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Functional characterization of *kurtz*, a *Drosophila* non-visual arrestin, reveals conservation of GPCR desensitization mechanisms

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ABSTRACT

The arrestins are a family of molecules that terminate signaling from many different G protein-coupled receptors, by inhibiting the association between receptor and downstream effectors. We recently employed a human βarrestin2-GFP fusion protein to explore the dynamics of different neuropeptide receptors in *Drosophila* and have previously used a βarrestin translocation assay to identify ligands at orphan receptors. Here, we report that the *Drosophila* arrestin encoded by *kurtz* functions in a similar fashion and can be employed to investigate GPCR–arrestin associations. Specifically, a GFP–*krz* fusion protein, upon co-expression with various *Drosophila* peptide receptors, an amine receptor, and a mammalian peptide receptor translocates to the plasma membrane in specific response to ligand application. This molecular phenotype is exhibited in a mammalian cell line as well as in a *Drosophila* cell line. Notably, the details of receptor–arrestin associations in terms of endocytotic patterns are functionally conserved between the mammalian arrestins and *kurtz*. Furthermore, we report that *kurtz* mutants exhibit hypersensitivity to osmotic stress, implicating GPCR desensitization as an important feature of the endocrine events that shape this stress response.

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1. Introduction

The molecular details by which the signals elicited by activation of G protein-coupled receptors (GPCRs) are attenuated involve a cohort of different regulatory proteins. Specifically, the active conformation of a GPCR is recognized by specific kinases (G protein-coupled receptor kinase (GRK)), which subsequently phosphorylate the receptor (Kohout and Lefkowitz, 2003). The arrestins, normally cytoplasmic resident proteins, have higher affinity for the phosphorylated form of the receptor and translocate to the plasma membrane where they bind to the receptor. Arrestin–receptor binding blocks further interactions between the GPCR and heterotrimeric G proteins, and consequently, signaling from the receptor–G protein complex is inhibited. Arrestins also recruit components of the endocytotic machinery, thereby culminating in the removal of the GPCR complex from the membrane and cessation of signaling (Pierce and Lefkowitz, 2001).

These molecular events apply to a wide range of different GPCRs as well as other signaling molecules (Mukherjee et al., 2005; Gavard and Gutkind, 2006). The kinetics of GPCR desensitization has been monitored by employing an arrestin–GFP fusion protein to witness the translocation of the arrestin from the cytoplasm to the membrane (Barak et al., 1997). We applied such methods to identify ligands for orphan receptors recently identified through genomic sequence mining in *Drosophila* (Johnson et al., 2003b). These previous descriptions of GPCR desensitization have identified differences in the basal association of arrestins with receptors, as well as differences during endocytosis. Specifically, two classes of receptors have been delineated on the basis of their association with the arrestin following receptor activation (Oakley et al., 2001). Class A receptors have lower affinity for the arrestin; the arrestins are released and stay at the membrane as these receptors are internalized. Class B receptors have greater affinity for the arrestin and remains associated with the receptor as the GPCR undergoes endocytosis. This nomenclature does not refer to the classification of different subfamilies based on sequence similarities of the GPCR superfamily (*e.g.*, Family A – rhodopsin-like, Family B – secretin-like) but rather describes the differential affinities that the arrestins have for specific receptor molecules (Oakley et al., 2001).

Are these events of GPCR desensitization universal? Available molecular evidence suggests that GRK and arrestin actions are common features of GPCR regulation. Within the genome of *Drosophila*, two GRKs have been identified (Cassill et al., 1991) as well as four genes with homology to the arrestins (Adams et al., 2000; Zdobnov and Apweiler, 2001). Of these four, two are the visual arrestins, arrestin1 and arrestin2. A third gene, *CG32683*, is

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interesting in that it contains both the amino-terminal and carboxy-terminal arrestin-like domains, but is up to twice the length of and quite divergent from the other *Drosophila* arrestins. The *kurtz* gene also possesses both amino- and carboxy-terminal arrestin domains and is 72% similar to the mammalian βarr2 and 74% similar to βarr1 (Roman et al., 2000). Loss-of-function mutations in this gene lead to lethality, and phenotypic analysis of the krz^1 mutation has demonstrated a role for this arrestin in antenna development, maintaining olfactory sensitivity, and a requirement for exploratory activity (Roman et al., 2000; Ge et al., 2006; Liu et al., 2007). We report herein that *kurtz* is a functional arrestin by virtue of translocation upon receptor activation, and akin to mammalian arrestins, delineates two classes of receptor molecules. These results argue that the molecular details of receptor desensitization are highly conserved.

2. Materials and methods

2.1. Molecular cloning

We generated full-length receptors' constructs for the following neuropeptide receptor-encoding genes using methods described in Johnson et al., 2003a: *FR* (*CG2114*) (NM139501) (Cazzamali and Grimmelikhuijzen, 2002), *CCAP-R* (*CG6111*) (NM206574) (Park et al., 2002), *Proc-R* (*CG6986*) (AY128481) (Johnson et al., 2003a), DH_{44}*-R1* (*CG8422*) (NM137116) (Johnson et al., 2004), *LK-R* (*CG10626*) (Radford et al., 2002) (NM139711), *Crz-R* (*CG10698*) (NM140314) (Park et al., 2002), and the *SIF-R* (*CG10823*) (BT009988) (Jorgensen et al., 2006). The mammalian CCK-R2 receptor was a kind gift from Alan Kopin. In addition, we included a *Drosophila* dopamine receptor (*CG9652*) (NM057659) (Feng et al., 1996). RACE PCR was performed using ESTs or fly head cDNA as templates. Primers flanking the predicted ORFs incorporated restriction sites to facilitate directional cloning into the pcDNA5/FRT vector (Invitrogen) and a 5′ Kozak sequence to facilitate expression in mammalian cells. All receptor constructs were fully sequenced. The LK-R ORF was also placed into the pMT vector for expression in S2 cells, and we are grateful to Julian Dow for providing this reagent. We generated a GFP–*krz* fusion construct, using primers that placed the *krz* initiation codon in frame with GFP. This was cloned into the pMT vector for S2 cells and also subcloned into the pcDNA3.1 vector (Invitrogen) for studies with mammalian cells.

2.2. Transfections and cell culture

HEK-293 cells were transiently transfected using lipofectamine (Invitrogen) according to manufacturer's recommendations and using a total of 10 μg DNA per 4×10^6 cells. Cells were transfected with a 5:1 ratio of *GPCR* DNA and *βarrestin2–GFP* (*βarr2–GFP*) DNAs or *GFP–krz* DNA. Cells were maintained in a humidified incubator in a 5% CO_2 atmosphere at 37 °C and split every 3 days at 1:5. Growth media was DMEM supplemented with 10% FBS and antibiotics. S2 cells were transfected using the PolyFect reagent (Qiagen) and were grown in Schneider's medium supplemented with 10% FBS and antibiotics.

2.3. Translocation assay

HEK-293 and S2 cells were transfected as described above and plated onto 35 mm dishes with a centered glass coverslip to facilitate imaging. Growth media was removed and replaced with serum-free media (Minimum Essential Media (MEM)) without phenol red 30 min prior to assays. Peptides were dissolved in the same media and 100 μl (equal to 1/10 the volume of culture medium) was added at room temperature without mixing. Images were collected using 488 nm excitation and a 505 nm long-pass filter on a Olympus laser-scanning microscope, or on an Axioskop inverted microscope equipped with Volocity deconvolution software. Images were imported into Adobe Photoshop and adjusted for contrast. At least twenty cells were imaged from each of two independent transfections. Cells displaying the extreme levels of GFP fluorescence as well as weak levels were excluded from analysis.

2.4. Peptides

Adipokinetic Hormone (AKH), Leucokinin (LK), NPLP1, DH31, and DH_{44} were purchased from Multiple Peptide Systems, San Diego. Proctolin, dopamine, and corazonin were purchased from Sigma (Saint Louis, MO). Crustacean Cardioactive Peptide (CCAP), NPLP2, CCK, and NPLP3 were purchased from Phoenix Pharmaceuticals. Dromyosuppressin (DMS), Tachykinin-1 (TK-1), and DPKQDFMRFamide were generous gifts from Paul Taghert. SIFamide was a generous gift from Elwyn Isaac. All peptides were tested at two doses (10^{-6} and 10^{-8} M).

2.5. Osmotic stress assays

The genotypes for these experiments were generated as in Ge et al. (2006). Briefly, the krz^1 mutants were rescued through development by the periodic heat induction of a *krz* cDNA with a heatshock-Gal4 driver. After the larvae began the wandering stage, the heat induction stopped, and *kurtz* levels were allowed to decay (Ge et al., 2006). Heterozygous and wild type controls were raised in parallel, under the same conditions, to the krz^1 mutants. Within 6 h after eclosion, the flies were briefly anaesthetized with CO_2 and placed in the designated hyperosmotic or control vials. The hyperosmotic media was generated with Formula 4-24 instant *Drosophila* food (Carolina Biological Supply, Burlington, NC) according to the methods of Huang et al. (2002). Viability was assessed twice daily over the course of the experiment. Significance was assessed with ANOVA, followed by Bonferroni Dunn post-hoc analysis where appropriate.

2.6. Data analysis

The translocation assay originally developed by Barak et al. (1997) can be used to assess dose–responsiveness of transmitter–ligand pairs with sophisticated confocal platforms (Oakley et al., 2002). Given that our hypothesis was to address whether or not there were fundamental differences between the translocation of the GFP–*krz vs.* the βarr2–GFP molecules, we choose to employ the assay in a qualitative all-or-none fashion as described in Johnson et al. (2003b). Translocation events were quantified in twenty GFP-expressing cells. Images were imported into Image Pro Plus (Media Cybernetics, Silver Springs, MD) for analysis of the GFP signal. Two measures were used to assess the distribution of GFP, which either is localized within vesicles or at the cell membrane: clumpiness and counting the number of vesicles. For both analyses, an area of interest (AOI) was manually drawn around the cytoplasm of the cell. Clumpiness is an analysis tool which reports the fraction of pixels deviating from the average pixel intensity following dilation, and thus, is a metric that reflects variation and the heterogeneous distribution of signal. Greater clumpiness values indicate greater variation within the image. Following spatial filtering with a 3 × 3 lo-pass filter, vesicles numbers were counted. Mean pixel intensity and standard deviations for the AOI were assessed and vesicles were defined by applying a threshold pixel intensity of two standard deviations above the mean intensity. To test for variation in vesicle numbers that correlate with receptor or arrestin identity, we employed a two-way ANOVA.

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