-length odorant receptor clones identified in catfish, and none of these receptors have been shown to functionally respond to odorant stimulation. The specific aim of the experiments conducted in Chapter 4 was to identify a catfish odorant receptor that mediates odorant detection. These experiments were prompted by the identification of the goldfish 5.24 basic amino acid receptor (Speca et al. 1999), since amino acids also represent powerful odorants for catfish. Obtaining a functional catfish odorant receptor clone would provide a mechanism to study olfactory signal transduction at the level of a single odorant receptor, provide information about axonal projections to the olfactory bulb for ORNs expressing this receptor, as well as information about receptor modulation.
MAPK SIGNALING

Mitogen activated protein kinase (MAPK) cascades are major components in signaling networks that mediate embryogenesis, cell differentiation, cell proliferation, and cell death (for recent reviews, Sweat 2001; Pearson et al. 2001; Adams and Sweat 2002). MAPKs were first identified in the yeast pheromone response pathway (Courchesne et al. 1989). The discovery of the extra-cellular receptor kinases (ERKs, Boulton et al. 1991), c-Jun N-Terminal kinases/stress-activated protein kinases (JNK/SAPKs, Kyriakis et al. 1994), p38 enzymes (Lee et al. 1994), as well as others revealed that these multiple kinase cascades displayed a variety of functions and were regulated distinctly. The JNK/SAPK cascade is associated with cellular stress signals. It can be activated by cytokines, agents that regulate DNA and protein synthesis, and many other stresses (Gupta et al. 1996). The p38 cascade is activated by cytokines, hormones, osmotic and heat shock, as well as other stresses (Rouse et al. 1994; Kultz 1998). The involvement of the ERK cascade in cell growth and proliferation has been the most studied function of this cascade (Hoshi et al 1988; Ahn and Krebs 1990), and both isoforms of ERK (ERK1 corresonds to the 42 kDa protein while ERK2 corresponds to the 44 kDa protein) were the first MAPKs to be cloned by Boulton et al. (1990). Given the variety of functional tasks, it is not surprising that the ERK module is evolutionarily conserved among species.

Each MAPK cascade is composed of a module of three kinases that sequentially phosphorylate one another resulting in an activated MAPK; thus, MAPK modules are comprised of an initial kinase called a MAPKKK, a second kinase called a MAPKK, and a third kinase called a MAPK. In the ERK module, Raf corresponds to the initial kinase in the module, MEK(1/2) is the second kinase, and ERK(1/2) is the third kinase in the module. In
most cases, initial activation of the MAPKKK is accomplished by other cellular kinases, such
as Ras, PKC, or Src (Rosen et al. 1994). Once Raf is phosphorylated on serine, it becomes
activated, and in turn phosphorylates MEK(1/2) on two serine residues. Phospho-MEK(1/2)
is a dual specificity kinase that concomitantly phosphorylates ERK(1/2) on a threonine and a
tyrosine residue. Phospho-ERK is a proline-directed serine/threonine kinase, preferring
proline at the +1 position within a potential substrate site. Potential ERK substrates include
transcription factors, such as Elk (Marais et al. 1993), or other downstream kinases.

The most well-defined pathway leading from an extracellular signal to ERK
activation is transduced by receptor tyrosine kinases (RTKs, for review Pawson and Scott
1997). This pathway involves activation of the RTK by ligand binding, receptor
dimerization, and autophosphorylation within the carboxy-terminal regions. The
phosphorylated tyrosine residues on the receptor dimer provide docking sites for SH2-
domain containing proteins. Binding of adaptor proteins, such as Shc and Grb2 to the
phosphotyrosines allows recruitment of SOS, a guanine nucleotide exchange factor, that
induces the exchange of GDP for GTP on Ras. Ras-GTP can then interact with Raf,
ultimately resulting in the activation of Raf, probably by localizing Raf to the plasma
membrane where it may become phosphorylated by kinases such as Src or PKC.
Phosphorylation of Raf induces the activation of the ERK module as described above.

Extra-cellular signals may also activate the ERK module through GPCR activation.
With the existence of multiple classes of G proteins, the ability of some receptors to bind to
more than one G protein, and the ability of GPCRs to activate RTK signaling, ERK
activation by this family of receptors is quite diverse. The mechanisms of activation used by
these receptors, including the cross-talk between them, to induce ERK activation are beyond
the scope of this introduction. There are several recent reviews that cover the overwhelming complexity of this topic (Schaeffer and Weber 1999; Cobb 1999; Pearson et al. 2001; Belcheva and Coscia 2002). A few pathways are presented here to illustrate how GPCRs accomplish ERK activation.

\( \text{G}_\alpha \)-mediated, cAMP ERK activation can be accomplished in neuronal cells through the activation of Rap1 by PKA phosphorylation (Wu et al. 1993). Rap1, in turn, activates the neuronal-specific B-Raf, leading to activation of the ERK cascade. In some cells, cAMP is associated with a reduction in ERK activation through the inhibitory effect of PKA on the ubiquitously expressed Raf1 (Vossler et al. 1997). \( \text{G}_i \)-mediated ERK activation is predominantly accomplished by the \( \beta_\gamma \) subunits (Hedin et al. 1999). The \( \beta_\gamma \) dimer is proposed to stimulate a Src family kinase activity that may then phosphorylate a tyrosine kinase receptor, such as PYK2 or FAK to create SH2 binding domains. The SH2-containing adaptor proteins (Shc, Grb2, SOS) would then assemble as described above for RTK signaling to culminate in Raf activation through Ras (Lopez-Illasaca et al. 1997). \( \text{G}_q \)-mediated signaling is usually dependent on PKC and may or may not involve Ras. \( \text{G}_q \) activation of PLC\( \beta \) produces IP\(_3\) and DAG that ultimately activate PKC. PKC may then phosphorylate Raf directly, initiating the ERK cascade (Kolch et al. 1993).

MAPK signaling is a complex process in that it occurs in the context of a multitude of extracellular signals. Several observations indicated that stimulation of a particular receptor was insufficient for eliciting a specific cellular response. For example, NIH 3T3 cells grown in suspension were unable to activate ERK after growth factor stimulation (RTK). It was observed that c-Raf1 was active under these conditions, but was unable to propagate activity to the downstream kinases MEK and ERK (Renshaw et al. 1997). The use of scaffolds and
adapters are one way that has evolved to specifically direct MAPK signaling cascades. Based on observations in yeast, scaffolding of MAPK modules organizes the components of the signaling module for efficient serial phosphorylation, restricts signal-crosstalk by only binding and organizing particular module members, and participates in localization of the modules to particular cellular regions (reviewed in Pearson et al. 2001).

Scaffolding molecules have been identified for the ERK module. MP1 is a 13 kDa protein that interacts with MEK1 within its proline-rich docking domain. This docking domain, located in the amino-terminal of MEK1 and MEK2, mediates specific interactions between these MAPKKs and ERK(1/2) (Catling et al. 1995). Interestingly, MP1 binds specifically to MEK1 and ERK1, resulting in the increased activation of ERK1 over ERK2 (Schaeffer et al. 1998). Such an interaction illustrates a potential site for regulating the activity of both ERK isotypes. Conversely, Raf kinase inhibitor protein (RKIP) appears to disrupt the interaction between Raf and MEK, shutting down activation of ERK (Yeung et al. 1999).

Once activated, ERK(1/2) phosphorylates a variety of cellular effectors. One such group of effectors is protein kinases. ERK has been shown to activate p90RSK, a ribosomal S6 kinase by phosphorylating Ser363 in the RSK activation domain. Once activated, RSK can phosphorylate targets involved in gene expression such as the transcription factors CREB and c-Fos (Xing et al. 1998; Chen et al. 1993). Interestingly, activation of RSK by ERK may lead to inhibitory feedback regulation of the module in that RSK can phosphorylate SOS, preventing activation of Ras (Douville and Downward 1997).

In neuronal cells, ERK activation has been associated with long-term potentiation (LTP), cellular memory, and synaptic plasticity (reviewed by Sweatt 2001; Adams and...
With regard to LTP, NMDA receptor-dependent LTP in CA1 neurons is divided into two phases: early which lasts about 60-90 min, and late, which is blocked by inhibitors of protein and RNA synthesis (English and Sweatt 1996). ERK association with late-LTP was identified pharmacologically with the use of MEK inhibitors—MEK inhibition blocked late-LTP. Conditioned taste aversion (CTA) occurs when an organism eats something that causes illness. After that exposure, the organism avoids eating the substance again since that food-stuff is associated with illness. Changes in the insular cortex region of the brain are necessary for mediating CTA. MEK inhibitors applied to this region through a stable infusion blocked CTA learning in rats (Berman et al. 1998). The authors went on to observe that activation of ERK led to phosphorylation of Elk1 in the insular cortex during CTA. This creates a direct link between chemosensory stimulation and transcription factor modulation.

Another interesting aspect of ERK signaling in neurons is the connection to learning. One attribute of most forms of learning is that the repetitive presentation of stimuli leads to the most robust learning (Adams and Sweatt 2002). Wu et al. (2001) observed that repetitively spaced membrane depolarization caused sustained ERK activation, and this sustained activation was associated with translocation of ERK to the nucleus along with concomitant structural changes in cultured hippocampal neurons. This observation suggests that the structural changes in the neurons were mediated by ERK activity. Similarly, dual stimulation of β-adrenergic receptors and muscarinic acetylcholine receptors in hippocampal neurons elicited ERK activation, while individual stimulation of either of these GPCRs was unable to activate ERK (Watabe et al. 2000). These observations link repetitive stimulation to ERK activation in neurons.
The specific aim of the work presented in Chapter 3 was to determine if odorant stimulation in catfish could initiate activation of the ERK cascade. Olfactory cues elicit particular behaviors in catfish (Sorenson and Caprio 1998); thus, various odorant stimuli are recognized by catfish and result in an appropriate response. Recognition of stimuli is a form of memory, and several processes involved in memory have been linked to ERK activation (Adams and Sweatt 2002). Since ERK activation is involved in a variety of cellular processes, it was important to study the effects of odorant stimulation on ERK activation where it would normally occur—in the olfactory epithelium. The data presented in Chapter 3 support most of the findings observed with primary cultures of rat olfactory neurons; however, the in vivo experiments performed in chapter 3 reveal that the pattern of stimulus presentation mimics the stimulation regime required for cellular memory processes, and that in the catfish olfactory system, ERK activation is modulated by PKC.

**G PROTEIN-COUPLED RECEPTOR INTERNALIZATION**

GPCRs initiate a variety of signaling pathways upon agonist binding and activation of G protein, and in the process, initiate events that result in their own desensitization. For many GPCRs, this process involves sequestration of the receptor and endocytosis, effectively removing the molecule from the plasma membrane, abolishing any additional ligand binding. Once internalized, receptors can be dephosphorylated and agonist removed for recycling to the membrane while others are targeted to lysosomes for degradation. One of the first steps in this process involves phosphorylation of the ligand-bound receptor by G protein receptor kinases (GRKs) after the release of G protein, termed homologous desensitization (Benovic et al. 1986). Seven mammalian GRK genes have been identified (Premont et al. 1995). Of these, GRK3/βARK2 has been identified in single catfish ORNs by RT-PCR (Bruch et al.
In the process of heterologous desensitization, active (ligand-bound) or inactive receptors become phosphorylated by second messenger kinases such as PKA or PKC. The effect that each type of kinase has on GPCRs varies, but inhibition of either kinase family results in the complete abolition of odorant receptor desensitization in rat (Schleicher et al. 1993; Boekhoff et al. 1994).

Phosphorylation of receptors by GRK alone is not sufficient for desensitization, rather, the phosphorylated receptor becomes a substrate for binding of arrestin proteins that do ensure desensitization (Wilden et al. 1986; Lohse et al. 1990). There are four mammalian arrestins, two of which are associated with the phototransduction pathway (visual and cone arrestins), and β-arrestin1/arrestin 2 and β-arrestin 2/arrestin 3 (Sterne-Marr and Benovic 1995). The latter two are ubiquitously expressed, but only β-arrestin2 has been found in olfactory epithelium (Dawson et al. 1993). Arrestins can bind GPCRs in any activation state. Arrestins bind GRK-phosphorylated GPCRs through the arrestin phosphorylation-recognition domain. Arrestins can also bind to activated GPCRs via the activation-recognition domain. When the GPCR is both activated and GRK-phosphorylated, arrestin can also bind to the receptor via the hydrophobic domain which increases the binding affinity of the other two domains (Gurevich et al. 1995).

The idea that GRK phosphorylation and arrestin binding was associated with receptor internalization was first proposed by Sibley et al (1986) when they observed that phosphorylation of the β2-adrenergic receptor (β2AR) resulted in removal of this receptor from the cell surface to internal vesicles. The receptors located in the vesicles became dephosphorylated and were recycled back to the cell surface. Others showed that mutant β2-
adenergic receptor that was unable to be phosphorylated by GRK was still able to internalize, and that this was accomplished by arrestin binding (Ferguson et al. 1995).

Subsequent research has shown that a membrane-associated phosphatase of the PP-2A family was present in internalized vesicles, and was active on phosphorylated β2-adrenergic receptor at acidic pH (Krueger et al. 1997). Removal of an agonist-stimulated GPCR from the plasma membrane to an intracellular compartment has been observed for a variety of other GPCRs in addition to the β2-adrenergic receptor (Ferguson et al. 1996; Claing et al. 2002). Currently there are three plasma membrane-associated regions that participate in receptor sequestration: clathrin-coated pits, caveolae, and a third that has no identified coat or adapter proteins associated with it (Claing et al. 2002).

Clathrin is a heterohexamer that forms a three-legged triskelion through the carboxy terminal association of three heavy chain proteins, each associated with a light chain (Pishvaee and Payne 1998). Adaptor proteins (AP) are heterotetramers comprised of four distinct subunits. AP-1 is localized to the trans-Golgi network, AP-2 is found at the plasma membrane, and AP-3 was first characterized in neuronal cells but is ubiquitously expressed (Le Borgne and Hoflack 1998). Clathrin and APs are the major constituents of clathrin coats. Coated vesicle formation is initiated with the binding of APs to appropriate membrane sites, in some cases through the recognition of sorting sequences present in transmembrane proteins (Trowbridge et al. 1993). In this way, APs select the cargo that will be included in the coated vesicle. Once located at the plasma membrane, APs bind clathrin and initiate polyhedral coat formation that drives membrane budding (Pishvaee and Payne 1998). β-arrestin 1 and 2 display high affinity binding (nM range) to clathrin cages in vitro (Goodman
et al. 1996), and β-arrestin 2 can bind to AP-2 directly (Laporte et al. 1999), providing a mechanism for translocation of GPCRs to clathrin-coated pits.

Dynamin is a large GTPase that plays an essential role in the release of endocytic vesicles from the plasma membrane to the interior of the cell. The GTPase activity is located in the amino-terminal region of dynamin. Dynamin also contains a pleckstrin homology domain that binds phosphoinositides as well as an assembly domain in the carboxy-terminal region that enables dynamin to form spiral oligomers (Hinshaw and Schmid 1995). Within the assembly domain, dynamin exhibits a proline-rich domain that can interact with proteins that contain SH3 domains. Dynamin colocalizes with clathrin through binding of α-adaptin (Wang et al. 1995). Dynamin was originally labeled as a “pinchase” that catalyzed the release of membrane invaginations, forming endocytic vesicles through GTP hydrolysis (Warnock et al. 1996). Current evidence suggests that this may not be the case. Stowell et al. (1999) have suggested that dynamin-GTP oligomerizes around the neck of the membrane invagination, and in this form, the dynamin structure is tightly compact. Activation of the GTPase activity causes a change in conformation in the dynamin oligomer such that it stretches like a spring, popping the neck of the invagination to form a vesicle. Others propose that dynamin does not directly catalyze vesicle formation, but activates another protein that performs this task (Kirchhausen 1999; Sever et al. 1999; Schmidt et al. 1999). Endophilin I has been proposed to be activated by dynamin, and once activated, converts lysophosphatidic acid LPA to PA. Such a change in the membrane phospholipid LPA would result in a change in the membrane curvature that would contribute to vesicle formation. Whatever the mechanism, dynamin is required for receptor endocytosis.
The variety of duties performed by clathrin-coated vesicles suggests that vesicles destined for different intracellular targets should be distinguishable. Cao et al. (1998) studied the endocytosis of two receptors via clathrin coated pits: the β2AR GPCR and the recycling nutrient transferrin receptor (TfnR, non-G protein-coupled). Their results showed that TfnR and β2AR were located in distinct populations of clathrin-coated pits that differ both functionally (temperature dependence for endocytosis) and biochemically (composition of coat-associated proteins). These distinct sites of endocytosis for each receptor resulted in different primary endocytic vesicles that fused within 10 minutes of formation with other endosomes that contained both b2-adrenergic and transferrin receptors. Oakley et al. (2001) observed that a particular cluster of serine residues in the carboxy-terminus of the vasopressin type 2 receptor promoted a stable interaction between the receptor and arrestin. Upon internalization, the vasopressin receptor was targeted to endosomes where they remained for long periods of time before being recycled to the cell surface. Chimeric receptors containing the vasopressin carboxy-terminus exhibited vasopressin recycling kinetics. Additional studies with β2AR reveal that there exist binding domains within the carboxy-tail region for the Na+/H+ exchanger regulatory factor (NHERF) (Cao et al. 1999) and NSF proteins (Cong et al. 2001). Ablation of binding of NSF or NHERF results in the trapping of the receptor in endocytic vesicles or mistargeting of the receptor, respectively. This evidence reveals that the duties performed by different receptor families are reflected in the composition of the clathrin-coated pit as well as in the receptor itself, and the combination of these factors result in vesicles that can be functionally distinguished during endocytosis.
Additional proteins have also been identified that interact with GRK or arrestin to modulate receptor internalization. The GRK-interactor proteins (GIT) are GAPs for the monomeric G protein ARF (Premont et al. 1998). Co-expression of GIT with β₁-adrenergic receptor, µ-opioid receptor, or adenosine 2B receptors impairs endocytosis (Claing et al. 2000). ARF6 has been correlated with a variety of cellular processes (reviewed in Claing et al. 2002), but with respect to endocytosis, ARF6-GTP was observed to be necessary for β₂AR internalization (Claing et al. 2001). There are six isoforms of ARF(1-6). The first five are located in intracellular locations, while ARF6-GTP is located at the plasma membrane. ARF6 is activated by its GEF ARNO—ARNO can directly interact with arrestin. ARF6 becomes associated with ARNO-arrestin upon arrestin binding to agonist-bound receptor. ARF6 is activated by ARNO to coordinate endocytosis of GPCRs and is promptly deactivated by the GAP GIT for vesicle budding.

In addition to mediating internalization of GPCRs, GRK, dynamin, and arrestin can also act as scaffolds for the assembly of additional proteins that participate in peripheral signaling pathways. Src has been shown to interact with β-arrestin 1 (Luttrell et al. 1999). Recruitment of Src to the plasma membrane has been shown to be involved in β₂AR endocytosis (Ahn et al. 1999) as well as participating in MAPK signal transduction (see previous section). Dynamin contains a proline-rich domain, as discussed above, that provides docking for SH3-containing proteins such as GRB2 and PLCγ (Seedorf et al. 1994). GRB2 plays a well-established role in the activation of Ras-dependent MAPK signaling cascades (previous section).

An alternative pathway for GPCR internalization is mediated by caveolae. Caveolae were originally identified as flask-shaped invaginations of the plasma membrane in
endothelial and epithelial cells (Bruns and Palade 1968), but caveolae can also exist as a flat planar structure within the plasma membrane, as detached intracellular vesicles, or in the form of grape-like structures that results from fusion of the intracellular vesicles (Smart et al. 1999). Caveolae are composed mainly of cholesterol and sphingolipids whereas non-caveolar regions are composed mainly of phospholipids. This unique composition renders caveolar structures resistant to detergent solubilization. Consequently, caveolae have been identified in the literature as detergent-insoluble glycolipid-rich membranes (DIGs), cholesterol-sphingolipid rafts, glycolipid-enriched membranes (GEMs), detergent resistant membranes, caveolin-enriched membranes, low-density Triton-insoluble domains, and caveola-like domains (Smart et al. 1999). Caveolins are proteins that define liquid-ordered domains as caveolae. For simplicity, I will refer to them as such and all other liquid-ordered domains lacking caveolins as caveolae-like domains.

Caveolae and caveolae-like domains are enriched in signaling molecules such as GPCRs, heterotrimeric G proteins, receptor tyrosine kinases (RTKs), components of the Ras-mitogen activated protein kinase pathway (MAPK), Src family of tyrosine kinases, PKCs, and nitric oxide synthase (NOS) (Smart et al. 1999). Such an accumulation of molecules enables caveolae and caveola-like domains to act as preassembled signaling complexes or chemical switchboards for integration of signal transduction cascades. Parton et al. (1994) showed that cholera toxin, which acts via caveolae by targeting GM1 gangliosides present in these domains, was internalized by a caveolae-mediated process distinct from clathrin-coated pits.

Caveolae-mediated internalization differs from the clathrin-mediated process in several ways. The caveolae-mediated pathway seems to be slower by approximately two- to
four-fold (Tran et al. 1987). The phosphatase inhibitor okadaic acid stimulates caveolae-mediated endocytosis but inhibits clathrin-coated vesicle formation (Parton et al. 1994). The sterol binding agent filipin has little effect on clathrin-mediated endocytosis, but inhibits caveolae-mediated internalization (Schnitzer et al. 1994). Given these differences, both caveolae and caveolae-related domains as well as clathrin-coated pits serve as docking sites for GPCRs and their modulators; and both methods of endocytosis involve cleavage of the invaginated vesicle from the plasma membrane by dynamin (Sweitzer and Hinshaw 1998; Oh et al. 1998). The particular mechanism of endocytosis utilized by a particular GPCR is probably dependent on the receptor itself as well as the cellular environment where it is expressed (Ferguson et al. 1996). While desensitization attenuates the responsiveness of the GPCR, evidence suggests that it is intimately involved in resensitization of the receptor; thus activation and desensitization should not be viewed only as opposing processes but as processes intimately linked in receptor modulation (Lefkowitz 1998).

GPCR endocytosis participates in desensitization/resensitization and downregulation of the receptors involved. For each receptor, the kinetics involved in the desensitization/resensitization pathway is different depending on the function of each individual receptor (Koenig and Edwardson 1997; Claing et al. 2002). For the β2-adrenergic receptor, endocytosis represents a means of removing a desensitized receptor from the cell surface to internal compartments where it can be dephosphorylated (resensitized) and recycled back to cell membrane (Krueger et al. 1997), while others are targeted to lysosomes where they are degraded. Little is known about what happens to odorant receptors once the primary olfactory response is initiated. The data presented in chapter 2 presents evidence
that there is a functional internalization pathway in dissociated catfish olfactory neurons, and that this process is mostly likely mediated by clathrin-coated pits.
CHAPTER 2: INTERNALIZATION OF G PROTEIN-COUPLED RECEPTORS IN SINGLE OLFAC TORY RECEPTOR NEURONS*

INTRODUCTION

After ligand binding, many G protein-coupled receptors are phosphorylated and internalized by a clathrin-dependent, receptor-mediated endocytic pathway (for review, see Ferguson et al., 1997). G protein-coupled receptor kinases phosphorylate agonist-bound receptors and trigger a series of molecular events leading to receptor desensitization and internalization. Receptor phosphorylation is followed by arrestin binding to the ligand-bound, phosphorylated receptor, thereby uncoupling the receptor from G proteins. Arrestin also acts to target the ligand-receptor-arrestin complex to clathrin-coated pits. Internalization by clathrin-coated endocytic vesicles also requires the participation of the GTPase dynamin, which acts to pinch off internalized vesicles (Sweitzer and Hinshaw 1998). Clathrin-coated vesicles eventually fuse with endosomes where ligand is separated from the receptor and the receptor is dephosphorylated. Receptors are then recycled to the plasma membrane by a poorly understood mechanism, or are degraded in lysosomes. Although the predominant internalization mechanism for G protein-coupled receptors appears to involve a clathrin-dependent pathway, some G protein-coupled receptors may also be internalized by an alternative pathway mediated by caveolae (Roettger et al. 1995). Caveolae are plasma membrane invaginations that contain the structural protein caveolin and act as molecular scaffolds that provide binding sites for a variety of signaling proteins including G protein-coupled receptors (for review, see Okamoto et al. 1998).

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Olfactory receptor neurons from channel catfish express members of the multigene G protein-coupled odorant receptor family (Ngai et al. 1993a). We have also recently shown that these neurons express at least two metabotropic glutamate receptor subtypes (Medler et al. 1998). We have also shown that these neurons express a G protein-coupled receptor kinase that shares 92% amino acid sequence identity to the mammalian β-adrenergic receptor kinase (βARK2) (Bruch et al. 1997b). The expression of βARK2 in these cells was consistent with previous data showing that βARK2 immunoreactivity was localized in the dendrites and cilia of rat olfactory receptor neurons (Dawson et al. 1993). A βARK2-specific antibody also prevented termination of odorant-stimulated responses in isolated rat olfactory cilia (Schleicher et al. 1993). In addition, the translocation and activation of βARK in isolated rat olfactory cilia were mediated by G protein βγ subunits (Boekhoff et al. 1994). Catfish olfactory receptor neurons express at least two G protein β (β1 and β2) and γ (γ2 and γ3) subunits (Bruch et al. 1997a). All possible dimeric combinations of these subunits are capable of activating βARK (Simonds 1994).

It is clear that the expression of βARK in olfactory receptor neurons is a consistent finding from various laboratories. However, it has been reported that complete desensitization of odorant responses may require the sequential action of second messenger-dependent protein kinases as well as βARK (Schleicher et al. 1993). In catfish, odorant amino acids stimulate the inositol phospholipid signaling pathway (Bruch 1996), thus implicating protein kinase C (PKC) as the relevant second messenger-dependent protein kinase in this system. In a previous study in catfish, the calcium-sensitive PKCβ isotype was shown to be expressed in olfactory neurons and was implicated in mediating odorant-stimulated phosphorylation in isolated olfactory cilia (Bruch et al. 1997b).
In addition to βARK and second messenger-dependent protein kinases, olfactory receptor neurons also express β-arrestin. Like βARK, β-arrestin 2 immunoreactivity was localized in the dendrites and cilia of rat olfactory receptor cells. Furthermore, a β-arrestin 2 antibody attenuated odorant-dependent desensitization in isolated cilia (Dawson et al. 1993). Thus, the molecular components required for initiation of desensitization and internalization of many G protein-coupled receptors are expressed in olfactory receptor neurons. In this report, we show that clathrin and dynamin, additional downstream molecular components known to be required for internalization of many G protein-coupled receptors (Ferguson et al. 1997), are also expressed in olfactory receptor neurons. In addition, using the activity-dependent fluorescent dye FM1-43 to monitor internalization in single neurons stimulated with odorant amino acids, we provide evidence that suggests that a clathrin-dependent receptor internalization pathway is functional in olfactory receptor neurons.

MATERIALS AND METHODS

Materials

Mouse monoclonal antibody to clathrin heavy chain, mouse monoclonal antibody to dynamin 1, and rabbit polyclonal antibody to caveolin were obtained from Transduction Laboratories (Lexington, KY, U.S.A.). Cyanine Cy3-conjugated goat anti-mouse antibody was obtained from Chemicon International (Temecula, CA, U.S.A.). FM1-43 was obtained from Molecular Probes (Eugene, OR, U.S.A.). Okadaic acid and glucose were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). The group I metabotropic glutamate receptor antagonist (S)-4-carboxyphenylglycine (S-4CPG) was obtained from Tocris (Ballwin, MO, U.S.A.). NaCl was obtained from Fisher Scientific (Fail Lawn, NJ, U.S.A.). KCl, MgCl₂, and CaCl₂ was obtained from Curtin Matheson Scientific (Houston, TX, U.S.A.) and HEPES
was purchased from Amresco (Solon, OH, U.S.A.). TetraMin was purchased from Wal-Mart distributed by Tetra Sales (Blacksburg, VA, U.S.A.) and 0.2 µm Nalgene filter syringes were obtained from VWR Scientific (Plainfield, NJ, U.S.A.).

**Western Blotting and Immunocytochemistry**

Western blotting was performed as described previously (Bruch and Abogadie 1995). For Western blotting, the clathrin and dynamin 1 antibodies were used at 1:1,000 dilution and the caveolin antibody was used at 1:5,000 dilution. Immunocytochemistry with isolated neurons was performed by indirect immunofluorescent microscopy (Gleason et al. 1993). Isolated neurons were plated on polyornithine-coated (0.1 mg/ml) Petri dishes and allowed to adhere to the dishes for 1 h on ice. Cells were then fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 1 h on ice. After three washes for 10 min each with PBS, cells were incubated with 5% normal goat serum in PBS for 1 h at room temperature. After removal of the goat serum solution, cells were incubated with primary antibodies to clathrin heavy chain and dynamin 1 for 1 h at room temperature. Primary antibodies were diluted in PBS containing 1% bovine serum albumin and 0.1% saponin and were used at 1:100 dilution for the clathrin antibody and at 1:500 dilution for the dynamin 1 antibody. After three washes for 10 min each in PBS, cells were incubated with Cy3-conjugated goat anti-mouse secondary antibody diluted 1:500 in PBS containing 1% bovine serum albumin and 0.1% saponin for 1 h at room temperature. Negative controls consisted of similarly processed cells that were exposed to secondary antibody only. After washing with PBS as before, cells were covered with mounting media (2% n-propyl gallate, 70% glycerol, and 28% PBS). Immunoreactivity was visualized by the fluorescence microscopy, using a 510-550-nm excitation band-pass filter on an Olympus IX70 inverted microscope.
PCR Amplification of Dynamin 1

Total RNA was isolated and reverse transcribed as described previously (Bruch and Medler 1996). The resulting cDNA from rat olfactory tissue was used in the PCR to amplify a 500-bp dynamin fragment as described by Cook et al. (1994). Negative controls consisted of similarly processed samples that lacked reverse transcriptase. These controls did not produce a 500-bp PCR product. The PCR product was gel purified and cloned into pCR2.1-TOPO (Invitrogen Corporation, Carlsbad, CA, U.S.A.). Identification of the PCR product as dynamin was performed by DNA sequencing on both strands and comparison of the sequence data with sequences in the databanks.

Visualization of Internalization by Fluorescence Microscopy

Olfactory rosettes were enzymatically dissociated by the method of Restrepo and Teeter (1990) as described previously (Bruch and Medler 1996). All procedures involving vertebrate animals were approved by the Louisiana State University Institutional Animal Care and Use Committee. Olfactory receptor neurons were identified by their characteristic bipolar morphology by using phase-contrast optics (Restrepo and Teeter 1990; Bruch and Medler 1996). Cell viability was estimated to be 80-90% by using trypan blue exclusion. Aliquots (~1 ml) of the cell suspension were transferred to 35-mm Petri dishes coated with polyornithine. The activity-dependent fluorescent dye FM1-43 was added to isolated cells at a final concentration of 1 μM. After 15 min at room temperature, L-glutamate was added to the cells at a final concentration of 1 mM. Isolated neurons were similarly stimulated with 1 mL of TetraMin extract instead of 1 mM L-glutamate. TetraMin extract was prepared by adding 2.5 g Tetramin to 50 mL of standard solution (121 mM NaCl, 3 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂, 10 mM HEPES, pH 7.6, and 15 mM glucose) and incubating this in a
37°C shaking incubator for 1 hr. The suspension was then centrifuged at 20,000xg for 20 min and filtered through a 0.2 μm Nalgene syringe filter prior to use. After a 45-min incubation at 37°C, the cells were perfused with 15 ml of fish Ringer’s solution over a period of several minutes to remove unincorporated FM1-43 and examined under phase contrast and fluorescence microscopy to visualize internalization. The effect of okadaic acid (5 µM) was determined by exposing cells to okadaic acid for 30 min at 37°C. The effect of the metabotropic glutamate receptor antagonist S-4CPG was assessed by pretreating cells with antagonist (0.1 mM) for 30 min at 37°C before stimulation with L-glutamate or the TetraMin extract, and was present throughout the experiment.

**Electro-olfactogram (EOG) Recording**

EOG analysis was performed by James Parker, Department of Biological Sciences, Louisiana State University. The EOG was recorded *in vivo* using calomel electrodes via Ringer’s-agar-filled capillary pipettes with tip diameters of 100-500 μm (Silver et al. 1976; Caprio 1995). Catfish were prepared as described above. The pipette of the active electrode was positioned near the midline raphe of the olfactory rosette at a location that maximized the EOG response; the pipette of the reference electrode was placed against the skin adjacent to the olfactory cavity. The fish was grounded via a hypodermic needle inserted into the flank musculature. The olfactory rosette was stimulated with 10⁻⁴ M L-alanine as a reference and a 1:100 dilution of TetraMin extract in charcoal-filtered tap water. The EOG was amplified (Grass P-18; Astro-Med Inc., West Warwick, RI, U.S.A.), displayed on an oscilloscope, digitized (Model DR-390, Neuro Data Instruments Corp.), stored on a video channel of a Hi-fi video recorder, and recorded on a chart recorder for visual analysis.
RESULTS

Identification and Localization of Clathrin and Dynamin in Olfactory Neurons

The expression of clathrin and dynamin proteins in olfactory neurons was initially investigated by Western blotting. When Western blots were probed with a monoclonal antibody to clathrin heavy chains, a single 180-kDa immunoreactive polypeptide was identified in isolated olfactory cilia preparations and in deciliated epithelium homogenates (Fig. 2.1). The 180-kDa polypeptide comigrated with a clathrin positive control, suggesting that it corresponded to clathrin heavy chain. Dynamin expression was initially investigated by PCR analysis of olfactory tissue to determine the specific dynamin isotype found in this tissue. A 500-bp PCR product was amplified from olfactory tissue that was 94% identical at

Figure 2.1. Western blot of clathrin. Five micrograms of protein from a HeLa cell lysate (lane H), 50 µg of protein from deciliated olfactory epithelium (lane D), and isolated cilia (lane C) were loaded on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose. The HeLa cell lysate was provided by the vendor as a positive control with the clathrin antibody. The numbers to the left of the blot indicate molecular mass markers in kilodaltons.
the amino acid level to dynamin 1 (Fig. 2.2). Western blots probed with a dynamin 1 antibody showed that an immunoreactive 80-kDa polypeptide that comigrated with a dynamin 1 positive control, was also found in olfactory tissue (Fig. 2.3). The 80-kDa polypeptide corresponds to a proteolytic fragment of dynamin that lacks the proline-rich domain (Sweitzer and Hinshaw 1998). The nature of the lower molecular weight polypeptide that was also recognized by the dynamin antibody is not known, but it may also represent a proteolytic fragment. As the Western blots showed that both antibodies were specific for their respective antigens, localization of clathrin and dynamin was performed by immunocytochemistry with isolated olfactory neurons. Both clathrin (Fig. 2.4) and dynamin (Fig. 2.5) immunoreactivities were found in the cell bodies, dendrites, and dendritic knobs of the neurons. It was not possible to visualize localization of clathrin and dynamin in receptor cell cilia or axons under the conditions of these experiments.

**Figure 2.2.** Predicted amino acid sequence of dynamin 1 PCR product amplified from olfactory tissue RNA. The upper sequence is rat dynamin 1 (DYN1) and corresponds to EMBL accession no. X54531. The lower sequence is the dynamin 1 fragment amplified from olfactory epithelium (OE DYN). Differences between the sequences are shown in boldfaced type.
**Figure 2.3.** Western blot of dynamin 1. Five micrograms of protein from a PC12 cell lysate (+ lane) were loaded on a 10% sodium dodecyl sulfate-polyacrylamide gel together with 50 µg of protein from brain (fish brain lane) and olfactory epithelium (FOE lane) homogenates. The PC12 cell lysate was provided by the vendor as a positive control with the dynamin 1 antibody. Numbers to the left of the blot indicate molecular mass markers in kilodaltons.

**Visualization of Receptor Internalization by Fluorescence Microscopy**

The identification of clathrin and dynamin in olfactory neurons suggested that a receptor internalization pathway involving these proteins might be functional in olfactory receptor neurons. To test this hypothesis, isolated olfactory neurons were treated with the fluorescent dye FM1-43 and subsequently stimulated with odorant amino acids. FM1-43 inserts into the plasma membrane but does not cross the membrane. Because membrane-associated dye is easily removed by washing, the retention of cellular fluorescence after washing indicates that the dye has been internalized in endocytic vesicles (Betz et al. 1992; Ryan et al. 1993). As our recent study suggested that metabotropic glutamate receptors were expressed at relatively high levels in catfish olfactory neurons (Medler et al. 1998), L-glutamate was used as a receptor ligand. Based on preliminary experiments, the stimulus...
Figure 2.4. Localization of clathrin immunoreactivity in olfactory neurons by indirect immunofluorescence microscopy. Panels A show three individual neurons, using phase-contrast optics. Panels B show the same neurons as panels A, after labeling with clathrin antibody visualized by fluorescence microscopy. Clathrin immunoreactivity was localized in the cell bodies, dendrites, and dendritic knobs of the neurons. Panels C show three individual neurons from the same preparation used as negative controls, using phase-contrast optics. Panels D show the same neurons as panels C after immunocytochemistry in the absence of the primary antibody, using fluorescence microscopy. The scale bar is 50 µm.
Figure 2.5. Localization of dynamin 1 immunoreactivity in olfactory neurons by indirect immunofluorescence microscopy. Panels A show three individual neurons, using phase-contrast optics. Panels B show the same neurons as panels A, after labeling with dynamin 1 antibody visualized by fluorescence microscopy. Dynamin 1 immunoreactivity was localized in the cell bodies, dendrites, and dendritic knobs of the neurons. Panels C show three individual neurons from the same preparation used as negative controls, using phase-contrast optics. Panels D show the same neurons as panels C after immunocytochemistry in the absence of primary antibody, using fluorescence microscopy. The scale bar is 50 µm.
concentration and time of exposure to the stimulus were chosen to give maximal internalization. After L-glutamate stimulation, isolated olfactory neurons were extensively perfused, as described in Materials and Methods, to remove unincorporated dye. Cells exposed to L-glutamate became fluorescent, indicating that the dye had been internalized in an agonist-dependent manner. In several independent experiments, numerous neurons showed a consistent pattern of fluorescence in the cell bodies and dendritic knobs (Fig. 2.6). This pattern of fluorescence suggested specific internalization in response to receptor stimulation. In addition, neurons that were not stimulated with L-glutamate were not fluorescent (Fig. 2.6). To confirm that L-glutamate was acting as a specific receptor ligand, cells were pretreated with the metabotropic glutamate receptor antagonist S-4CPG. Neurons treated with the antagonist exhibited significantly reduced fluorescence compared with control cells not treated with the antagonist (Fig. 2.6). To test if other odorant stimulation could also induce FM1-43 retention, a TetraMin extract (Wellerdieck et al. 1997) was used to activate other olfactory receptor neurons (ORNs) not sensitive to L-glutamate stimulation. The TetraMin extract was tested by electro-olfactogram (EOG) analysis relative to L-alanine and was observed to be an effective odorant (Fig. 2.7). Stimulation of dissociated neurons with TetraMin extract in the presence of S-4CPG also displayed FM1-43 dye retention, indicating that other odorant stimuli in addition to L-glutamate can induce internalization (Fig. 2.8). This result also indicated that receptor activation was responsible for FM1-43 internalization.

Additional experiments were performed to determine whether receptor internalization was specifically mediated by a clathrin-dependent pathway. Hypertonic medium has been reported to inhibit clathrin-dependent endocytosis in some cells (Heuser and Anderson 1989;
Figure 2.6. Visualization of FM1-43 internalization in olfactory neurons. Panels A show two neurons by phase-contrast optics (first and third panels) and by fluorescence microscopy (second and fourth panels) after stimulation with L-glutamate. Note FM1-43 fluorescence in the cell bodies and dendritic knobs. Panels B show two neurons by phase-contrast optics (first and third panels) and by fluorescence microscopy (second and fourth panels) that were not stimulated with L-glutamate. Note the lack of fluorescence in these cells. Panels C show two neurons by phase-contrast optics (first and third panels) and by fluorescence (second and fourth panels) that were pretreated with S-4CPG and then stimulated with L-glutamate. Note that FM1-43 fluorescence is markedly decreased in these cells compared with the cells shown in panels A. The scale bar is 50 µm.

Slowiejko et al. 1996). However, exposure of olfactory receptor neurons to hypertonic media containing various concentrations of sucrose (200-450 mM) resulted in altered morphology even after a brief (15 min) exposure. Olfactory neurons in hypertonic media exhibited enlarged cell bodies and loss of a well-defined olfactory knob (data not shown).
Figure 2.7. EOG analysis of TetraMin extract. Peak A represents the response to L-alanine at $10^{-4}$ M and peak B represents the response to a 1:100 dilution of TetraMin extract. The TetraMin extract induces a similar EOG response as the well-established L-alanine odorant.

These cells also exhibited bright uniform fluorescence throughout the cells in the absence of L-glutamate, suggesting that dye entered the cells by a nonspecific mechanism.

As some G protein-coupled receptors may be internalized by a clathrin-independent pathway that uses caveolae (Roettger et al. 1995), the possible role of a caveolae-mediated receptor internalization pathway in olfactory receptor neurons was also investigated. Okadaic acid has been reported to activate internalization by caveolae (Parton et al. 1994). Treatment of olfactory receptor neurons with okadaic using the conditions described by Parton et al. (1994) did not induce FM1-43 internalization (data not shown), suggesting that a caveolae-mediated internalization pathway may be absent in olfactory neurons. However, a 23 kDa polypeptide that was immunoreactive with an antibody to caveolin was detected by Western blotting of rat olfactory epithelium homogenates; however, the antibody did not cross-react with catfish proteins (Fig. 2.9). It is therefore possible that a caveolae-mediated receptor internalization pathway exists in olfactory tissue but may be localized in non-neuronal cells.
Figure 2.8. Visualization of FM1-43 internalization in olfactory neurons using TetraMin extract. Column A shows four neurons by phase-contrast optics and column B shows these same neurons by fluorescence microscopy pretreated with S-4CPG and stimulated with TetraMin in the presence of FM1-43. Note the fluorescence in the dendrites and cell bodies of the first three cells. The last cell probably does not express the appropriate receptor for odorant stimulation. Column C depicts four neurons by phase-contrast optics and column D represents the same four neurons as in C by fluorescence microscopy. In column D, neurons were treated with S-4CPG and FM1-43 as in A and B; however, were not stimulated with TetraMin extract. Note the lack of fluorescence in the neurons in column D. The scale bar represents 50 µm.
Figure 2.9. Western blot of caveolin. Five micrograms of protein from a human endothelial cell lysate (+ lane) were loaded on a 15% sodium dodecyl sulfate-polyacrylamide gel. Fifty micrograms of protein from rat brain, rat olfactory epithelium (ROE lane), catfish brain, and catfish olfactory epithelium (FOE lane) homogenates were also analyzed. The human endothelial cell lysate was provided by the vendor as a positive control with the caveolin antibody. Numbers to the left of the blot indicate molecular mass markers in kilodaltons.

DISCUSSION

Previous studies have shown that a G protein-coupled receptor kinase and arrestin are expressed in rat and catfish olfactory receptor neurons (Dawson et al. 1993; Schleicher et al. 1993; Bruch et al. 1997b). Using subtype-selective antibodies, Dawson et al. (1993) also showed that βARK and β-arrestin 2 antibodies attenuated desensitization of odorant responses in isolated olfactory cilia preparations. These combined observations suggest that olfactory receptor neurons express the molecular components required for initiation of receptor internalization by βARK-mediated phosphorylation and for targeting of the phosphorylated receptors to clathrin-coated pits by arrestin (Ferguson et al. 1997). We have extended these previous observations by identifying clathrin and dynamin in olfactory neurons, additional downstream molecular components required for a clathrin-dependent
receptor internalization pathway (Ferguson et al. 1997). Thus, olfactory receptor neurons express βARK, arrestin, clathrin and dynamin, four major components of a clathrin-dependent receptor internalization pathway. In this regard, it is noteworthy that these cells appear to express the neuronal dynamin 1 isotype (Nakata et al. 1991). Dynamin 1 GTPase activity is modulated by phosphoinositide lipids (Barylko et al. 1998). Dynamin 1 is also a substrate for PKC, and its translocation to and association with the plasma membrane are modulated by calcium (Liu et al. 1994). Thus, considerable potential exists for regulation of dynamin membrane association and GTPase activity, and hence, for regulation of receptor internalization. This may be particularly relevant in catfish olfactory receptor neurons, as the predominant response to odorant amino acids in these cells is the generation of phosphoinositide-derived second messengers (Bruch 1996). In addition, catfish olfactory neurons express the calcium-sensitive PKCβ isotype (Bruch et al. 1997b) and often respond to odorants by increasing intracellular calcium (Restrepo et al. 1990).

As the major molecular components required for a clathrin-dependent receptor internalization pathway were expressed in olfactory receptor neurons, we also investigated the possibility that such a pathway might be functional in these cells. Using the activity-dependent fluorescent dye FM1-43 (Betz et al. 1992), we were able to demonstrate that an odorant amino acid, L-glutamate, as well as a complex odorant extract derived from TetraMin, induced internalization of the dye by the neurons. As we wished to visualize internalization using the native complement of receptors in these cells without receptor over-expression, a high stimulus concentration was used in these experiments to saturate the receptor binding sites. Neurons stimulated with L-glutamate and TetraMin extract exhibited a consistent pattern of fluorescence in their cell bodies and dendritic knobs, consistent with
the conclusion that internalization was due to a specific receptor-mediated mechanism. The time course of FM1-43 internalization was also similar to that observed for other G protein-coupled receptors expressed in heterologous cells (see Kallal et al. 1998). Fluorescence was not observed in neurons in the absence of odorant stimuli, suggesting that dye internalization was due to receptor activation. This conclusion was further strengthened by the significant decrease in L-glutamate-induced cell fluorescence in the presence of S-4CPG. In a previous study, we showed that the metabotropic glutamate receptor group 1 subtype was expressed in the dendritic knobs and cilia of catfish olfactory neurons and that S-4CPG significantly decreased the \textit{in vivo} electrophysiological response to L-glutamate (Medler et al. 1998). Thus, the coincidence of metabotropic glutamate receptor protein expression in the dendritic knobs of the receptor cells and the L-glutamate-induced fluorescence in this region of the cells is consistent with the conclusion that FM1-43 cell-associated fluorescence was due, at least in part, to activation of metabotropic glutamate receptors. The effect of S-4CPG on cell-associated fluorescence is also consistent with this conclusion. As S-4CPG is a specific group I metabotropic glutamate receptor antagonist (Conn and Pin 1997), it is unlikely that L-glutamate-induced FM1-43 internalization was due to activation of ionotropic glutamate receptors. However, we cannot rule out the possibility that odorant receptors that recognize L-glutamate were also activated. In preliminary experiments, neurons exposed to a mixture of three odorant amino acids (L-alanine, L-arginine, and L-cysteine, each at 1 mM) did not exhibit FM1-43 fluorescence after perfusion. This result suggests that our methodology was not sensitive enough to visualize internalization of odorant receptors due to their low expression levels (Ngai et al. 1993a) and the inability to visualize the receptor cell cilia under the conditions of these experiments. An alternative possibility is that the cells stimulated
with the odorant mixture did not express the appropriate subset of odorant receptors. TetraMin extract was used to overcome this problem by increasing the probability that a greater number of odorant receptors would be activated due to the larger number of odorant ligands present in the extract. This extract has been used successfully as an odorant stimulus by Wellerdieck et al. (1997). Stimulation of dissociated olfactory neurons with the TetraMin extract induced FM1-43 dye retention in the majority of neurons tested. It is also possible that desensitization of odorant receptors involved an alternative pathway that is not dependent on clathrin and dynamin. Ongoing experiments are addressing these possibilities.

Several experiments were performed to attempt to inhibit clathrin-dependent internalization. Hypertonic medium has been shown to inhibit clathrin-dependent internalization in fibroblasts (Heuser and Anderson 1989) and in neuroblasts (Slowiejko et al. 1996). However, hypertonic media containing various concentrations of sucrose presented to olfactory neurons resulted in altered morphology and loss of the consistent pattern of cell-associated fluorescence observed in the absence of hypertonic media. Neurons exposed to hypertonic media exhibited enlarged cell bodies, suggesting that hypotonic media may stimulate exocytosis in these neurons. This suggestion is consistent with the stimulation of exocytosis by hypertonic medium in hippocampal neurons (Stevens and Tsujimoto 1995). The uniform fluorescence exhibited by olfactory neurons in hypertonic media also suggested that FM1-43 was internalized by a non-receptor-mediated mechanism.

As some G protein-coupled receptors may be internalized by an alternative pathway that depends on caveolae (see Roettger et al. 1995), we also investigated the possibility that such a pathway might exist in olfactory receptor neurons. Western blotting showed that a 23-kDa polypeptide that was immunoreactive to a caveolin antibody was present in rat olfactory
epithelium homogenates. However, okadaic acid, an activator of caveolae-mediated internalization (Parton et al. 1994), had no obvious effect on FM1-43 internalization in olfactory neurons. These observations suggest that a caveolae-mediated pathway may be present in olfactory epithelium but may be associated with cell types other than olfactory neurons. An alternative possibility is that the caveolae pathway is present in olfactory neurons at low levels that could not be detected by our methodology. Irrespective of these possibilities, our data are most consistent with the conclusion that G protein-coupled receptors are internalized primarily by a clathrin-dependent pathway in olfactory receptor neurons.
INTRODUCTION

Odorant stimulation of the olfactory epithelium involves binding of the odorant to G protein-coupled receptors located in the cilia or microvilli of olfactory neurons located in the sensory region of the epithelium. In catfish, the odorant-bound receptor probably activates G_q, that in turn initiates the inositol phospholipid second messenger pathway (Bruch 1996). This pathway results in depolarization of the neuron and the generation of action potentials, relaying odorant-encoded information to the olfactory bulb and higher centers of the brain. This sequence of events comprises the primary olfactory response, conveying odorant information within the environment to the organism and results in odor recognition.

ORNs located in the olfactory epithelium are the sites where the primary olfactory response is initiated. In contrast to other neurons, ORNs are regenerated throughout the lifespan of the organism, and this turnover is initiated by both genetic and environmental cues (Hinds et al. 1984, Evans and Hara 1985, Saucier and Astic 1995). In addition, most mature ORNs continue to express juvenile forms of microtubule associated proteins (Viereck et al. 1989) with only a very small fraction of these neurons expressing proteins typically found in intermediate filaments of mature neurons (Schwob et al. 1986). Synaptic activity with the olfactory bulb is also necessary for maturation of developing neurons, since the development of cilia is incomplete in cells lacking direct contact with the bulb (Hinds et al. 1984). Maintenance of the ORN population in the olfactory epithelium is most likely controlled locally since the thickness of the epithelium and the rate of proliferation can vary within an individual animal (Farbman 1990).
MAPK cascades are evolutionarily conserved among eukaryotes and participate in a variety of cellular mechanisms including growth, differentiation, movement, cellular division, and cell death (for reviews see Lewis et al. 1998 and Pearson et al. 2001). MAPKs represent a family of proteins that are organized into modules that consist of at least three members. Each member of the module is serially linked and regulates one another by sequential phosphorylation. The third member of the module is the MAPK and this kinase, once dually phosphorylated on adjacent threonine and tyrosine residues, goes on to phosphorylate various cellular effectors such as protein kinases (RSK and MNK; Zhao et al. 1996; Waskiewicz et al. 1997) and transcription factors (Elk, c-Jun, c-Fos, and ATF-2; Hibi et al. 1993; Chen et al. 1993; Gupta et al. 1995), as well as other membrane and cytoplasmic substrates. There are currently 15 MAPK family members in Caenorhabditis elegans and 20 mammalian members of which ERK, p38, and c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK) comprise the better defined constituents (Plowman et al. 1999).

The ERK signaling system has been extensively studied in the regulation of cell proliferation (Hoshi et al. 1988) and is composed of Raf (MAPKKK), MEK (MAPKK), and ERK1/2 (p44 MAPK and p42 MAPK, respectively). ERK1 and ERK2 are ubiquitously expressed, however, the relative abundance of each protein varies among different cell types. In immune cells, ERK2 is the predominant species but in neuroendocrine cells both isotypes are equally expressed (Pearson et al. 2001; Lewis, et al. 1998). ERKs are also present in mature neurons of the central nervous system where this signaling cascade has been harnessed to participate in synaptic plasticity and memory (reviewed in Sweatt 2001). Of particular interest is the participation of ERK in conditioned taste aversion (CTA). This response describes the lifelong aversion an organism develops in response to eating a new
food source that causes the animal to become ill (Gallo et al. 1992; Berman et al. 1998).

CTA provides a link between chemosensory information and molecular processes that result in long-term behavioral changes, and thus provide a possible link of ERK to olfaction.

Several observations in mammals indicate that ORNs display neuronal plasticity when exposed to odorant stimuli (Wang et al. 1993; Dittman et al. 1997). The primary olfactory response in mammals is a cAMP-mediated process that opens cyclic nucleotide-gated channels (CNGCs) leading to membrane depolarization and action potential generation (Nakamura and Gold 1987). Indeed Watt and Storm (2001) were able to show in primary cultures of rat ORNs that odorant stimulation induced ERK phosphorylation. They also showed that the signaling cascade of Raf, MEK, ERK, and RSK 2 were all sequentially activated upon odorant stimulation, and that this activity was diminished with inhibition of CaMKII, but unaltered with PKA, PKC, and PKG inhibitors. Odorant stimulation of transgenic mice with the lacZ operon under cAMP response element (CRE) promoter control displayed beta-galactosidase activity, indicating that cAMP response element binding protein (CREB) was an ultimate substrate of ERK activation.

Studies in Pacific Coho salmon showed that juveniles exposed to phenylethyl alcohol displayed a preference for this compound and an increased guanylyl cyclase activity in olfactory cilia upon exposure to the compound two years later (Dittman et al. 1997). This suggests that odorant information is stored in cellular memory and that an ERK pathway similar to that described in mammals may be active in fish. In this report, we present data showing that odorant stimulation activates the ERK signaling cascade in catfish olfactory epithelia in vivo, and that the signaling cascade in catfish differs from that observed in primary cultures of rat olfactory neurons.
MATERIALS AND METHODS

Materials

Juvenile channel catfish were supplied by Dr. John Hawke from the LSU School of Veterinary Medicine or were raised in floating cages held in ponds at the Louisiana State University Aquaculture Center (Baton Rouge, LA, U.S.A.). All procedures involving vertebrate animals were approved by the Louisiana State University Institutional Animal Care and Use Committee. Mouse monoclonal antibody to clathrin heavy chain was obtained from Transduction Laboratories (Lexington, KY, U.S.A.). Rabbit polyclonal phospho-specific antibodies to ERK (1/2), Raf-1, MEK, and p90RSK were obtained from Cell Signaling Technology (Beverly, MA, U.S.A.). Peroxidase conjugated goat anti-mouse and anti-rabbit secondary antibodies as well as amino acids, bile salts, nucleotides, polyamines, and 3-aminobenzoic acid ethyl ester were obtained from Sigma (St. Louis, MO, U.S.A.). KN-93 was obtained from Seikagaku America (Falmouth, MA, U.S.A.) and GF109203X was obtained from Tocris Cookson Incorporated (Ellisville, MO, U.S.A.). Flaxedil was obtained from American Cyanamid Company (Wayne, NJ, U.S.A.). The following chemicals were obtained from the indicated sources: Tris and DTT were obtained from, Invitrogen/Life Technologies (Carlsbad, CA, U.S.A), EDTA and Tween 20 from Amresco (Solon, OH, U.S.A.), NaCl from Fischer Scientific (Fair Lawn, NJ, U.S.A.), and MgCl$_2$ from Curtin Matheson Scientific (Houston, TX, U.S.A.).

Catfish Preparation

Channel catfish were prepared essentially as described in Kang and Caprio (1991). Catfish were immobilized with a 50 µL intramuscular injection of the neuromuscular blocking agent Flaxedil (gallamine triethiodide, 1.3mg/mL). The immobilized fish were
wrapped in wet Kim-Wipes, placed in a Plexiglas container, and stabilized using a pair of orbital ridge clamps. The gills were irrigated using an orally inserted glass tube supplying a constant flow of aerated, charcoal-filtered city tap water containing 1 mL of 10% 3-aminobenzoic acid ethyl ester anesthetic per 2 L of water. Access to both olfactory rosettes was achieved by removing the overlying skin and connective tissue.

**Olfactory Epithelia Stimulation**

One rosette was exposed to stimuli while the other was exposed only to charcoal-filtered tap water, providing an internal control for each animal. All stimuli were prepared using charcoal-filtered city tap water, pH adjusted to 8.5-9.0 to match both the control water bathing the olfactory rosettes and the natural pH of the local ponds where the fish were maintained. Stimulus delivery was via a “gravity-feed” system using a spring-loaded valve (Model 5301, Rheodyne Inc., Cotati, CA, U.S.A.) driven by a pneumatic actuator (Model 5300) at 40 psi. Stimulus solutions and charcoal-filtered tap water used to bathe the olfactory rosettes were delivered through separate Teflon tubes (1.06 mm diameter) at a rate of 6-8 mL/min. Both olfactory cavities were perfused continuously with charcoal-filtered tap water to facilitate stimulus delivery, protect the mucosa from desiccation, avoid the introduction of mechanical artifacts associated with stimulus presentation, and thoroughly rinse the olfactory tissue between stimuli. A foot switch connected to an electronic timer (Model 645, GraLab Instruments Division Dimco-Gray Corporation, Centerville, OH, U.S.A.) triggered the valve to introduce the stimulus to the experimental rosette for a 5 sec duration. After stimulus delivery, the experimental olfactory tissue was perfused with charcoal-filtered tap water until the next stimulus delivery and prior to animal termination. In general, stimuli were delivered five times with each stimulus presentation lasting 5 sec with a 30 sec charcoal-filtered tap
water rinse in between, and as well as a 7 min post-stimulation perfusion with charcoal-filtered tap water prior to animal termination. For those experiments involving the CamKII inhibitor KN-93 and the PKC inhibitor GF109203X, both the control and experimental rosettes were manually perfused with the inhibitor 15 min prior to stimulation. The inhibitors were present in all stimulation and perfusion solutions throughout the experiment. KN-93 was used at 10 µM and GF109203X was used at 5 µM concentration. Catfish were terminated by severing the spinal cord and each olfactory rosette was removed separately to corresponding glass homogenizers chilled on ice. Control and experimental rosettes were homogenized separately in 200-250 µL of homogenization buffer (20 mM Tris, pH 8, 2 mM EDTA, 2 mM DTT, 1% Triton X-100, 15 mM NaCl, 3 mM MgCl$_2$ supplemented with phosphatase inhibitors 1 and 2 (P 2850 and P5726 Sigma, St. Louis, MO, U.S.A.) and protease inhibitor cocktail (P 2714, Sigma, St. Louis, MO, U.S.A.) or Complete protease inhibitor cocktail tablets (Roche, Indianapolis, IN, U.S.A.) with 12-14 strokes on ice. Homogenates were transferred to 1.5 mL microcentrifuge tubes and centrifuged for 5 min at 4°C at 14,000 rpm. Supernatants were transferred to fresh microcentrifuge tubes and 1:10 dilutions of supernatants in water were quantitated using BioRad Protein Assay Reagent (BioRad, Hercules, CA, U.S.A.) according to the manufacturer’s instructions.

**Western Blotting**

Western blotting was performed as described previously (Bruch and Abogadie, 1995) with the following amendments. Equal quantities of all control and experimental rosette homogenates were resolved on 10% SDS-PAGE and transferred to nitrocellulose membranes. All membranes were blocked in 100 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween, 1% bovine serum albumin, and 2% nonfat dried milk for two hours at room
temperature prior to incubation in primary antibody. Primary antibodies were used at 1:1000 dilution at 4°C overnight and secondary antibodies were used at 1:5000 dilution at room temperature for one hour. Membranes were washed after primary and secondary antibody incubations with TBST (100 mM Tris, pH 7.4, 150 mM NaCl, and 0.05% Tween) and visualized with ECL Western blotting detection reagents from Amersham Biosciences Corporation (Piscataway, NJ, U.S.A.).

**Electro-olfactogram (EOG) Recording**

The EOG was recorded *in vivo* using calomel electrodes via Ringer’s-agar-filled capillary pipettes with tip diameters of 100-500 µm (Silver et al., 1976; Caprio 1995). Catfish were prepared as described above. The pipette of the active electrode was positioned near the midline raphe of the olfactory rosette at a location that maximized the EOG response; the pipette of the reference electrode was placed against the skin adjacent to the olfactory cavity. The fish was grounded via a hypodermic needle inserted into the flank musculature. The olfactory rosette was stimulated with five, 5 sec stimuli comprised of L-methionine and L-lysine at $10^{-5}$ M, separated by 30 sec of perfusion with charcoal-filtered tap water between each stimulus delivery. The EOG was amplified (Grass P-18; Astro-Med Inc., West Warwick, RI, U.S.A.), displayed on an oscilloscope, digitized (Model DR-390, Neuro Data Instruments Corp.), stored on a video channel of a Hi-fi video recorder, and recorded on a chart recorder for visual analysis.

**Pixel Density Determination for Western Blots**

Western blots were scanned by an Eagle Eye Still Video System from Stratagene (La Jolla, CA, U.S.A.) and pixel density was measured using Zero D-Scan 1.0 Image Analysis Software by Scanalytics (Fairfax, VA, U.S.A.). Fold increase in pixel density of ERK1 and
ERK2 for experimental rosettes was determined by dividing the density value of the experimental rosette by the corresponding value for the control rosette for that animal.

RESULTS

Odorant Stimulation of Olfactory Epithelia in vivo Activates ERK

In order to test if odorants could elicit a MAPK response, olfactory rosettes in anesthetized catfish were exposed individually to complex mixtures of four classes of odorants as described in Materials and Methods. Bile salts (taurocholic acid, taurolithocholic acid, and lithocholic acid at $10^{-5}$ M), nucleotides (ATP, ITP, and IMP at $10^{-4}$ M), polyamines (putrescine, spermine, and cadaverine at $10^{-6}$ M), or amino acids (L-methionine, L-alanine, L-arginine, and L-lysine at $10^{-4}$ M) were delivered to experimental rosettes for 30 sec then perfused with charcoal-filtered tap water for 10 minutes before sacrifice and isolation of the control and experimental olfactory rosettes. Equal quantities of rosette homogenates were analyzed by Western blot as described using anti-phospho-specific ERK(1/2) primary antibody, peroxidase-conjugated goat anti-rabbit secondary antibody, and visualized by enhanced chemiluminescence (Fig. 3.1). Only in the case of the amino acid mixture did the amount of phosphorylated (activated) ERK increase (~ 3 fold) over control. Although clathrin was used as an equal loading control in this experiment, sufficient tissue was not always obtained in smaller fish in later experiments.

Characterization of the Amino Acid Stimulus

Since it was observed that amino acids mediate an increase in phosphorylation of ERK, further characterization of the stimulus mixture was performed. Each amino acid in the original stimulus mixture (L-methionine, L-alanine, L-arginine, and L-lysine) was introduced individually as well as with several other amino acids (Fig. 3.2) and amino acid
derivatives. In no case did a single amino acid delivered at $10^{-5}$ or $10^{-4}$ M increase the amount of phosphorylated ERK. L-methionine and L-lysine together at $10^{-5}$ M could, however, elicit results similar to those obtained with the original amino acid mixture. No other combinations of the original mixture increased phospho-ERK. L-cysteine could be substituted for L-methionine, and in combination with L-lysine increased the phosphorylated ERK signal (Fig. 3.2).

Having established that the L-methionine and L-lysine mixture (M/K) could repeatedly increase the amount of phosphorylated ERK, the effect of concentration (Fig. 3.3a), number of stimuli (Fig. 3.3b), length of stimulus (data not shown), and time post-stimulation (Fig. 3.3c) were explored. Dilute concentrations of M/K to $10^{-8}$ M could elicit an increase in phospho-ERK in some fish (Fig. 3.3a), however, a $10^{-5}$ M concentration of M/K was more consistent over the majority of catfish tested. Since this concentration is similar to

**Figure 3.1.** Olfactory experimental rosettes (lanes labeled S) were stimulated with amino acids (MARK), bile salts (BS), nucleotides (NTPs), or polyamines (poly) as described in the text, while the corresponding control rosette was perfused with charcoal-filtered tap water (lanes designated with 0). Rosettes were isolated, homogenized, and quantitated by the Bradford method as described in Materials and Methods. Each lane was loaded with 50 µg of total homogenate. An increase in phospho-ERK is defined as an increase in Western signal in the experimental (S) lane as compared to the control lane (0). Clathrin signal is presented here as an equal protein loading control.
what a catfish might encounter in the natural environment, $10^{-5}$ M stimulus concentration was used for the remainder of the experiments. Further characterization of olfactory epithelial stimulation revealed that as little as a single 5 sec stimulus could activate ERK in some fish. However, multiple 5 sec stimuli were consistently more effective, specifically five, 5 sec stimuli (Fig. 3.3b). The length of time for a single stimulus delivery was also tested, and consistently a duration of 5 sec was most effective (data not shown). The length of time the olfactory tissue was perfused after the last stimulus delivery was also manipulated, and an increase in phospho-ERK could be detected in as little as 1 min, but the largest increase in signal was observed after 7-10 min post-stimulation (Fig. 3.3c).

To determine if the stimulation regime resulted in adaptation and attenuation of the odorant signal, electro-olfactogram analysis was performed. The EOG is a slow negative potential measured in the water immediately above the olfactory mucosa as a result of
Figure 3.3. Olfactory experimental rosettes (lanes labeled S) were stimulated with L-methionine and L-lysine as described in Materials and Methods except that the following variables were manipulated (a) concentration of the M/K mixture, (b) number of 5 second M/K 10^{-5} M stimulations, and (c) amount of time post-stimulation before tissue was collected. The corresponding control rosette was perfused with charcoal-filtered tap water (lanes designated with 0). Each lane was loaded with 50 µg of total homogenate.

odorant exposure and is thought to reflect the summated olfactory receptor neuron generator potentials that result (Ottoson 1971). Catfish olfactory rosettes were prepared as described in Materials and Methods and stimulated with M/K at 10^{-5} M for five, 5 sec stimulus deliveries
separated by 30 sec of charcoal-filtered tap water perfusion (Fig. 3.4). If attenuation of the odorant signal was occurring, a decrease in peak amplitude from the first to the last stimulus delivery would be observed. However, peak amplitude for each stimulus delivery remained unchanged, suggesting that MAPK activation was not associated with an adaptive response to repeated odorant stimulation.

**ERK Pathway Components are also Phosphorylated**

Catfish olfactory rosettes were stimulated as described for the EOG analysis, then collected after 5, 7, and 10 min post-stimulation. The tissue was homogenized as described in the Material and Methods and exposed to Western analysis using primary phospho-specific antibodies against Raf, MEK, ERK, and p90RSK (Fig. 3.5). The phospho-Raf signal (~3 fold over control) peaks between 5 and 7 min post-stimulation, while phospho-MEK and phospho-ERK peak between 7 and 10 min post-stimulation. Phospho-MEK and phospho-ERK were increased about 3 and 5 fold respectively over control. The approximately 2 fold increase in phospho-p90RSK signal begins to peak at 10 min post-stimulation. This order of
Figure 3.5. Olfactory experimental rosettes (lanes labeled S) were stimulated with L-methionine and L-lysine as described in the text, while the corresponding control rosette was perfused with charcoal-filtered tap water (lanes designated with 0). After stimulation, both rosettes were perfused with charcoal-filtered tap water for 5, 7, and 10 min before they were isolated, homogenized, and quantitated by the Bradford method as described in Materials and Methods. MEK and ERK Westerns were loaded with 50 µg of total homogenate per lane while Raf and RSK Westerns had 70 µg per lane.

events reflects the order of phosphorylation with the ERK cascade with Raf being activated first, followed by phosphorylation of MEK by activated Raf, then phosphorylation of ERK by MEK, and finally phosphorylation of p90RSK by activated ERK. The absence of a phospho-RSK signal in the control lane for the 5 minute homogenate is unknown given that the same homogenate produced signals for phosphorylated Raf, MEK, and ERK in the same figure. Similar homogenates derived from the same stimulation regime showed a slight de-
phosphorylation of Src between the 7 and 10 min homogenates (Figure 3.6). The phospho-Src antibody was directed against a peptide containing Tyr418 of Src. Phosphorylation of this residue in Src is necessary for maximal kinase activity (Schwartzberg 1998). There was a slight increase of phospho-Src in the 5 min experimental homogenate. Western analyses of similarly stimulated homogenates enriched in nuclei were probed with several different anti-phospho-CREB antibodies produced anomalous results, while those probed with anti-phospho-Elk antibodies showed signals too weak to detect reliably (data not shown).

**Figure 3.6.** Olfactory experimental rosettes (lanes labeled S) were stimulated with L-methionine and L-lysine as described in the text, while the corresponding control rosette was perfused with charcoal-filtered tap water (lanes designated with 0). After stimulation, both rosettes were perfused with charcoal-filtered tap water for 5, 7, and 10 min before they were isolated, homogenized, and quantitated by the Bradford method as described in Materials and Methods. 70 µg of total homogenate were loaded per lane and the resulting Western blot was probed with anti-phospho-Src antibodies.

**Inhibition of CamKII and PKC Alters ERK Activation**

To determine if inhibition of CamKII interfered with an increase in phosphorylated ERK, the CamKII inhibitor KN-93 was used throughout the preparation, stimulation, and perfusion of the olfactory rosettes. Each rosette was isolated as described in Materials and Methods and analyzed by Western blotting using anti-phospho-ERK primary antibody. Rosettes exposed to the CamKII inhibitor KN-93 failed to produce an increase in phospho-
ERK signal compared to control (Fig. 3.7). This suggests that CamKII must be active for ERK to be phosphorylated.

![Graphical representation of Western blots and histograms showing the fold increase in pixel density.](image)

**Figure 3.7.** (Top) L-M/K stimulation regime as described in Materials and Methods in the presence (+) or absence (-) of CaMKII inhibitor KN-93 or PKC inhibitor GF109203X as indicated with 7 min perfusion prior to tissue isolation and homogenization. The DMSO lanes represent a control for GF109203X (see text). (Bottom) Graph of average pixel density of Western blots representing three experiments depicted in the (top). The third bar represents the fold increase in pixel density of the experimental rosette over the control rosette for that animal. The first and second bars represent the L-M/K stimulation regime in the presence of KN-93 and GF109203X inhibitors, respectively. P<0.001 for MK Avg compared to each inhibitor.

To determine the role, if any, that PKC plays in ERK phosphorylation, the PKC inhibitor GF109203X was used in a similar manner as the CamKII inhibitor. An additional control using DMSO was also performed since GF109203X must first be dissolved in DMSO before it is soluble in aqueous solution. Exposure of the olfactory epithelium to DMSO +
M/K causes an increase in phosho-ERK as compared to the rosette exposed to DMSO only, showing that DMSO does not interfere with the ability of the M/K stimulus solution to elicit phosphorylation of ERK. However, exposure of the experimental rosette to GF109203X + M/K did not increase the phosphorylation of ERK as compared to the control tissue exposed to a similar concentration of GF109203X without the amino acid mixture. Examination of the Western blot shows that both the control and experimental signals do not differ in intensity, but the signal intensity of the control homogenate was consistently larger than any obtained from control rosettes that were not exposed to GF109203X (Fig. 3.7). There are several possibilities for this observation. First, inhibition of PKC by GF109203X inhibits phosphorylation of ERK relative to control; however, this seems unlikely given that the GF109203X control consistently produces Western signal larger than that obtained from control rosettes exposed to charcoal-filtered tap water alone. This would imply that PKC provides a negative regulatory effect on the phosphorylation of ERK, and when PKC is pharmacologically inhibited with GF109203X, this inhibition is removed and maximal ERK phosphorylation occurs. Lastly, GF109203X may be acting as an agonist for an unidentified pathway that increases ERK phosphorylation. Based on the available data, the latter two possibilities cannot be distinguished.

DISCUSSION

Signaling through the ERK cascade has been linked to cell proliferation in a variety of cell types. Studies performed in neurons revealed that the ERK cascade has been harnessed to perform additional functions including participating in synaptic plasticity and cellular memory. Studies on CTA in mammals and guanylyl cyclase induction upon odorant stimulation in salmon revealed that these animals displayed an increased sensitivity to
compounds they had been exposed to as much as two years ago, demonstrating a connection between chemosensory perception and cellular memory. Such a connection was confirmed by Watt and Storm (2001) where they showed that odorant stimulation of primary cultures of rat olfactory neurons induced ERK activation. A similar connection was also observed by Hirotsu et al. (2000) when mutation of LET-60 Ras impaired the chemotactic behavior normally observed in *C. elegans* upon odorant stimulation.

The mammalian primary olfactory response is driven by cAMP second messenger cascades. We were interested in determining if ERK activation was universally linked to olfactory signaling, since the primary olfactory response in catfish is driven by inositol phospholipid signaling. Given that MAPK signaling, in general, is context dependent (reviewed in Pearson et al., 2001), we performed our studies on anesthetized catfish. Catfish make an ideal model for studying olfactory stimulation since they have two olfactory rosettes that equally express odorant receptors, yet these structures are not synaptically linked. This anatomical feature provides for both control tissue and experimental tissue within the same animal that can easily be treated independently.

We report that ERK is activated above basal level upon stimulation with a combination of amino acids, specifically L-methionine and L-lysine, in catfish olfactory epithelia. This effect is not elicited with any other group of known odorants such as bile salts, nucleotides, or polyamines. L-cysteine may be substituted for L-methionine in the methionine-lysine mixture; however, no single amino acid stimulus can elicit a detectable increase in ERK phosphorylation. ERK activation can be elicited by a single stimulation with this amino acid mixture, however, multiple stimulations with the L-methionine and L-lysine mixture were consistently more effective from fish to fish. This is probably due to the
fact that catfish ORNs are highly sensitive to amino acids with unbranched, uncharged side
chains, with L-cysteine being the most effective olfactory stimulus (Caprio 1978). The
olfactory epithelia is uniformly sensitive to various stimuli (Caprio and Byrd 1984; Bruch
and Rulli 1988; Caprio et al. 1989; Kang and Caprio 1991); thus, the use of a combination of
amino acids would activate a denser region of the epithelium, activating more ORNs,
producing a homogenate with a detectable increase in ERK activation. The fact that multiple
stimulations are necessary probably reflects a requirement for a threshold of stimulation prior
to ERK activation. The EOG data (Fig. 3.4) show that this effect is not due to adaptation to
the stimulation paradigm. It is interesting to note that the requirement for five L-
methionine/lysine stimulations parallels the serotonin stimulation regime required to exhibit
an increase in long-lasting neurotransmitter release and a long-term increase in the
excitability of cultured Aplysia sensory neurons (Dale et al. 1987; Mercer et al. 1991). Both
of these events require transcription of genes under the control of CRE and signal through
ERK. These observations suggest that ERK activation upon odorant stimulation may also
affect gene transcription.

The presence of activated ERK in control homogenates is not surprising given the
diversity of molecules that signal through the ERK module. The amount of activated ERK in
control homogenates varies from animal to animal. Such variability is a consequence of in
vivo experiments since no two animals are exposed to exactly the same environment cues and
conditions prior to experimentation. Weather conditions, transportation of the catfish, and
time of handling prior to experimentation, as well as a multitude of other factors, may affect
the basal level of ERK activation. Such factors cannot be controlled for in each set of
experiments; however, these difficulties do not detract from the overall observation since
ERK activation was always observed to increase above basal levels upon odorant stimulation with L-methionine and L-lysine. These lysates were also probed for other phospho-MAPKs, but no detectable phospho-SAPK or phospho-p38 were detected (data not shown).

Catfish olfactory rosettes were stimulated with the L-methionine/lysine regime and were harvested to produce homogenates that were analyzed by Western blotting using phospho-specific antibodies to Raf, MEK, and p90RSK. All the members of the ERK MAPK module were activated sequentially including the downstream target p90RSK. RSK is a well-established downstream substrate for activated ERK (reviewed in Frodin and Gammeltoft 1999). A common result of RSK activation is the resulting activation of CREB (Xing et al. 1996). This supports the data obtained by Watt and Storm (2001) in rat primary olfactory cultures; however, phosphorylation of the transcription factors CREB and ELK could not be detected in our system. This may be because the number of nuclei obtained from a single olfactory rosette was insufficient to produce a signal by Western analysis.

Many GPCRs that stimulate MAPK activation internalize through clathrin-coated pits (Daaka et al. 1998; Luttrell et al. 1997) and this process is dependent on Src activation of dynamin (Ahn et al. 1999). We have shown that an internalization pathway is active in isolated catfish olfactory neurons specifically under odorant stimulation (Rankin et al. 1999). Homogenates from stimulated rosettes were also probed for phospho-Src, and a slight increase was observed in the 5 min experimental homogenate, although this was difficult to detect. The phosphorylated peptide used to generate the phospho-Src antibody is also conserved in the Src-family kinases Fyn and Yes. It does remain possible that a Src-like tyrosine kinase activity may be involved in the ERK cascade; however, the data presented here is not definitive. In this same vein, phospho-regulation of the kinase Akt/PKB has been
shown to effect ERK signaling by reducing Raf-1 activity (Rommel et al. 1999), however, this kinase was not detected by Western blot analysis of amino acid stimulated catfish olfactory rosettes. It is still possible that Akt/PKB is involved since the mammalian derived antibody may simply not interact with the catfish homolog.

To explore the roles of CaMKII and PKC in the activation of ERK, catfish olfactory rosettes were exposed to either the CaMKII inhibitor KN-93 or the PKC inhibitor GF109203X throughout the entire M/K stimulation regime. Inhibition of CaMKII blocked activation of ERK placing it upstream in the signaling cascade, supporting the observation in rat olfactory neuron primary culture (Watt and Storm 2001). Inhibition of PKC produced homogenates with little difference in density between the control and experimental rosette; however, the density associated with the control rosette alone was much larger than those obtained from control rosettes in all other experiments. This was not due to the DMSO used to dissolve the GF109203X, since DMSO treated rosettes were able to activate ERK as compared to rosettes treated with DMSO alone. This finding is in contrast to the findings of Watt and Storm (2001), as PKC inhibition had no effect on ERK activation in the rat primary olfactory neuron cultures. This may reflect differences in ERK signaling between species or may be associated with odorant stimulation in the context of intact tissue versus in tissue culture.

Data described here show that the ERK MAPK module can be activated in ORNs specifically upon odorant stimulation regardless of the second messenger-mediated pathway initiated during the primary olfactory response, and that activation of the module involves activation of RSK and is dependent upon CaMKII activity. In contrast, catfish olfactory stimulation also appears sensitive to PKC activity. Our data seem to indicate that PKC plays
an inhibitory role in ERK activation, since inhibition of this kinase produces control phospho-ERK bands with a larger pixel density than those obtained in the absence of PKC inhibitor. We also found no definitive data that CREB was phosphorylated upon ERK activation or that Src activity was necessary for ERK activation. It may be that our system was not sensitive enough to detect CREB phosphorylation or Src participation in ERK activation and that these mediators are best studied in primary neuronal cultures.

The data presented here in anesthetized animals support much of the data obtained from rat primary olfactory neuron cultures; however, our in vivo study identified unique features of ERK activation in olfaction. Given the context dependence of MAPK signaling, and that ORN-turnover is ongoing, we believe our data to be especially reflective of the contextual signaling paradigms active within ORNs. The data presented here support the hypothesis that the ERK module in channel catfish olfactory epithelia acts as a signal detector, balancing inputs of both odorant detection and neuronal plasticity.
CHAPTER 4:
IDENTIFICATION OF A PUTATIVE ODORANT RECEPTOR IN
ICTALURUS PUNCTATUS OLFATORY EPITHELIUM

INTRODUCTION

The olfactory system enables an organism to detect a multitude of chemically diverse compounds through binding of odorants to receptor proteins located in dendritic extensions of neurons in the sensory region of the olfactory epithelium. Odorant binding to the olfactory receptor (OR) activates G proteins, initiating second messenger cascades that result in depolarization of the membrane and the generation of action potentials, conveying the signal to the olfactory bulb and higher centers of the brain. Identification of the ORs in several species (Buck and Axel 1991; Ngai et al. 1993a; Sengupta et al. 1996; Clyne et al. 1999) revealed that ORs comprise the largest gene family identified to date, and with very few exceptions, lack introns (for reviews see Mombaerts 1999 and Firestein 2001). Mammals were proposed to have about 1000 individual OR genes; however, with the release of the mouse and human genomes, data mining efforts revealed that humans have 70% pseudogenes with 347 full-length ORs (Zozulya et al. 2001) and mouse has 20% pseudogenes with about 900 full-length ORs (Zhang and Firestein 2002). Catfish are predicted to express about 100 different ORs (Ngai et al. 1993a). Identification of the invertebrate OR genes indicate that C. elegans (Sengupta et al 1996) has about 500 and Drosophila has about 60 members (Clyne et al. 1999). Neither of the invertebrate groups exhibits much homology to the vertebrate ORs.

Analysis of the primary sequences of the available ORs revealed that mammals have two classes of receptors, with class I being similar to the fish sequences and class II representing the majority of mammalian ORs identified to date (Freitag et al. 1995; Glusman
et al. 2000; Zhang and Firestein 2002). Both class I and class II receptors have the characteristic motif of a short amino-terminal, extracellular domain (about 40 amino acids). A given OR is expressed in a mosaic fashion within a small subset of ORNs organized into zones in the olfactory epithelium. This zonal topography has been observed in mouse (Ressler et al. 1993), rat (Vassar et al. 1993), and zebrafish (Weth et al. 1996); however, it has not been clearly defined in catfish (Ngai et al. 1993a). Evidence gathered from several labs indicates that ORNs expressing the same OR project to the same glomerulus within the olfactory bulb (Ressler et al. 1994 and Vassar et al. 1994).

Identification of receptor 5.24 in goldfish by Speca et al. (1999) revealed this receptor to bind specifically to basic amino acids. The primary sequence of receptor 5.24 was 500 amino acids longer than class I and class II ORs, exhibiting a very large extracellular amino-terminal domain similar to that identified in metabotropic glutamate receptors, calcium sensing receptors, and putative pheromone receptors. Moreover, receptor 5.24 was localized by in situ hybridization to ORNs in the sensory region of the goldfish olfactory epithelium that exhibited different morphology than the neurons where class I odorant receptors were expressed. Identification of the goldfish 5.24 receptor revealed that the fish OR repertoire displays unexpected diversity at the structural level previously thought to be associated with pheromone signaling.

We were interested in identifying a catfish homolog to the goldfish 5.24 OR because none of the available ORs for catfish have been shown to functionally respond to odorant stimulation and amino acids are well-established odorants for catfish (Caprio et al. 1989; Kang and Caprio 1991). Catfish are predicted to contain a tenth of the OR genes found in mammals (Ngai et al. 1993a) with no obvious zonal expression making them a less complex
system to study olfaction. Data presented here indicate that channel catfish express a receptor that exhibits similarity to goldfish 5.24 and putative pheromone receptors. In addition, the catfish putative receptor exhibits limited expression in tissues other than the olfactory epithelium and is expressed in neurons with morphology similar to those that express goldfish 5.24.

MATERIALS AND METHODS

RT-PCR Amplification of aa64 Fragment

Total RNA was isolated from goldfish and catfish olfactory epithelium using RNAzol B (Tel-Test, Inc., Friendswood, TX, U.S.A.) according to manufacturer’s instructions. First strand cDNA synthesis was performed on both goldfish and catfish total RNA with random hexamers and Superscript II RT from Invitrogen Life Technologies (Carlsbad, CA, U.S.A.). Second strand synthesis was performed using the resulting first strand reactions with the Failsafe PCR system from Epicentre (Madison, WI, U.S.A.) and combinations of the following primers: (5’Gold 10.8) 5’-GACAATGACTATGGAAATAATGGAATGG-3’, (3’ Gold 10.8) 5’-GAGCCACAGCATAAACTGCTTTGTAAAC-3’, and (3.2’ Gold 10.8) 5’-GGACAACTCTCATGACACACAGACC-3’. These primers were derived from the goldfish 10.8 partial clone identified by Speca et al. (1999) and correspond to the nucleotide positions 13-40, 526-498, and 860-836 respectively. All primers were synthesized by MWG Biotech, Inc. (Highpoint, NC, U.S.A.). Second strand synthesis reactions were performed in a PTC-100 Programmable Thermal Controller from MJ Research Inc. (Watertown, MA, U.S.A.) as follows: 95°C denaturation for 1 min, 63°C or 64°C annealing for 1.5 min, and 70°C extension for 1 min for a total of 40 cycles with an additional 70°C extension for 10 min before termination of the amplification. PCR reactions were resolved on a 1% agarose/TAE
gel, gel purified with Qiagen’s QIAEX II Gel Extraction kit (Valencia, CA, U.S.A.), cloned using the TOPO TA cloning kit from Invitrogen (Carlsbad, CA, U.S.A.), and sequenced using the Thermo Sequenase $^{33}$P Terminator Cycle Sequencing kit from USB Corporation (Cleveland, OH, U.S.A.). The nucleotide sequences obtained were submitted to BLAST (www.ncbi.nlm.nih.gov) for identification.

**In Situ Hybridization in Catfish Olfactory Rosettes with aa64 Fragment**

In situ hybridization was performed by Dr. Karl Anderson in Dr. Tom Finger’s laboratory in the Department of Cellular and Structural Biology, University of Colorado Health Sciences Center, Denver, CO, U.S.A. Anesthetized channel catfish were perfused transcardially with 4% paraformaldehyde/PBS and sectioned using a cryostat at -15 to -20°C. RNA in situ hybridizations were carried out on 12-15 µm thick cryostat sections of dissected catfish olfactory organ collected onto Superfrost plus slides (Fisher Scientific, Fair Lawn, NJ, U.S.A.). The sections were pretreated with proteinase K and hydrogen peroxide to aid in probe penetration and quenching endogenous peroxidase activity, respectively. Digoxigenin or fluorescein labeled cRNA probes were hybridized at 58-60°C overnight and the sections were high stringency washed up to 60-65°C in 0.2X SSC. Localization of the hybridized probes utilized either alkaline phosphatase labeled sheep anti-digoxigenin antibodies or peroxidase labeled sheep anti-digoxigenin antibodies (Roche, Indianapolis, IN, U.S.A.). A light purple chromogenic signal was generated in alkaline conditions utilizing nitroblue tetrazolium chloride and X-phosphate/5-Bromo-4-chloro-3-indolyl-phosphate and the alkaline phosphatase antibody. Alternatively, an enhanced fluorescent signal using the fluorophore tyramide cyanine 3 was generated in the presence of the peroxide antibody. The
tyramide signal amplification (TSA) technique enables low copies of mRNAs to be visualized by enhancing the signal up to 1000 fold (Roche, Indianapolis, IN, U.S.A.).

**5’ and 3’ Rapid Amplification of cDNA Ends (RACE) of aa64 Putative Receptor**

The full-length aa64 clone was obtained using the 5’ RACE and 3’ RACE kits from Invitrogen Life Technologies (Carlsbad, CA, U.S.A.) according to the manufacturer’s instructions with the following changes. Briefly, total RNA isolated from olfactory epithelium as described above was treated with DNAse I to remove genomic DNA contamination before first strand synthesis was performed for both the 5’ and the 3’ RACE procedures. The first strand synthesis was performed at 50°C using Superscript II RT (Invitrogen, Carlsbad, CA, U.S.A.) for both procedures to remove secondary structure barriers present in the mRNA.

The 5’ RACE procedure was performed using the following gene specific primers (GSP) designed antisense to the coding sequence obtained for the aa64 600 bp fragment: (aa5’RACEGSP1) 5’-CCAAGTCATTCTCATCTTG-3’ was used to prime the first strand synthesis reaction, (aa5’RACEGSP2) 5’-GCATCAATCACCTTGATGATGATG-3’ was used along with the AAP primer provided in the kit for second strand synthesis, and (aa5’RACEGSP3) 5’-CTTGATAATATCCACCACATTTAGAAAC-3’ was used along with the AUAP primer provided in the kit to perform the final nested PCR. All GSPs were synthesized by MWG Biotech, Inc. (Highpoint, NC, U.S.A.). All PCR reactions were performed using the Failsafe PCR system from Epicentre (Madison, WI, U.S.A.) and the following thermal cycler program: 94°C for 1 min denaturation, 68°C for 1 min annealing, 72°C for 2.5 min extension for a total of 35 cycles. A final 72°C incubation for 10 min was
performed before reactions were analyzed on a 1% agarose/TAE gel. Fragments were cloned as described above and verified by sequence analysis as described below.

For 3’ RACE, the manufacture’s protocol was followed with the following adaptations. First strand synthesis was performed using AP oligo(dT) supplied in the kit with Superscript II RT at 50°C. Second strand synthesis and the final nested PCR reaction were performed using (aa3RACEGSP1) 5’-CCGGTGAGGAGTCTCTTGAGAATAC-3’ and (aa3RACEGSP2) 5’-CACGCTTCACAGATGTGTCAGATG-3’, respectively, with the AUAP primer provided in the kit with the Epicentre Failsafe PCR System and the same thermal cycler program listed for 5’ RACE except that the annealing temperature was changed to 65°C. All GSPs were synthesized by MWG Biotech, Inc. (Highpoint, NC, U.S.A.). The ~1500 bp amplification product was cloned as described above and verified by sequence analysis as listed below.

The nucleotide sequences of the amplification products were verified using the ABI PRISM BigDye Terminator Cycle Sequencing Kit from Applied Biosystems (Foster City, CA, U.S.A.). Sequencing reactions performed with the BigDye kit were analyzed by the Genomics Core Facility at the Pennington Biomedical Research Center (Baton Rouge, LA, U.S.A.). Nucleotide sequences for some of the amplification products were also obtained using the Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ, U.S.A.) BigDye sequencing reactions were primed with T7 and M13 reverse sequencing primers (Invitrogen, Carlsbad, CA, U.S.A.) along with several GSPs to obtain the full-length nucleotide sequence. The Thermo Sequenase Fluorescent Primer kit was used with fluorescently tagged M13 reverse and T7 sequencing primers synthesized by LI-COR, Inc. (Lincoln, NE, U.S.A.).
Computer Analysis of Full-length aa64 Clone

Protein structure prediction software SOSUI and TMpred used on the full-length aa64 cDNA was accessed on the Baylor College of Medicine Search Launcher site. (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). Multiple alignment of aa64 BLAST search results was performed with ClustalW from the BCM search launcher and BoxShade 3.21 (http://www.ch.embnet.org/software/BOX_form.html) as well as BioEdit Sequence Alignment Editor 5.0.9, Tom Hall, Department of Microbiology, North Carolina State University, Raleigh, NC, U.S.A. Protein motifs present in the aa64 clone were identified with Gene Runner 3.04 by Hastings Software, Inc. (http://www.generunner.com).

Northern Blot Analysis of aa64 Expression in Multiple Catfish Tissues

Total RNA was isolated from barbel, brain, olfactory epithelium, gill, liver, muscle, and egg tissue of two 55 cm channel catfish using the TOTALLY RNA Isolation kit from Ambion (Austin, TX, U.S.A.) according to manufacturer’s instruction for each tissue type and stored in The RNA Storage solution from Ambion (Austin, TX, U.S.A.). Quantitation and purity were estimated using A$_{260}$ and A$_{280}$ absorbance values. All tissues produced an A$_{260}$/A$_{280}$ ratio of 1.9-2.2. 25 µg of total RNA from each tissue was dissolved in an equal volume of gel loading solution (62.5% formamide, 1.14 M formaldehyde pH > 4, 1.25X MOPS, 200 µg/mL bromophenol blue, and 50 µg/mL ethidium bromide) and separated on a denaturing 1% agarose/formaldehyde gel (2.5 g agarose, 185 mL sterile water, 40 mL formaldehyde, and 25 mL 10X MOPS) in 1X MOPS in a Kodak BioMax HR 2025 gel apparatus and transferred to Zeta Probe GT Genomic Membrane from BioRad (Hercules, CA, U.S.A.) overnight by capillary action. RNA was cross-linked on a 312 nm Variable Transilluminator (Fisher Scientific, Pittsburgh, PA, U.S.A.) for 60 seconds, then in a UV
Stratalinker 1800 (Stratagene, La Jolla, CA, U.S.A.). Northern membranes were photographed and allowed to dry before probing.

The probe for Northern analysis was synthesized using the Strip-EZ DNA kit from Ambion (Austin, TX, U.S.A.). 100 ng of the gel-purified (Qiagen’s QIAEX II Gel Extraction Kit, Valencia, CA, U.S.A.) 600 bp aa64 fragment described above was used as template. The resulting probe solution was purified using a Micro Bio-Spin P-30 Tris Chromatography Column from Bio-Rad (Hercules, CA, U.S.A) to remove unincorporated α[^32]P]-dATP and counted in a Liquid Scintillation Analyzer (Packard Biosciences Co., Meriden, CN, U.S.A.).

Northern membranes were pre-hybridized in 5 mL of hybridization solution (50% formamide, 0.12 M Na₂HPO₄, pH 7.2, 0.25 M NaCl, and 7% SDS) at 42°C in a Labnet Hybaid oven (Scientific Consultants, Inc., Baton Rouge, LA, U.S.A.) for 3 hours, then hybridized in 5 mL of fresh hybridization solution containing 5 x 10⁶ cpm of denatured aa64 probe in a Hybaid oven at 42°C overnight. Membranes were washed for 15 min at room temperature with 2X SSC + 0.1% SDS, then for 15 min at room temperature with 0.5X SSC + 0.1% SDS, then for 15 min at 42°C with 0.1X SSC + 0.1% SDS, and finally for 15 min at 67°C with 0.1X SSC + 1% SDS. Washed membranes were wrapped in polyvinyl chloride plastic wrap and placed under Kodak BioMax MS film (Rochester, NY, U.S.A.) with an intensifying screen (Kodak BioMax Transcreen HE, Rochester, NY, U.S.A.) and stored at -80°C for one week before developing the film.

RESULTS

Identification of a Homolog in Catfish of the Goldfish 10.8 Partial Clone

In order to determine if a catfish homolog of the goldfish 5.24 amino acid receptor existed, the goldfish full-length 5.24 receptor sequence and the partial clones identified by
Speca et al. (1999) were aligned to identify similarities in the amino acid sequences. Degenerate primers were designed based on this alignment, however, these primers never produced definitive RT-PCR amplification products even after PCR optimization with several buffers and annealing temperatures. The goldfish partial clone 10.8 displayed regions of amino acid sequences that were repeated within the other partial clones as well as in the 5.24 full-length clone. Primers were designed in these regions of the 10.8 clone and used in RT-PCR reactions using total RNA isolated from both goldfish and catfish olfactory epithelia as template. The particular primer pair of (5’ Gold 10.8) and (3’ Gold 10.8) produced definitive amplification products ~600 bp in both goldfish and catfish olfactory tissue (Fig. 4.1). The catfish and goldfish amplification products were cloned and sequenced then submitted to BLAST for identification. All goldfish and catfish products were identified from BLAST as the 10.8 receptor with sequence similarity to other putative goldfish odorant receptors. This ~600 bp catfish amplification product will be referred to as the aa64 amplification product throughout the remainder of the text.

**In situ Hybridization in Catfish Olfactory Epithelia Using the aa64 Amplification Product as Probe**

The aa64 amplification product was cloned into the plasmid pBluescriptII to provide a template for synthesis of an anti-sense probe for *in situ* hybridization experiments in fixed catfish olfactory epithelium. The aa64 probe was localized throughout the sensory region of the epithelia with no apparent zonal expression (Fig. 4.2A). Interestingly, higher magnification of the aa64 signal revealed that the probe localized to areas near the surface of the epithelium (Fig. 4.2B). Closer analysis of these signals revealed that the aa64 probe was localized to olfactory receptor neurons with short, flat dendritic extensions and cell
Figure 4.1. Generation of aa64, 600 bp fragment. RT-PCR was performed using total RNA isolated from both goldfish and catfish olfactory epithelium as template. Three primer combinations were used for each RNA pool from each animal at two different annealing temperatures (63°C or 64°C, see Materials and Methods for details). Products were separated on an agarose gel containing ethidium bromide. Only the 3’ lane primer combination produced amplification products in both the goldfish and catfish reactions. The leftmost lane is a 100 bp ladder.

bodies located near the surface of the epithelium—attributes associated with microvillar olfactory neurons (Fig. 4.2C and D).

Given that the aa64 probe was localized within the sensory region of the catfish olfactory epithelium to what appeared to be microvillar olfactory receptor neurons, the aa64 clone became a good candidate for an odorant receptor. 5’ and 3’ RACE were performed on total RNA isolated from catfish olfactory epithelium as described in Materials and Methods, and a full-length cDNA clone was obtained (Fig. 4.3).

Computer Analysis of the Putative aa64 Receptor

BLAST search of the complete catfish aa64 cDNA sequence displayed high similarity to odorant receptors identified in goldfish, mouse, and pufferfish. SOSUI and TMpred protein structure software predicted a membrane protein with eight-nine transmembrane helices. Gene Runner 3.04 analysis revealed eight possible casein kinase II and nine possible possible myristoylation post-translational modifications. ClustalW multiple alignment and homology calculations revealed that the aa64 protein sequence was only ~20% homologous
Figure 4.2. *In situ* hybridization using the aa64, 600 bp product as probe. (A) Fluorescently labeled probe is localized throughout the sensory region of the epithelium with no apparent zonal expression; bar = 100 µm. (B) Higher resolution image of lamellae with aa64 digoxigenin-labeled probe localized to neurons near the surface of the epithelium; bar = 25 µm. (C) and (D) Fluorescently labeled probe localized to neurons, some exhibiting flat dendritic extensions; bars = 25 and 10 µm, respectively. Micrographs were generously provided by Dr. Karl Anderson.

Figure 4.3. Nucleic acid sequence of the full-length putative aa64 catfish receptor. The cDNA isolated by RACE was 2804 bp with a base count of A = 806, C = 545, G = 613, and T = 840. The start codon corresponds to base pair 35 and the first stop codon to base pair 2597. The translation product is 854 amino acids in length. (see following page)
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to catfish and goldfish class I odorant receptors, while it was ~50% homologous to goldfish
GFB and pufferfish Ca12 and Ca13 receptors (Fig. 4.4). These latter receptors display a long
extracellular domain unlike the class I odorant receptors (Table 4.1).

Table 4.1. Estimated Homology of Catfish aa64 Protein to Other Receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Accession Number</th>
<th>Length in Amino acids</th>
<th>No. AA Similar To aa64</th>
<th>Estimated % Homology</th>
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<tr>
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<td>344</td>
<td>58</td>
<td>17</td>
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<tr>
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<td>AB008857</td>
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<td>M61099</td>
<td>1199</td>
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</table>

Estimated homology values were calculated by counting the number of amino acids that were
identical or homologous to the corresponding amino acids in catfish aa64 protein sequence
based on ClustalW alignment. This sum was divided by the length of the shortest protein in
the comparison (the query or aa64). Only full-length query sequences were used CF =
Ictalurus punctatus, FG = Takifugu rubripes, GF = Carassius auratus, M = Mus musculus,
and R = Rattus norvegicus.

Northern Blot Analysis Using the aa64 Amplification Product

To determine the tissue expression pattern of the putative catfish aa64 receptor, total
RNA from catfish barbel, brain, olfactory epithelium, gill, liver, muscle, and egg tissue was
screened by Northern analysis using the 600 bp aa64 amplification product as probe (Fig.
Figure 4.4. ClustalW multiple alignment of the catfish aa64 protein sequence with full-length sequences obtained from the BLAST homology search. Labels of each protein are the same as in the legend for Table 4.1. Transmembrane regions are labeled.
4.5). The aa64 probe displayed a highly restrictive expression pattern, with signal present only in the olfactory epithelium lane.

Figure 4.5. Northern blot analysis using the 600 bp aa64 amplification product as probe for total RNA isolated from multiple catfish tissues. 25 µg of total RNA from each tissue was separated on a 1% denaturing agarose gel as described in Materials and Methods. FOE = olfactory epithelium (A) Ethidium bromide photograph of the separated RNA before probing with labeled aa64 probe (B). Positions for the 28S (4.7 kb) and 18S (1.9 kb) rRNAs are indicated.

DISCUSSION

The olfactory system is extraordinary in its ability to detect a diverse molecular pool of odorants with a variety of functional groups, including compounds to which an organism has never been exposed. One way the olfactory system has evolved to accomplish this task is by maintaining a large repertoire of diverse odorant receptors. The first report of this multigene family in rat by Buck and Axel (1991) rendered the study of olfaction accessible to molecular techniques. Since then, much has been learned about the signal transduction events that comprise the primary olfactory response; however, functional evidence that the multigene family of odorant receptors mediated responses to particular odors was not obtained until seven years after their initial identification (Zhao et al. 1998).
The release of the genomic sequences of human, mouse, and fly has revealed that, consistent among the species, odorant receptors comprise the largest identified gene group and that the proteins comprising this family are diverse at the sequence level (reviewed in Kratz et al. 2002; Zhang and Firestein 2002), providing a glimpse as to how the olfactory system can detect such a large array of ligands. The genomic sequence information has also revealed a larger than anticipated pool of pseudogenes (60-70%), especially in humans (Sosinsky et al. 2000). This may reflect that some vertebrates such as mouse with 20% pseudogenes have a greater dependence on olfaction than do humans; hence, the human pool has evolutionarily been reduced in number of receptors. Zhang and Firestein (2002) have proposed that although the number of functional OR genes is a third of what is predicted in mouse, the human pool still expresses members that are as diverse as mouse. The authors suggest that humans have retained the ability to detect as broad a spectrum of chemicals as mouse; however, humans would not be as discriminating within a group of odorants with similar functional groups.

The identification by Speca et al. (1999) of goldfish receptor 5.24 that responds to odorant cues of basic amino acids added an additional level of complexity to odorant receptor structure. Vertebrate odorant receptors identified to date have a characteristically short amino-terminal extra-cellular domain (~40 amino acids) (Buck and Axel 1991; Ngai et al. 1993a; Freitag et al. 1995; Cao et al. 1998). The goldfish 5.24 receptor exhibits an extended amino-terminal domain of 566 residues and shares homology to calcium sensing receptors (CaSR, Hebert and Brown 1995), metabotropic glutamate receptors (mGluR, Tanabe et al 1992), and the V2R class of vomernasal receptors (Herrada and Dulac 1997). Furthermore, when Speca et al. (1999) expressed the goldfish 5.24 receptor in HEK293 cells and *Xenopus*
oocytes, they did not detect an increase in cAMP, but rather saw an arginine-induced increase in IP$_3$. This suggests that ligand binding to 5.24 receptor initiates a phospholipase-mediated signal transduction pathway.

Few odorant receptors have been identified for the channel catfish beyond those published by Ngai et al. (1993a), and none of those receptors have been shown to respond functionally to odorant stimulation when expressed in a variety of cell lines. Amino acids represent powerful odorant cues for channel catfish (Caprio 1978) and odorant stimulation in catfish results in a rapid increase in the cellular pool of IP$_3$ (Huque and Bruch 1986; Restrepo et al. 1993). Based on this information, it is highly likely that channel catfish express a homolog to the goldfish 5.24 receptor because catfish respond to basic amino acids and generate an IP$_3$ second messenger cascade upon stimulation (preceding paragraph). Primers designed from the sequences presented by Speca et al. (1999) produced RT-PCR amplification products from total RNA isolated from the olfactory epithelia from both goldfish and channel catfish (Fig. 4.1). Sequence analysis and BLAST homology searches identified the amplification product from catfish to be highly homologous to the goldfish 10.8 and 5.24 sequences reported by Speca et al. (1999).

To determine if the catfish amplification product was expressed in sensory neurons within the olfactory epithelia, *in situ* hybridization experiments were performed on sections of olfactory rosettes by our colleague Dr. Karl Anderson. The aa64 probe was localized to the sensory region within the olfactory epithelium as indicated by its presence near the raphe of the rosette; however, it did not exhibit any identifiable zonal expression pattern (Fig. 4.2A). Higher magnification of the aa64 probe in thin sections of the olfactory lamellae reveals localization to neurons near the surface of the epithelium (Fig. 4.2B and C). These
neurons exhibit a short cell body, and some of them display flat dendritic extensions (Fig. 4.2D). These data support expression of aa64 in microvillar neurons; however, definitive identification of neuronal morphology awaits electron microscopy experiments. These are the same type of neurons that the goldfish 5.24 receptor was localized to in goldfish olfactory epithelium. This is in contrast to the localization patterns obtained with in situ hybridizations using the catfish “Ngai-type” odorant receptor probes (Ngai et al. 1993a). The “Ngai-type” probes localize to olfactory receptor neurons that have long, bulbular dendritic extensions with cell bodies located deep in the olfactory epithelium—attributes associated with ciliated olfactory neurons. These data are interesting since no olfactory receptor proteins have been identified in catfish microvillar neurons.

Using the sequence information obtained from the aa64 product, non-overlapping gene-specific primers were designed outside of the original primer regions for use in RACE. 5’ and 3’ RACE procedures produced a complete amplification product of 2804 bp that encoded a protein of 854 amino acids. SOSUI and TMpred software predicted that the catfish clone contained multiple transmembrane domains (8-9). BLAST and ClustalW Multiple Alignment software revealed that the aa64 full-length clone was highly similar to goldfish GFB and 5.24 receptors, as well as mouse V2R receptors and pufferfish putative pheromone receptors Ca12 and Ca13. Interestingly, aa64 is more similar to these receptors than to goldfish 5.24 receptor, and displays even less similarity to the catfish odorant receptors identified by Ngai et al. (1993a). Furthermore, the aa64 clone lacks the extended second extra-cellular loop present in mammalian class I and class II receptors, as well as many of the other characteristics present in mammalian odorant receptors (reviewed in Mombaerts 1999; Firestein 2001; Zhang and Firestein 2002); however, the goldfish 5.24
receptor also lacks these elements yet responds specifically to amino acid stimulation and is located within sensory region of the goldfish olfactory epithelium. Interestingly, aa64 also shares homology with mGluR1 (Table 4.1) and contains the identical sequence in intracellular loop three required for $G_q$ activation (Francesconi and Duvoisin 1998).

Northern blot analysis was performed to determine how widely expressed the aa64 clone was in multiple catfish tissues. Total RNA isolated from barbel, brain, olfactory epithelium, gill, liver, muscle, and egg sacks was probed with labeled aa64. The aa64 signal was only detected in total RNA isolated from olfactory epithelium (Fig. 4.5). This is in contrast to the expression pattern observed for the goldfish 5.24 receptor—the goldfish receptor was localized to the gills, lips, tongue, and palatal organ in addition to the olfactory epithelium by Northern analysis.

Based on this data, the catfish aa64 clone represents a good candidate for a chemosensory receptor. It is expressed predominantly in the olfactory tissue, localized to neurons that preside in the sensory region of the epithelium. Structural analysis and multiple alignments predict that the aa64 clone is a GPCR with high similarity to putative pheromone receptors from mouse, pufferfish, and goldfish, including the goldfish 5.24 odorant receptor. Identification of the ligand(s) for the aa64 clone await functional expression experiments; however, should the catfish aa64 protein respond to odorant stimulation, then the characteristic odorant receptor domains like those used in the data mining strategies employed in the human and mouse genomes (Zozulya et al. 2001; Zhang and Firestein 2002) will have to be broadened to include this new group of receptors.
CHAPTER 5:
SUMMARY AND CONCLUSIONS

The work presented here studies three important aspects of odorant receptor signaling in the channel catfish. In Chapter 2, data were presented that identified for the first time that a functional internalization pathway exists in olfactory neurons. This internalization event was specifically induced upon odorant stimulation. Internalization in catfish ORNs probably occurs via clathrin-coated pits. Key participants in clathrin-mediated endocytosis include clathrin, dynamin, arrestin, and GRK. Western blot analysis and immunocytochemistry presented here show that clathrin and dynamin are present in catfish ORNs. Work done previously in our lab identified a catfish homolog of GRK in ORNs (Bruch et al. 1997b). An alternate internalization pathway mediated by caveolae was also considered as the mediator of the observed internalization events. Western blot analysis with anti-caveolin antibody indicated that this protein was present in rat olfactory epithelium, but was not detected in the corresponding catfish tissue. Furthermore, okadaic acid, an activator of caveolae-mediated internalization (Parton et al. 1994) did not induce retention of FM1-43 in isolated catfish ORNs, suggesting that caveolae did not participate in endocytosis in these cells.

All of the evidence presented here indirectly supports clathrin-mediated internalization in catfish ORNs. Studies that directly assess the relationship of dynamin, clathrin, arrestin, and GRK on internalization of odorant receptors would greatly enhance the work presented in Chapter 2. Such studies would best be performed in cell culture since the viability of ORNs dissociated from catfish olfactory rosettes is greatly compromised four hours after isolation. This would render them useless for transfection experiments. HEK293 cells are a good candidate for such experiments since they support internalization of a variety
of receptors. These include $\beta_2$-adrenergic, $\mu$-opioid, dopamine type 1, and vasopressin type 2 receptors (Oakley et al. 2000). There also exists an olfactory cell line *odora* that would make an ideal system for such studies (Murrell and Hunter 1999). The *odora* cell line has been shown to properly express odorant receptors at the plasma membrane upon differentiation (Gimelbrant, et al. 2001). Cultured cells expressing odorant receptors as well as mutated forms of dynamin, arrestin, and GRK could be stimulated with odorants and monitored for endocytosis. This approach has been quite successful in delineating the $\beta_2$-adrenergic receptor internalization pathway (Ferguson et al. 1995; Laporte et al. 2000).

The third chapter addresses peripheral signaling pathways, namely ERK signaling, initiated upon odorant stimulation *in vivo*. MAPK signaling is highly conserved among species and different cell types. To achieve signaling specificity in the midst of a multitude of extracellular signals received by a variety of cell-surface receptors, MAPK cascades rely on temporal and spatial organization within a given cell type. The study presented in chapter 3 was performed on anesthetized animals, with stimulation directed at intact olfactory epithelium. Such an experimental design maintains the contextual environment where olfactory signaling occurs. Catfish are especially amenable to these studies in that they contain two analogous olfactory rosettes that are not neurally linked and anatomically separate, thus providing an internal control for each study within the experimental animal.

Olfaction in mammals is mediated through the cAMP signal transduction pathway, and primary cultures of rat olfactory neurons exhibit an increase in ERK(1/2) activation upon odorant stimulation (Watt and Storm 2001). Olfaction in the catfish system is mediated by the IP$_3$ and DAG signaling pathway, and data presented here show that ERK(1/2) is also activated. The stimulation regime that elicited this response in catfish consisted of a mixture
of L-methionine and L-lysine (L-cysteine could be substituted for L-methionine) delivered in five sequential 5 sec applications. These amino acids have been shown to be highly stimulatory in catfish (Caprio and Byrd 1984). The requirement for multiple stimuli mimics that observed in cellular memory processes observed in *Aplysia* (Dale et al. 1987; Mercer et al. 1991). The requirement for a combination of amino acids to elicit detection of ERK activation may reflect a need to stimulate a larger pool of ORNs for the signal to be detected by Western analysis, since stimulation with a single amino acid never produced a convincing increase in activated ERK. Furthermore, amplification of the signal is not achieved at the ERK activation step, but rather occurs at the Raf-MEK step since MEK and ERK are roughly expressed equally within cells (Ferrell et al. 1996), hence a need for a larger portion of the epithelium to be stimulated.

As expected, all three members of the ERK signaling module were activated in response to odorant stimulation: Raf, MEK, and ERK. RSK is a well-established downstream substrate for activated ERK (reviewed in Frodin and Gammeltoft 1999) and this protein was also observed to be activated upon odorant stimulation. A common result of RSK activation is the resulting activation of CREB (Xing et al. 1996). In rat ORNs, odorant stimulation results in the activation of ERK followed by phosphorylation and activation of CREB; however, in catfish, CREB phosphorylation was never conclusively observed. This is most likely due to the inability of the phospho-CREB antibody to recognize the catfish isotype, so CREB activation cannot be completely ruled out. Another well-established link between GPCR activation and ERK stimulation is the participation of Src (Luttrel et al. 1996). Src has three phosphorylation sites that regulate its activity (Schwartzberg 1998). Experiments with catfish olfactory epithelium showed that the Src phosphorylation state was
altered upon odorant stimulation. Initially, there was more phospho-Src in the stimulated homogenates at the 5 min interval; however, between the 7 and 10 min intervals, phospho-Src diminished in the experimental lysate as compared to the control. Furthermore, the antibody may not be specific enough to distinguish between Src, Fyn, and Yes kinase family members. The data here are not definitive, and additional experiments, perhaps using more selective antibodies, should be performed to establish the role and identity of the Src-family of kinases involved in odorant induced activation of ERK.

Treatment of the olfactory epithelium with CamKII inhibitor (KN-93) or with PKC inhibitor (GF109203X) prior to odorant stimulation altered ERK activation. Inhibition of CamKII abolished odorant activation of ERK, while inhibition of PKC seemed to increase it. The results obtained here with the inhibitor of CamKII support the results obtained in rat (Watt and Storm 2001). In contrast, PKC inhibition had no effect on ERK activation in rat, where in catfish, even non-stimulated olfactory epithelia showed a noticeable increase in phosho-ERK in the presence of the inhibitor.

Additional experiments should be performed to better delineate the roles of Src, PKC, and CREB in ERK activation in catfish. The Src and PKC experiments could be done using anesthetized catfish to maintain the appropriate signaling environment. Use of the individual Src antibodies that recognize each of the three Src phosphorylation sites should be used to determine when Src is being activated or inhibited during the signaling process. Additional Src and PKC inhibitors should be used to see if similar results are obtained. CREB experiments could be performed on dissociated ORNs to see if a change in phospho-CREB can be detected in the nuclei between stimulated and control cells.
Of prime interest is the identification of the ultimate downstream effectors of odorant-induced ERK activation. These experiments could also be performed in anesthetized catfish by stimulating the olfactory epithelium as described in chapter 3, then extracting the mRNA from the olfactory rosettes. Quantitative RT-PCR could be performed on the mRNA isolates to determine a shift in gene expression in stimulated versus control rosettes. Alternatively, microarrays could be screened with probes generated from the mRNA isolates. Given that ORNs turnover throughout the lifetime of the organism, I expect housekeeping genes to be turned on as well as genes necessary to induce neuronal maturation of the basal cells and genes that maintain the neural connections from the epithelium to the bulb.

In Chapter 4, data were presented in support of the identification of a putative chemosensory receptor in catfish olfactory epithelium. Identification of the catfish clone stemmed from the discovery by Speca et al. (1999) of the basic amino acid receptor 5.24 in goldfish. The identification of goldfish 5.24 receptor was surprising since this receptor exhibited a large extracellular amino-terminal tail (~500 residues) that resembled mGluRs and pheromone receptors rather than odorant receptors, since the latter contain a much smaller amino-terminus (~40 residues). It was expected that receptor 5.24 would respond to pheromones, but instead was specifically activated with basic amino acids. Since amino acids represent powerful odorants for catfish olfaction, it seemed reasonable that catfish should express a similar receptor. Primers were designed using the sequences reported by Speca et al. (1999) for amplification of total RNA isolated from catfish and goldfish olfactory epithelium by RT-PCR. Sequence analysis of the amplification products confirmed that these sequences were homologous to the goldfish odorant receptors.
In situ hybridization was performed using the RT-PCR amplification product described above as probe (called aa64) to determine if the product was expressed in olfactory sensory neurons. Hybridization results showed that the probe localized within the sensory region of the olfactory epithelium to what appeared to be microvillar neurons. The probe was evenly distributed over the olfactory epithelium, with no apparent zonal expression pattern. A similar localization pattern was obtained for the goldfish 5.24 receptor. This is in contrast to the localization pattern obtained with probes derived from the original catfish odorant receptors identified by Ngai et al. (1993a). The “Ngai-type” odorant receptors localized to neurons with cell bodies located deeper in the olfactory epithelium, corresponding to ciliated neurons. The aa64 clone represents the first putative receptor localized to catfish neurons with cell bodies close to the surface of the epithelium, suggesting microvillar ORN expression.

5’ and 3’ RACE was performed on total RNA isolated from catfish olfactory epithelium and a full-length clone corresponding to 2804 bp and 854 amino acids was obtained. TMpred and SOSUI software both predict a transmembrane protein based on the amino acid sequence of the coding region of aa64. ClustalW multiple alignment and BLAST indicate that catfish aa64 receptor is more similar to mGluR, CaSR, and pheromone receptors than it is to the catfish “Ngai-type” odorant receptors. In fact, the aa64 receptor is more similar to pheromone receptors than to goldfish 5.24. Northern blot analysis also showed that aa64 exhibits a more restricted pattern of expression in catfish tissue than does receptor 5.24 in goldfish tissue. The goldfish 5.24 receptor was located in regions outside of the olfactory epithelium, like gill and oral palate, where aa64 was restricted to the olfactory epithelium. Interestingly, aa64 contains the conserved residues in intracellular loop III that
are associated with \( G_q \) coupling in mGluR1\(\alpha \). This is the same G protein that is likely to mediate the primary olfactory response in catfish.

Clearly, the determination of the ligand binding properties of aa64 is crucial for categorizing the protein as an odorant receptor. Given that aa64 is more similar to vomeronasal receptors than is goldfish 5.24 receptor, it cannot be discounted that aa64 may respond to pheromonal cues; thus I am referring to aa64 by the more broad term of chemosensory receptor. Expression of the aa64 protein in HEK293 cells should provide a means of determining ligand binding properties. These cells contain the appropriate G proteins for signal transduction as assessed by goldfish 5.24 heterologous expression. In HEK293 cells, goldfish 5.24 preferentially activated \( G_q \) with a concomitant increase in intracellular \( Ca^{2+} \); thus, a rise in intracellular calcium can be related to the efficiency of ligand binding. Since aa64 is similar to golfish 5.24 and displays the necessary amino acids in intracellular loop III attributed with \( G_q \) coupling in mGluR1, the aa64 receptor is likely to be functional in HEK293 cells. Should these cells prove to be difficult for expressing aa64, the \textit{odora} cell line would make an excellent alternative. In addition to exploring the ligand binding properties of the receptor, the second messenger system initiated by activated aa64 could also be evaluated by heterologous expression. Incubation of the cell media with labeled phosphate would allow incorporation of the label into second messengers upon receptor stimulation for detection of those second messengers.

Successful heterologous expression and determination of the ligand(s) for aa64 would provide an additional tool for exploration of the data presented in chapters 2 and 3. For example, having a functional odorant receptor clone would allow for mutation of particular sites within the receptor to observe the effect on internalization. Such mutational analyses
could elucidate arrestin binding sites or interactions with other proteins (NSF) involved in both internalization kinetics and signaling scaffold formation (Src).
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Wilden, U., S. W. Hall, and H. Kuhn (1986). Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48 kDa protein of rod outer segments. *Proceedings of the National Academy of Science USA* 83, 1174-1178.


APPENDIX: LETTER OF PERMISSION

From:"Doyle, Lindsay" <Lindsay.Doyle@blacksci.co.uk> on 04/29/2002 10:04 AM CET
Sent by: "Doyle, Lindsay" <Lindsay.Doyle@blacksci.co.uk>

To: <mrankin@lsu.edu>
cc: <jneurochem-feedback@forsythe.stanford.edu>
Subject: RE: Publishing to dissertation (JNEUROCHEM Feedback Form)

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Michele Rankin
VITA

Michele Rankin obtained a Bachelor of Science degree with a major in chemistry and a minor in English in 1990 from Louisiana State University. Michele accepted a research and development position at CoPolymer Chemical Plant in Baton Rouge in the summer of 1990. She continued her education at Louisiana State University by joining Dr. Pat DiMario’s laboratory in the Department of Biochemistry, studying the protein nucleolin and pre-ribosomal RNA processing in Xenopus laevis and received her Master of Science degree in 1997. Michele also worked as a research associate for Dr. Richard Bruch in the Department of Biological Sciences at Louisiana State University from 1996-1998 studying catfish RGSs before joining his laboratory as a doctoral student. Michele’s dissertation work focused on the modulation of odorant receptors in the channel catfish Ictalurus punctatus. Michele will complete her studies and earn the degree of Doctor of Philosophy in August of 2002.