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RECEPTOR KINASE GENE EXPRESSION IN OLFACTORY RECEPTOR NEURONS

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ABSTRACT

Amino acids, potent olfactory stimuli for channel catfish (Ictalurus punctatus), elicit rapid and transient increases in phospholipid-derived second messengers. Activation of the second messenger signaling cascade is initiated by the binding of odorants to G-protein coupled odorant receptor proteins. Biochemical evidence in mammals suggests that signaling through the odorant receptors is terminated by the action of protein kinases that act to desensitize the receptors by phosphorylation. We have therefore investigated the expression of protein kinase genes in catfish olfactory tissue and neurons as a first step in studying the mechanisms of olfactory desensitization and signal termination in this organism. Random hexamers, oligo-dT and degenerate primers were used in the polymerase chain reaction (PCR) to amplify sequences encoding members of the G-protein coupled receptor kinase (GRK) multigene family. Sequence analysis showed that catfish GRK PCR products shared 86% and 92% amino acid sequence identity to human βark1 and bovine βark2 respectively. These results clearly demonstrate that a βark gene is expressed in the catfish olfactory tissue and isolated olfactory neurons.

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ABBREVIATIONS

GDP : guanosine diphosphate

GTP : guanosine triphosphate

PLC : phospholipase C

PIP2 : phosphatidylinositol 4,5-bisphosphate

DAG : diacylglycerol

IP3 : inositol 1,4,5-triphosphate

PKC : protein kinase C

GRK : G-protein-coupled receptor kinase

βARK: β-adrenergic receptor kinase

PH : pleckstrin homology

RT : reverse transcroption

PCR : polymerase chain reaction

INTRODUCTION

The olfactory system in the channel catfish (*Ictalurus punctatus*) serves as an appropriate model for the study of olfaction due to the dependency of the catfish on their ability to respond to chemical stimulants. Catfish use their olfactory systems to help them locate food, avoid predators and choose their mates. Therefore, the catfish olfactory system has become highly adapted to insure the survival of the species and is capable of detecting stimuli in nanomolar concentrations (1). Olfactory stimuli for channel catfish include amino acids and bile salts (2).

As in other vertebrate olfactory systems, chemical stimulants are recognized upon binding to a receptor protein from the seven transmembrane domain superfamily. The binding of an odorant to the receptor causes a dissociation of a heterotrimeric G-protein [figure 1]. The Gα subunit remains associated with the Gβ and Gγ subunits as long as it is bound to GDP. However, once the receptor is stimulated, the Gα subunit exchanges GTP for GDP causing the dissociation of the heterotrimer into a free GTP-bound α subunit and a βγ dimer (3). G-protein subunits are responsible for stimulating phospholipase C (PLC) which in turn is responsible for the cleavage of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) (4). PIP_2 is cleaved into two second messengers, diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). The function of IP3 in olfactory neurons is to generate a rapid and transient depolarization by binding to a receptor in the membrane that is also a nonselective cation channel that passes calcium. By binding to the receptor on the channel, IP3 causes calcium to enter the cell therefore causing membrane depolarization. Calcium may serve a dual role by not only causing membrane depolarization, but also by opening a calcium dependent chloride channel (3). The other second messenger, DAG, is responsible for activating a protein kinase C (PKC)

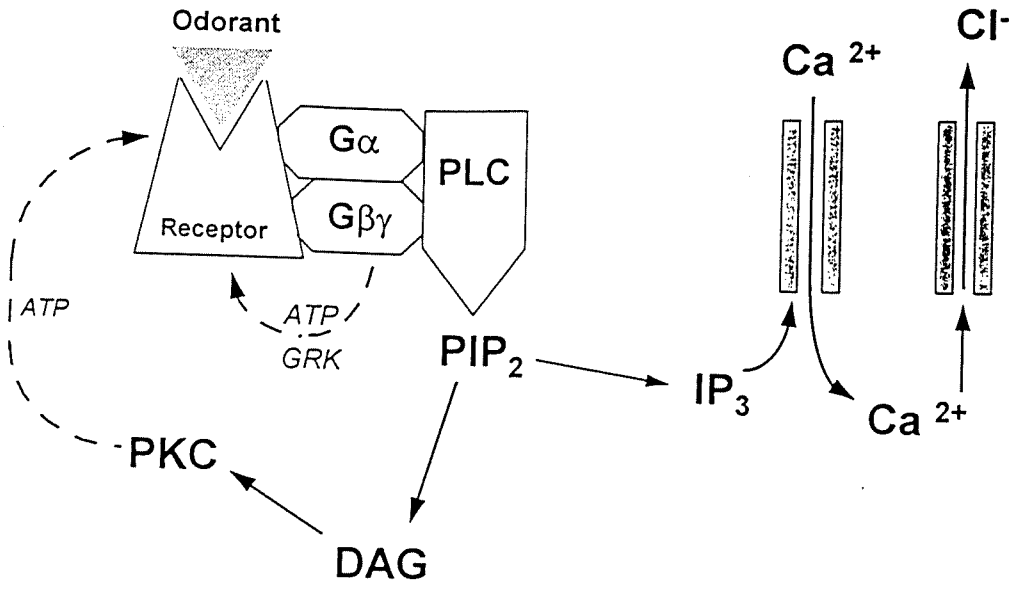

FIGURE 1. G-PROTEIN COUPLED RECEPTOR AND ASSOCIATED PROTEINS. Odorant binding to a member of the multigene odorant receptor family activates a G-protein which couples the receptor to a phospholipase C (PLC). PLC catalyzes the phosphodiesteratic cleavage of the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2) releasing the second messenger inositol 1,4,5-trisphosphate (IP3) and diaclyglycerol (DAG). IP3 binds to its receptor that is itself a calcium-permeable cation channel, resulting in influx of calcium and increased intracellular calcium levels that subsequently gate a calcium-activated chloride channel. DAG is expected to stimulate the activity of protein kinase C (PKC). PKC, together with G-protein coupled receptor kinases (GRK) and Gβγ, participates in signal termination by phosphorylating the odorant receptor (3).

which is then partially responsible for termination of the signaling event by phosphorylating the odorant receptor (3,5). Three other proteins work together with PKC to achieve this result. Both the G-protein-coupled receptor kinases (GRK) and the $G\beta\gamma$ dimer play a role in the desensitization and signal termination of the G-protein-coupled odorant receptor (3).

The focus of the present study is the GRK proteins that are responsible for the termination of the signaling event through the phosphorylation of a receptor from the seven transmembrane domain receptor superfamily. The second messenger cascade that results from the binding of an odorant to its receptor is turned off through a negative feedback mechanism involving GRKs (6). The G-protein-coupled receptor kinases phosphorylate receptors on multiple serine and threonine residues on the intracellular loops and carboxyl terminal tail (7). Once the receptor has been phosphorylated, arrestin proteins are responsible for the uncoupling of the GRK protein from the receptor (8). Six different proteins have been identified from the GRK family by molecular cloning [figure 2]. Each of the GRK proteins have a similar structure which includes a centrally located catalytic core that is surrounded by an amino-terminus of approximately 185 amino acids and a carboxyl-terminus of various lengths (7,9). The catalytic domain contains invariant amino acids that are present in all members of the protein kinase superfamily. The amino-terminus domain is probably responsible for the recognition of the receptor protein which is conserved among the six subfamilies. The carboxyl-terminus domain may be involved in receptor targeting and membrane association. This is the most variant region of the GRK sequence and may contain the signal for any post-translational modifications (9).

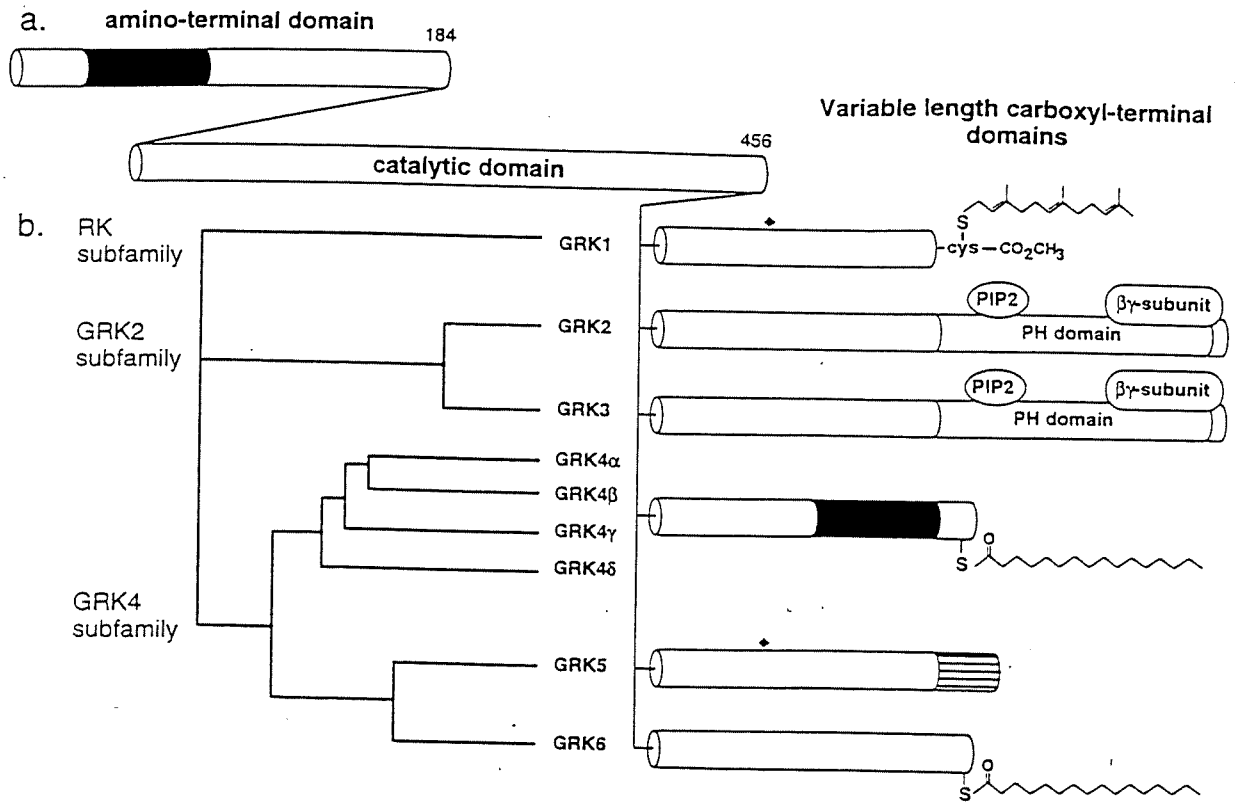

FIGURE 2. THE GRK FAMILY. This schematic representation of the GRK domain architecture shows the similarities and differences between the three GRK families (7).

In general, all six subfamilies of GRKs exhibit three fundamental characteristics which include a preference for activated receptors, a translocation from the cytoplasm to the plasma membrane and the phosphorylation of a receptor at multiple serine and threonine residues (10). The six members of the GRK family can be further subdivided into three subfamilies based on their carboxy-terminus domains (7).

Rhodopsin kinase is the sole member of the rhodopsin kinase subfamily and is also called GRK1. This protein is responsible for phosphorylating light-bleached rhodopsin in the visual system. This light-dependent kinase phosphorylates rhodopsin at up to nine different sites on the carboxyl-terminus. Rhodopsin kinase is exclusively found in the retina and in the pineal gland and is the most studied GRK because it was the first to be discovered. The carboxyl-terminus sequence of GRK1 contains regions that signal for two post-translational modifications including farnesylation and carboxymethylation which differs from the carboxyl-terminus domains of the other five GRKs. GRK1 also undergoes a translocation in response to the activation of the receptor from the cytoplasm to the plasma membrane (7,11).

The β-adrenergic receptor kinase (βARK) subfamily is composed of the GRK2 (βARK1) and GRK3 (βARK2) proteins. The original distinctions of βARK1 and βARK2 were derived from the substrates that these proteins target which are β1 adrenergic receptors and β2 adrenergic receptors respectively. These GRKs are not localized like rhodopsin kinase. Instead, they are found in multiple tissue types and are responsible for phosphorylating β-adrenergic receptors, which are found throughout the body (7,12). GRK2 is very abundant in the brain and peripheral tissues, while GRK3 is

found in high levels in olfactory neurons and in sperm. In a manner similar to that of rhodopsin kinase, the β-adrenergic receptor kinases undergo a cytoplasm to plasma membrane translocation. The sequences for GRK2 and GRK3 contain a pleckstrin homology (PH) domain of approximately 100 amino acids which is unique to this subfamily. The GRK2 and GRK3 proteins are also the only members of this gene family that are activated by G protein βγ subunits (9).The increased degree of complexity in this subfamily may be due to the necessity to increase its selectivity since multiple receptors will be present in the environment. This is very different from the rhodopsin kinases which will only be exposed to a single type of receptor (7). The amino acid sequences of GRK2 and GRK3 show 98% homology at the catalytic site and 85% homology overall. These sequences are approximately 34% homologous overall to rhodopsin kinase (11).

The third subfamily is composed of GRK4, GRK5 and GRK6. Less is known about this subfamily since these proteins were identified by molecular cloning instead of being characterized functionally (7). The proteins in the GRK4 subfamily are found in a wide variety of tissue types, but the substrates targeted by these proteins have not been identified. Only the substrate for GRK5 which is the β2 adrenergic receptor has been determined. GRK4 is primarily expressed in the testes, however, it is present in small amounts in many tissue types. GRK4 was discovered using cloning methods in the search for the Huntington's disease locus and was originally named IT-11 (10,12). GRK5 is also present in small amounts in many tissue types, but most highly in the heart, placenta, lung and retina. GRK5 is expressed in its lowest levels or absent in

brain, liver and kidney tissue. GRK6 is also expressed in a wide variety of tissues and is found in the highest levels in the brain and skeletal muscle (7,10,11). Unlike all of the other GRK proteins, members of the GRK4 subfamily are membrane associated proteins that do not undergo the cytoplasm to plasma membrane translocation (7,10). The GRK4 subfamily also differs from other GRKs by a consensus sequence of Asp-Leu-Gly present in the catalytic domain rather than the Asp-Phe-Gly present in the catalytic domain of other GRKs (11). The sequence of the GRK4 protein is unique from other GRKs. GRK4 exists in four splice variant forms that vary by alternative splicing of two exons. One exon encodes 32 amino acids on the amino-terminus and the other consists of 46 codons on the carboxyl-terminus. In its longest form the GRK4 protein is formed from 578 amino acids (10,12). The sequence of the GRK5 protein also contains a domain that is unique from all other GRKs. This domain of 46 amino acids on the carboxyl-terminus is highly basic and is responsible for the membrane association of the GRK5 protein (7,10). GRK6 has a sequence of 570 amino acids (10). The amino acid sequences of GRK4, GRK5 and GRK6 are 68-70% homologous to each other and they are 35-38% homologous to the β-adrenergic receptor kinase subfamily (11).

The purpose of the present study was to investigate which member(s) of the GRK gene family are expressed in olfactory neurons of channel catfish to identify candidate GRK protein(s) that may be responsible for the desensitization of the odorant receptors. Because biochemical data suggested that G-protein βγ-subunits are required for activation and translocation of a GRK in isolated rat olfactory cilia (6), only the expression of GRKs regulated in this manner was investigated (i.e. GRK2 and

GRK3). Using primers complementary to conserved sequences of GRK2 and GRK3, RT-PCR was used to study the expression of these genes in olfactory tissue and isolated olfactory neurons.

MATERIALS AND METHODS

Reverse transcription-PCR: Total RNA was isolated from intact olfactory rosettes using Trizol reagent following the protocol supplied with the reagent (Gibco, Grand Island, NY). Poly (A)+ RNA was isolated from olfactory neurons lysates using oligo-dT coupled to Dynabeads following the vendor's protocol (Dynal, Lake Success, NY). Reverse transcription primed with random hexamers, oligo-dT and GRK2 and GRK3 gene specific primers 3F (sense) and 3R (antisense)(13) and the GRK4 gene specific primers encoding PPFCPD (sense) and EPKQC* (antisense)(12) was performed with Superscript II following the protocol supplied with the enzyme (Gibco). PCR reactions were initiated by the addition of Taq polymerase at 90° C. Thermal cycling was performed for 40 cycles of 95°C for 1 minute, 60°C for 1 minute, 72°C for 2 minutes. Reactions were terminated by incubating the samples at 95°C for 15 minutes and slowly cooling over several minutes to 4°C. The approximately 600 base pair products were cloned into pCR II (Invitrogen, San Diego, CA) and propagated in DH5α competent cells on LB plates supplemented with 50 μg ml^{-1} ampicillin and 1 mg per plate X-Gal. Plasmids harboring inserts were identified by restriction digestion with *Eco*RI. Positive plasmids were grown in LB broth cultures and purified using columns and protocols supplied by Qiagen (Chatsworth, CA). DNA sequencing was performed with the Fidelity sequencing system (Oncor, Gaithersburg, MD). GRK sequences were identified by comparison to sequences in the databanks using the BLAST program (National Centre for Biotechnology Information, Bethesda, MD). Predicted amino acid sequences were obtained using the Gene Runner program (Hastings Software, Hastings NY).

Isolation of olfactory receptor neurons: Channel catfish, 15-20 cm in length, were deeply anesthetized in MS-222 (1:5000) and sacrificed. Olfactory rosettes were

rapidly excised and placed in 35 mm Sylgard-coated petri dishes containing fish Ringer's solution. The rosettes were immobilized with pins and dissociated with activated papain as described (14). Dissociated cells were visualized by phase contrast microscopy and were identified by their characteristic morphologies. Identified cells were harvested by applying gentle suction with a syringe connected to a 20 μm bore microcapillary. Microcapillaries were fabricated with a patch-clamp pipette puller and were siliconized (Sigmacote, Sigma Chemical Co., St Louis, MO) to prevent adherence of cells to the outside of the capillary. Isolated cells were transferred to 0.5 ml sterile microcentrifuge tubes containing 100 μl ice-cold lysis buffer. Lysis buffer contained 1% NP-40, 10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 5mM KCl, 5mM DTT and 40 units/ml RNAsin. Cell lysates were centrifuged at 12 000 X g for 2 minutes to remove cellular debris and the supernatants were transferred to new sterile microcentrifuge tubes. Cell lysates were used immediately or were stored frozen.

Southern Blotting: PCR products from olfactory neurons were resolved in a 2% agarose gel and transferred to a nylon membrane overnight with 10x SSC. Following baking of the membrane for 1 hour at 80°C, prehybridization was performed for 30 minutes at 65°C with Rapid-hyb buffer (Amersham, Arlington Heights, IL). Hybridization was performed at the same temperature for 2 hours with a digoxigenin-labeled cDNA probe (5 ng ml^{-1} in Rapid-hyb buffer) prepared from a GRK PCR product obtained from olfactory rosettes. The probe was gel purified and labeled overnight with the Klenow enzyme (Boehringer Mannheim, Indianapolis, IN). Following hybridization, the membrane was washed twice for 5 minutes each in 2x SSC/0.1% SDS at room temperature and twice for 15 minutes each at 65°C in 0.5x SSC / 0.1% SDS. Hybrids were detected using an alkaline phosphatase-conjugated

antibody to digoxigenin and the chemiluminescent substrate CSPD (Boehringer Mannheim).

RESULTS AND DISCUSSION

PCR of GRK sequences in olfactory tissue: Reverse transcription of total RNA isolated from olfactory tissue was primed using three types of primers. In order to assess the feasibility of using the PCR primers originally designed from mammalian sequence data to amplify the GRK sequences from catfish, the reverse transcribed cDNA was used in the PCR. Following PCR, agarose gel analysis showed bands corresponding to PCR products approximately 600 base pair in length in all lanes as expected [figure 3]. No bands were detected in the negative control and the lane corresponding to the sample primed with the GRK4 specific primers. These results suggested that GRK2 and/or GRK3 were expressed in olfactory tissue and that GRK4 was not expressed in this tissue. The PCR products were gel purified and ligated into the cloning vector. Following transformation of competent cells, colonies were screened for plasmids with 600 base pair inserts by restriction digestion. Partial sequence analysis of the cloned PCR products showed that these products were similar to members of the βARK subfamily.

PCR of GRK sequences in isolated olfactory neurons: In order to determine which GRK member was expressed in olfactory neurons, PCR was performed using the random primed cDNA from four groups of two cells each. The PCR products were analyzed by agarose gel electrophoresis, but the bands were not visible. Therefore they were visualized using Southern analysis [figure 4]. The Southern blot showed hybridization bands corresponding to a PCR product of approximately 600 base pair in all four groups of neurons. The PCR products obtained from each group of cells were excised from the gels and individually ligated into the cloning vector. Sequence analysis of six cloned GRK PCR products obtained from the isolated cells showed that the sequence was identical in all of the clones and was also identical to that obtained from olfactory tissue. The GRK sequence obtained from the 600 base pair PCR product shared 80% sequence similarity to mammalian βark genes. The predicted

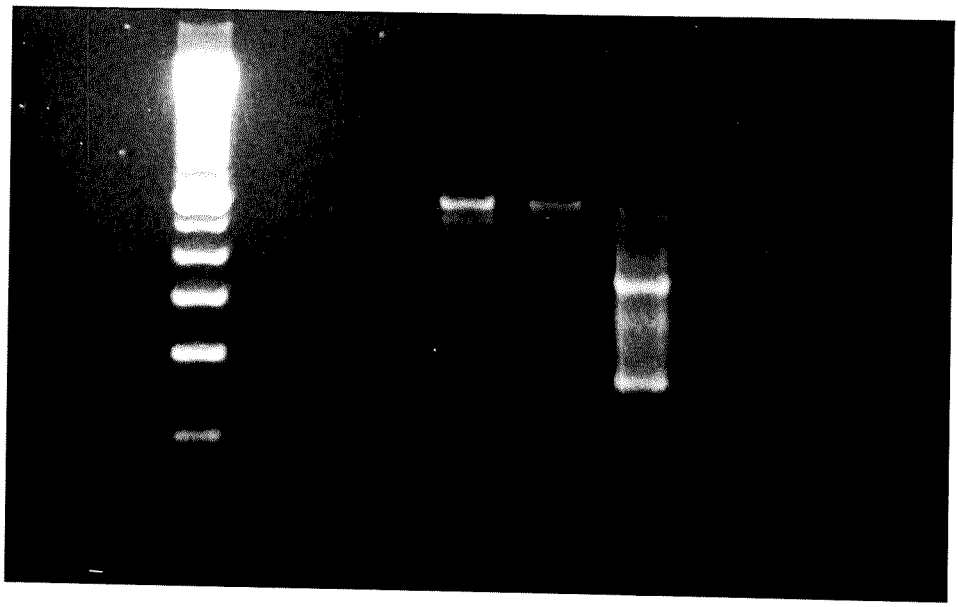

FIGURE 3. GRK PCR RESULTS. Reverse transcription was performed with random hexamers (lane 2), oligo dT (lane 3) and two gene-specific primers (lanes 4 and 5). A PCR product of the expected size of about 600 base pair was obtained with random hexamers, oligo dT and one of the gene specific primers. No bands were detected in the negative control (no reverse transcriptase, lane 1).

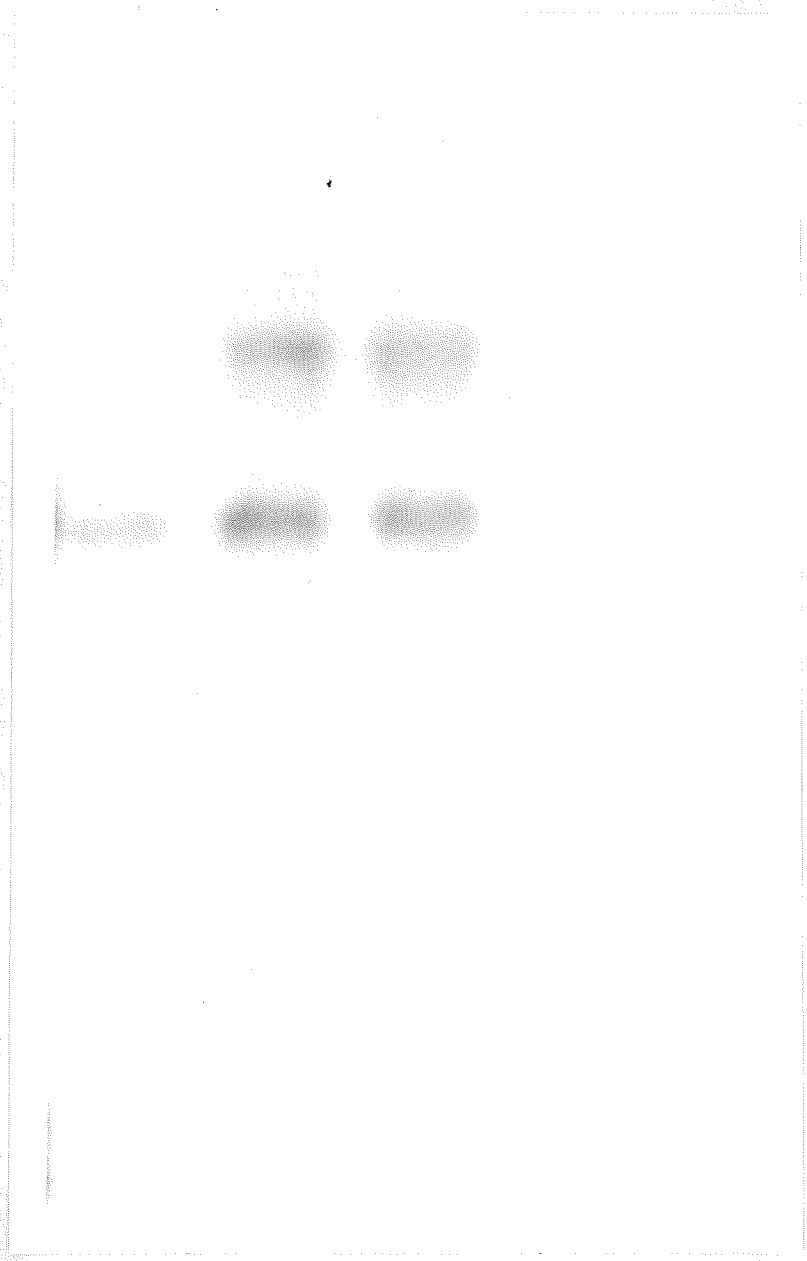

FIGURE 4. SOUTHERN ANALYSIS OF NEURON PCR RESULTS. The Southern blot showed hybridizaton bands corresponding to a PCR product of approximately 600 base pair in all four groups of neurons. The high molecular weight bands (lanes 2 and 3) are unidentified.

amino acid sequence indicated that the catfish GRK PCR product was 86% identical to human βark1 and 92% identical to bovine βark2 [figure 5]. Therefore, the GRK PCR products obtained from isolated olfactory neurons were most closely related to the βark2 gene.

Other identification experiments: In an attempt to prove that only one GRK sequence was being expressed in the olfactory neurons, restriction digestions of the PCR products using frequent cutting enzymes were analyzed by agarose gel electrophoresis. The results were noninformative due to the problem of resolution of small fragments on an agarose gel. All of the frequent cutting enzymes that cut GRK2 and GRK3 sequences differently, produced fragments less than 100 base pair which are difficult to resolve on an agarose gel. Since the βark1 and βark2 proteins are closely related at the amino acid level, we attempted to use comercially available specific antibodies to each βark on Western blots to determine which protein was expressed in isolated olfactory neurons. Unfortunately, these antibodies produced unacceptable background staining in our experiment (unpublished observations).

Summary: βark1 and βark2 proteins share 85% amino acid similarity overall and human βark1 shares 98% amino acid identity with the bovine enzyme (11). The GRK PCR product obtained from catfish olfactory tissue and from isolated olfactory neurons shared 86% and 92% amino acid sequence identity to human βark1 and bovine βark2 respectively. We were unable to use Western blot analysis or restriction digestion to definitively assess the identity of the βark protein expressed in catfish. However, our results clearly demonstrate that a βark gene is expressed in catfish olfactory receptor neurons that shares significant sequence identity to mammalian βark genes, most closely to bovine βark2. This result is consistent with previous

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hβARK1   DWFSLGCMLFKLLRGHSPFRQHKTKDKHEIDRMTLTMAVELPDSFSP
bβARK2   DWFSLGCMLFKLLRGHSPFRQHKTKDKHEIDRMTLTMNVELPDSFSP
fβARK    DWFSLGCMLFKLLRGHSPFRQHKTKDKHEIDRMTLTMNVELPDVFSP

         ELRSLLEGLLQRDVNRRLGCLGRGAQEVKESPFFRSLDWQMVFLQKY
         ELKSLLEGLLQRDVSKRLGCHGGSAQELKTHDFFRGIDWQHVYLQKY
         ELKSLLEGLLQRDVAKRLGCQGQGASEVKDHLFFKGIDWQQVYLQKY

         PPPLIPPRGEVNAADAFDIGSFDEEDTKGIKLLDSDQELYRNFPLTI
         PPPLIPPRGEVNAADAFDIGSFDEEDTKGIKLLDCDQELYKNFPLVI
         SPPLIPPRGEVNAADAFDIGSFDEEDTKGIKLLDSDQELYKNFPLVI

         SERWQQEVAETVFDTINAETDRLEARKKAKNKQLGHEEDYALGKDCI
         SERWQQEVAETVYEAVNADTDKIEARKRAKNKQLGHEEDYALGRDCI
         SERWQQEVAETVYEAVNSDTDKNEARQRAKNKQQGHEEDYALGKDCI

         MH
         MH
         VH
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FIGURE 5. AMINO ACID SEQUENCE DATA. The predicted amino acid sequence data of the catfish GRK PCR product (fβark) compared to the amino acid sequences of human βark1 (hβark) and bovine βark2 (bβark). The boldface amino acids show the differences between the three sequences.

immunocytochemical (15), biochemical (6) and rapid kinetic (16) data collected from mammalian systems.

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