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Protein Kinase Cβ and δ Selectively Phosphorylate Odorant and Metabotropic Glutamate Receptors

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Abstract

Recombinant protein segments from a metabotropic glutamate receptor and from an odorant receptor were used as substrates in protein kinase C phosphorylation assays. Protein kinase Cβ and δ phosphorylated an intracellular consensus phosphorylation site in the metabotropic glutamate receptor. Only protein kinase Cδ phosphorylated a novel extracellular consensus phosphorylation site in the odorant receptor. These results suggest differential regulation of these receptors by protein kinase C isotypes.

Introduction

It is well established that seven transmembrane domain (7TMD) receptors are regulated by protein kinases. Protein kinase-mediated phosphorylation of 7TMD receptors initiates a series of molecular events leading to desensitization and internalization of the receptors (reviewed in Bohm *et al.*, 1997; Ferguson *et al.*, 1997). Most 7TMD receptors are phosphorylated in a ligand-dependent manner by G-protein-coupled receptor kinases (GRKs). We (Bruch *et al.*, 1997) and others (Dawson *et al.*, 1993) have shown that GRK3 (βARK2) is expressed in vertebrate olfactory neurons. Targeted disruption of the GRK3 gene in transgenic knockout mice led to loss of odorant-induced desensitization, indicating a critical role for GRK3 in odorant receptor desensitization (Peppel *et al.*, 1997). In addition to GRKs, 7TMD receptors may also be phosphorylated in a ligand-independent manner by second messenger-dependent protein kinases such as the cyclic AMP-dependent protein kinase and protein kinase C (PKC). The PKC family consists of a dozen genes and splice variants (Liu, 1996). Two PKC family members, β and δ, are expressed in catfish olfactory receptor neurons (Bruch *et al.*, 1997).

Channel catfish (*Ictalurus punctatus*) olfactory receptor neurons express multiple 7TMD receptors such as odorant receptors (Ngai *et al.*, 1993) and metabotropic glutamate receptors (mGluRs; Medler *et al.*, 1998). Inspection of the originally published (Ngai *et al.*, 1993) catfish odorant receptor sequences indicates that half of the receptors contain a PKC consensus phosphorylation site located between transmembrane domains IV and V. Based on current understanding of 7TMD receptor structure and membrane topology, this region of the odorant receptors containing the consensus site is predicted to be extracellular loop 2. The presence of an extracellular PKC consensus site in these receptors is an apparently novel feature of their structures and may be a unique structural feature within the 7TMD receptor superfamily. In contrast, mGluR1, which is also expressed in catfish olfactory receptor neurons (Medler *et al.*, 1998), contains a PKC consensus site between transmembrane domains III and IV. This region of mGluR1 is predicted to be intracellular loop 2.

Given the different positions and predicted membrane orientation of PKC phosphorylation sites in odorant receptors and mGluRs, we investigated the ability of PKCβ and δ, previously shown to be expressed in catfish olfactory receptor neurons (Bruch *et al.*, 1997), to phosphorylate the consensus sequences in both receptors *in vitro.* Segments of the receptors containing the PKC consensus sequences were expressed as fusion proteins, purified and used as substrates in phosphorylation assays to determine whether PKC β and δ displayed differential substrate specificities and whether PKC would phosphorylate the unusual odorant receptor extracellular consensus site. Both PKC isotypes phosphorylated the intracellular mGluR1 consensus site. In contrast, only PKCδ phosphorylated the extracellular odorant receptor consensus site. The specificity of the phosphorylation assay was confirmed using mutant (S/T to A) fusion proteins that were not phosphorylated by either PKC isotype. These results show that PKC isotypes are capable of differential interaction with odorant receptors and mGluRs *in vitro* and suggest these receptors may be differentially regulated by PKC isotypes.

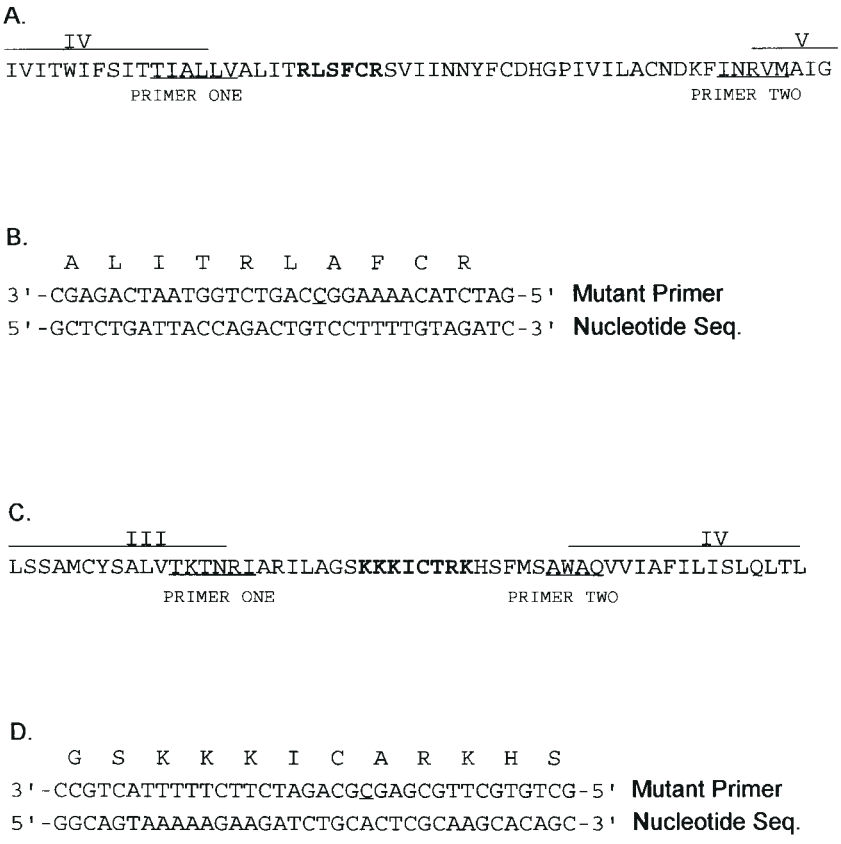

Figure 1 Hydrophilic loop domains containing PKC consensus phosphorylation sites in an odorant receptor **(A)** and mGluR1 **(C)**. Bold letters indicate the consensus sequences and underlined letters indicate amino acid sequences used to design PCR primers for amplification of the receptor loops. Transmembrane domains are indicated by Roman numerals. PCR primers used to mutate the serine and threonine residues within the consensus sequences to alanine in the odorant receptor **(B)** and in mGluR1 **(D)**. Nucleotide changes are underlined.

Methods, results and discussion

Wild type and mutant hydrophilic loops of the odorant receptor and mGluR1 containing the consensus PKC phosphorylation sequences were amplified from previously cloned receptor PCR products (Medler *et al.*, 1998) using specific primers for each receptor (Figure 1). Receptor segments were amplified with *Taq* polymerase using the following thermal cycling program: 90°C for 5 min, 95°C for 1 min, 48°C (or 54°C for mGluR1) for 1 min, and 72°C for 1 min for 40 cycles. After a final 10 min extension at 72°C, the reactions were terminated by heating to 95°C for 15 min and cooling of the samples to 4°C over several minutes. Amplified PCR products (~150 bp for the odorant receptor and 100 bp for mGluR1) were gel purified and ligated into pCR 2.1 (Invitrogen, Carlsbad, CA). Complete DNA sequence analysis on both strands confirmed that the products encoded the desired hydrophilic loops of the receptors (Figure 1).

Site-directed mutagenesis was used to mutate the serine and threonine residues within the consensus PKC sequences to alanine. Mutagenesis was performed using the Quikchange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). Specific primers for each receptor were used to obtain the desired mutations (Figure 1). The primers were used in the following thermal cycling program: 95°C for 1 min, 55°C for 1 min and 68°C for 12 min for 1 cycle followed by 15 cycles of the same program with the 95°C denaturation shortened to 30 s. Products were digested with *Dpn*I to remove non-mutated plasmids and transformed into Epicurean Coli XL1-Blue Supercompetent cells. Purified plasmids with inserts were sequenced to confirm mutagenesis of the desired nucleotide and to verify that no additional mutations were introduced by PCR.

Wild type and mutant receptor sequences in pCR2.1 were excised with *Eco*RI and subcloned into the pThioHis prokaryotic expression vector (Invitrogen). DNA sequence analysis confirmed the correct in frame orientation of the subcloned sequences. Protein expression was induced with 1 mM IPTG at 37°C and proceeded for 2 h. Because the thioredoxin moiety in the fusion protein localizes to osmotically sensitive adhesion zones, purification of the fusion proteins was performed by osmotic shock following a protocol provided by Invitrogen in the presence of 0.1 mg/ml PMSF. Proteins isolated by osmotic shock were separated on a 15% SDS–polyacrylamide gel. Fusion proteins were located by Western blotting with the anti-

Thio antibody (Invitrogen, 1:5000 dilution). The fusion proteins were excised from the gel, electroeluted and concentrated in Centricon-10 devices (Millipore, Bedford, MA). Due to a potential phosphorylation site in the fusion proteins upstream from the receptor sequences, enterokinase (Invitrogen) was used to cleave the fusion proteins. Following overnight incubation with 1 U of the enzyme at 4°C, the reaction mixtures were separated on an 18% SDS–polyacrylamide gel. Proteins were visualized with GELCODE Blue Stain Reagent (Pierce, Rockford, IL) and bands of the appropriate size (~9 kDa for the odorant receptor and 5.5 kDa for mGluR1) were excised, electroeluted, concentrated with Centricon-3 devices and exchanged into assay dilution buffer (20 mM MOPS, pH 7.2, 1 mM dithiothreitol, 1 mM $CaCl_2$).

A mixed micellar assay was used to evaluate PKC phosphorylation (Hannun *et al.*, 1985). Purified (>95%) recombinant PKCβII and δ (Upstate Biotechnology, Lake Placid, NY) were diluted tenfold with enzyme dilution buffer (supplied by the vendor). [γ-^{32}P]ATP (3000 Ci/mmol) was diluted tenfold with assay dilution buffer supplemented with 75 mM $MgCl_2$ and 500 μM ATP. Dioleoyl L-α-phosphatidyl-L-serine (PS) and 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) were diluted in chloroform and dried in a glass tube. The lipids were solubilized in 3% Triton X-100 in assay dilution buffer to final concentrations of 0.5 mg/ml PS and 0.05 mg/ml OAG. Assays contained ~400 ng of homogeneous substrate, 10 μl of lipids and 10 μl of diluted radiolabeled ATP in a total volume of 50 μl. Reactions were initiated by the addition of PKC (25 ng) and were incubated at room temperature for 25 min. Parallel samples lacking substrate were used to monitor PKC autophosphorylation. Reactions were stopped with 3 vol of 10% TCA or with 1 vol of 2× Laemmli buffer. Samples precipitated with TCA were analyzed in a filter assay (Bruch *et al.*, 1997). Samples in Laemmli buffer were separated on a 15% SDS–polyacrylamide gel. The dry gels were exposed to X-ray film at –80°C for at least 16 h.

The PKC phosphorylation sequences identified in the odorant receptor and in mGluR1 (Figure 1) conformed to the generalized consensus sequence $(R/K)_{1–3}$-$(X)_{0–2}$-S/T-$(X)_{0–2}$-$(R/K)_{1–3}$ where X represents any amino acid (Pinna and Ruzzene, 1996). The hydrophilic loops from both receptors containing the PKC consensus sequences were expressed as fusion proteins and used as substrates in phosphorylation assays. To verify that the PKC isotypes were active, assays were performed using histone (type III, Sigma Chemical Co., St Louis, MO), a generic PKC substrate. Enzyme activity was determined by measuring the incorporation of radiolabeled phosphate into TCA precipitable material. In triplicate assays, PKCβ had a sp. act. of 0.124 ± 0.017 nmol phosphate/μg histone/min while that of PKCδ was 0.196 ± 0.014 nmol phosphate/μg histone/min. These results confirmed that both PKC enzymes were catalytically active. Receptor hydrophilic loops were also

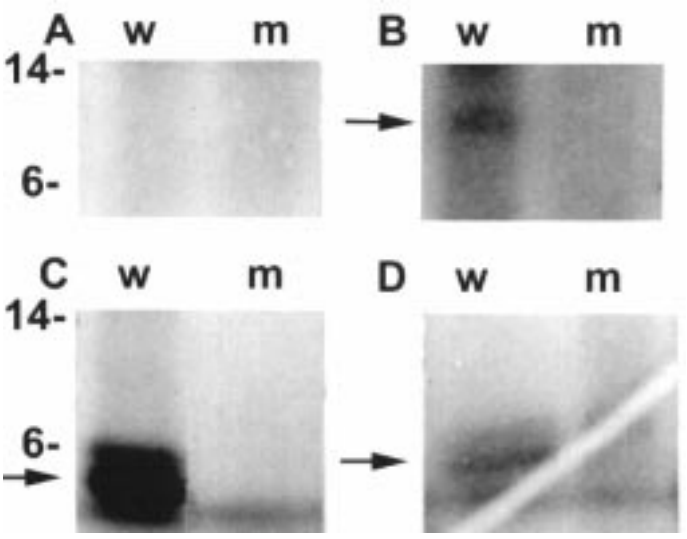

Figure 2 Autoradiograms of receptor phosphorylation assays. Lanes W represent wild type and lanes M represent mutant receptor substrates. PKCβ did not phosphorylate the odorant receptor substrate **(panel A)**, although it was phosphorylated by PKCδ **(panel B)**. Both PKCβ **(panel C)** and PKCδ **(panel D)** phosphorylated the mGluR1 substrate. Numbers to the left of the autoradiograms indicate molecular weights in kDa.

PKC substrates. Both PKCβ and δ phosphorylated the mGluR1 loop; however, only PKCδ was able to phosphorylate the odorant receptor loop (Figure 2). When mutant receptor loops were used as substrates no phosphorylation was detected, thereby confirming the specificity of the assay.

This study investigated *in vitro* the specific interactions between the PKC isotypes and 7TMD receptors expressed in olfactory receptor neurons. We have previously shown that the calcium-sensitive PKCβ and the calcium-insensitive PKCδ are expressed in olfactory neurons. We also showed that a specific inhibitor of calcium-sensitive PKC isotypes reduced odorant-induced phosphorylation to basal levels in isolated cilia preparations, suggesting that PKCβ mediated odorant-stimulated phosphorylation (Bruch *et al.*, 1997). These data, together with previous biochemical evidence (Boekhoff and Breer, 1992; Boekhoff *et al.*, 1992), suggested that PKC phosphorylated activated 7TMD receptors in olfactory neurons and played a role in receptor desensitization. Within this context, two 7TMD receptors known to be expressed in olfactory neurons—an odorant receptor (Ngai *et al.*, 1993) and mGluR1 (Medler *et al.*, 1998)—were used to test this hypothesis.

In this study we have shown that the mGluR1 PKC consensus sequence is phosphorylated by both PKCβ and δ, although the extent of phosphorylation catalyzed by PKCβ was qualitatively higher than that catalyzed by PKCδ. This apparent differential phosphorylation by the PKC isotypes may be indicative of their physiological roles *in vivo*. Activation of mGluR1 stimulates phospholipase C and increases intracellular calcium (Pin and Duvoisin, 1995). Increased intracellular calcium may activate the calcium-sensitive

PKCβ that could then phosphorylate and desensitize the receptor. Since there are no known interactions between GRKs and mGluRs (Gereau and Heinemann, 1998), PKC may play a critical role in desensitization of these receptors.

The mGluR1 consensus phosphorylation sequence that we studied resides within the second intracellular loop of the receptor, an important site for G-protein interaction in mGluRs (Francesconi and Duvoisin, 1998; Gereau and Heinemann, 1998). Deletion of residues C694 and T695 within the second intracellular loop significantly reduced the ability of the receptor to couple to second messenger signaling pathways (Francesconi and Duvoisin, 1998). Interestingly, T695 is the residue phosphorylated by PKCβ and δ in our study. The mGluR1 second intracellular loop is also critical for activation of a phospholipase C-coupled G-protein, and optimal coupling between the receptor and G-protein requires the 16 C-terminal residues of intracellular loop 2 (Gomeza *et al.*, 1996). The PKC consensus sequence tested in our study is also contained within the 16 C-terminal residues of the second intracellular loop. Taken with the results from other studies, the present study predicts that the PKC consensus sequence within intracellular loop 2 is a functional phosphorylation site that may play a role in receptor desensitization *in vivo*.

PKCδ, but not PKCβ, phosphorylated the PKC consensus sequence within the odorant receptor. This was a surprising result since our previous study suggested that the β isotype probably mediated odorant-stimulated phosphorylation (Bruch *et al.*, 1997). Since there are no additional PKC consensus sequences in the receptor, it is likely that PKCβ interacts with other non-receptor components following receptor activation. Although specific PKC substrates have not been identified in olfactory neurons, known substrates within the signaling pathway include phospholipase C, adenylate cyclase, IP_3 receptor, GRKs and dynamin (Liu, 1996; Pronin and Benovic, 1997).

In this study we have described the identification of a novel extracellular phosphorylation site in an odorant receptor. Although this site was differentially phosphorylated by PKCδ *in vitro*, the possible functional significance of phosphorylation at this site *in vivo* is unknown. Perhaps PKC phosphorylation in the extracellular loop is associated with receptor synthesis, processing or membrane targeting. Since these processes are not associated with acute increases in intracellular calcium, our observation of phosphorylation of the extracellular consensus site by the calcium-insensitive PKCδ may have physiological relevance. Ongoing heterologous expression experiments will test this hypothesis.

Recent evidence suggests that the extracellular domains of 7TMD receptors may have functional significance beyond merely acting as static structural components (reviewed by Ji *et al.*, 1998). For example, adrenergic receptor chimeras with mutations in the third extracellular loop exhibited altered affinity for agonist, presumably by affecting helical packing within the transmembrane domains (Zhao *et al.*, 1998). A mechanism for the binding of thyrotropin releasing hormone to its receptor has also been described in which the receptor extracellular domains may act as primary binding sites that guide the hormone into the binding site within the membrane (Colson *et al.*, 1998). A similar role for the second extracellular loop of the chemoattractant cAMP receptor of *Dictyostelium* has also been proposed (Kim *et al.*, 1997). It is of interest to note that the second extracellular loop of odorant receptors has also been proposed to function as a cell surface identifier for the particular receptor expressed in individual olfactory neurons (Singer *et al.*, 1995). Although it is tempting to speculate that the odorant receptor phosphorylation site in the second extracellular loop may affect the conformation and ligand binding properties of the receptor, or may serve some role as a cell surface marker, additional experiments are required to determine the functional role of this novel PKC consensus sequence.

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