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CAPA periviscerokinin-mediated activation of MAPK/ERK signaling through Gq-PLC-PKC-dependent cascade and reciprocal ERK activation-dependent internalized kinetics of *Bom*-CAPA-PVK receptor 2

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ABSTRACT

Bombyx mori neuropeptide G protein-coupled receptor (BNGR)-A27 is a specific receptor for *B. mori* capability (CAPA) periviscerokinin (PVK), that is, *Bom*-CAPA-PVK receptor 2. Upon stimulation of *Bom*-CAPA-PVK-1 or -PVK-2, *Bom*-CAPA-PVK receptor 2 significantly increases cAMP-response element-controlled luciferase activity and Ca^{2+} mobilization in a Gq inhibitor-sensitive manner. However, the underlying mechanism(s) for CAPA/CAPA receptor system mediation of extracellular signal-regulated kinases1/2 (ERK1/2) activation remains to be explained further. Here, we discovered that *Bom*-CAPA-PVK receptor 2 stimulated ERK1/2 phosphorylation in a dose- and time-dependent manner in response to *Bom*-CAPA-PVK-1 or -PVK-2 with similar potencies. Furthermore, ERK1/2 phosphorylation can be inhibited by Gq inhibitor UBO-QIC, PLC inhibitor U73122, protein kinase C (PKC) inhibitor Go 6983, phospholipase D (PLD) inhibitor FIPI and Ca^{2+} chelators EGTA and BAPTA-AM. Moreover, *Bom*-CAPA-PVK-R2-induced activation of ERK1/2 was significantly attenuated by treatment with the Gβγ-specific inhibitors, phosphatidylinositol 3-kinase (PI3K)-specific inhibitor Wortmannin and Src-specific inhibitor PP2. Our data also demonstrate that receptor tyrosine kinase (RTK) transactivation pathways are involved in the mechanisms of *Bom*-CAPA-PVK receptor to ERK1/2 phosphorylation. In addition, β-arrestin1/2 is not involved in *Bom*-CAPA-PVK-R2-mediated ERK1/2 activation but required for the agonist-independent, ERK1/2 activation-dependent internalization of the G protein-coupled receptor (GPCR).

1. Introduction

CAPA peptides are encoded by the capability (*capa*) gene, synthesized in median neurosecretory neurons of abdominal ganglia and released into the hemolymph (Tublitz and Truman, 1985; Kean et al., 2002). The first insect CAPA-related peptide was identified from the parasympathetic organs (PSOs) of tobacco hornworm, *Manduca sexta*, and showed active cardioactivity (Tublitz and Truman, 1985; Cheung et al., 1992). Subsequently, additional *Manduca* CAPA peptides were identified, named cardioacceleratory peptides (CAPs) and later specified as CAP1a,b and CAP2a,b,c (Cheung et al., 1992; Huesmann et al., 1995; Tublitz et al., 1992; Predel and Wegener, 2006). Moreover, CAP2a shared identity with crustacean cardioactive peptide (CCAP) (Cheung et al., 1992; Lehman et al., 1993), which was firstly isolated from the shore crab, *Carcinus maenas* (Stangier et al., 1987). CAP2b presents no sequence similarity with CAP2a but a novel member of the pheromone biosynthesis-activating neuropeptide (PBAN)/diapause hormone (DH)/pyrokinin (PK) family (Predel and Wegener, 2006; Jiang et al., 2014), that is, the periviscerokinins (PVKs) (Wegener et al., 2002). Moreover, CAPs not only affect heart rate in insects and crustaceans (Stangier et al., 1987) but also modulate hindgut contractions and promote fluid secretion from Malpighian tubules in insects (Predel and Wegener, 2006; Davies et al., 1995; Pollock et al., 2004).

Neuropeptides exert their biological functions through receptors present on the cell surface. The *Drosophila melanogaster* G protein-coupled receptor (GPCR) CG14575, was first identified as a cognate receptor for the CAPA peptides (Iversen et al., 2002; Hewes and Taghert, 2001), which is evolutionarily related to the human neuromedin U receptor (Park et al., 2002). The *D. melanogaster* neuropeptide gene *capa* at 99D on chromosome 3R encodes three CAPA peptides, *Drm*-CAPA-PVK-1, *Drm*-CAPA-PVK-2 and *Drm*-PK-1. *Drm*-CAPA-PVK-1 and *Drm*-CAPA-PVK-2, which are homologs of the first identified CAPA peptide, *M. sexta*-CAP2b (Huesmann et al., 1995). *Drm*-PK-1 is a member of the pheromone PBAN/PK peptide family and is closely related to *Bombyx* DH (Kean et al., 2002). In *D. melanogaster* Malpighian tubules, both *Drm*-CAPA-PVK-1 and -PVK-2 perform a diuretic role in increasing fluid

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secretion and muscle contraction rates by stimulating nitric oxide production; this stimulation is accomplished by elevating intracellular cGMP level and Ca^{2+} concentration via endoplasmic reticulum and nifedipine-sensitive L-type voltage-dependent Ca^{2+} channels (Kean et al., 2002; Rosay et al., 1997; Macpherson et al., 2001; Pollock et al., 2003; Wegener and Nässel, 2000; Davies et al., 1997; O'Donnell et al., 1996). *Drm*-PK-1 activates the *Drm*-PK-1 receptor (Cazzamali et al., 2005), but not CAPA receptor (Terhzaz et al., 2012). Moreover, *Drm*-PK-1 cooperates with Na^+/K^+ ATPase in feeding and/or cuticular and respiratory transpiration, which are associated with desiccation stress responses (Macmillan et al., 2015; Torrie et al., 2004; Terhzaz et al., 2015). CAPA/CAPA receptor signaling pathway is functionally conserved because CAPA receptors are found in representatives of the major orders, including Hymenoptera, Coleoptera, and Lepidoptera. Hence, CAPA signaling system is a potential pest control target for significant vectors of medical and veterinary pathogens, such as different mosquito species, flies, kissing bugs and ticks (Rosenkilde et al., 2003; Olsen et al., 2007; Paluzzi et al., 2010; Yang et al., 2013a,b,c; Yang Y et al., 2015; Hauser et al., 2006; Yamanaka et al., 2008; Hauser et al., 2010; Hauser et al., 2008; Veenstra, 2014; Gondalia et al., 2016; Veenstra et al., 2012; Christie and Chi., 2015; Roller et al., 2008).

The silkworm, *Bombyx mori*, is a typical Lepidoptera. *B. mori* contains two *capa* genes, namely *capa-a* and *capa-b*, which encode *Bom*-CAPA-PVK-1, -PVK-2 and two different extended forms of *Bom*-CAPA-PK (Roller et al., 2008). Our previous study demonstrated that *B. mori* neuropeptide G protein-coupled receptors (BNGR)-A25L and BNGR-A27 are two functional receptors activated by *B. mori* PVK neuropeptides (*Bom*-CAPA-PVK-1 and -PVK-2), which lead to a significant increase in Ca^{2+} mobilization and phosphorylation of extracellular signal-regulated kinase1/2 (ERK1/2) in a Gq-dependent manner (Shen et al., 2017). However, the underlying mechanism of regulating *Bom*-CAPA receptor-mediated ERK1/2 activation remains largely unknown. Further detailed downstream information of ERK1/2 activation via *Bom*-CAPA receptor needs to be elucidated.

As a member of the mitogen-activated protein kinase (MAPK) family, ERK1/2 exerts pronounced effects on diverse processes ranging from cell proliferation and differentiation to cell apoptosis and death (Lorenz et al., 2009; Schwindinger and Robishaw, 2001; Ahn et al., 2009; Thiels and Klann, 2001; Pierce et al., 2001). Activated GPCR signaling pathways typically involve the MAPK cascades, which traditionally crosstalk with RTK. When either RTK or GPCR is activated, ERK1/2, which functions as transcriptional regulator, is also activated (Hupfeld and Olefsky, 2007; Lin et al., 1998; Kim et al., 2003; Hollenberg, 1995). Here we investigate the mechanism (s) of *Bom*-CAPA-PVKs-mediated ERK1/2 activation by using HEK293 cells that stably and transiently express the *Bom*-CAPA-PVK receptor 2 (BNGR-A27). The results demonstrate that multiple signaling-linked pathways, such as the Gq protein-initiated Ca^{2+}, PLC/PKC-, PLD- and epidermal growth factor receptor (EGFR) transactivation-dependent RTK pathways, are involved in *Bom*-CAPA-PVK receptor to ERK1/2, which is also a reciprocal regulator of GPCR via β-arrestin1/2.

2. Material and methods

2.1. Experimental animals

Larvae of a polyvoltine strain, N4 strain of the silkworm, *Bombyx mori*, were provided by the National Center for Silkworm Genetic Resources Preservation, Chinese Academy of Agricultural Sciences. They were reared on fresh mulberry leaves at 25 °C under a 12 h-light and 12 h-dark photoperiod.

2.2. Materials

Cell culture media and fetal bovine serum (FBS) were purchased from HyClone (Beijing, China). G418 was purchased from Invitrogen (Carlsbad, CA). Opti-MEM® was purchased from Roche (Mannheim, Germany) and Invitrogen (Carlsbad, CA), respectively. The pCMV-FLAG vector, Go6983, and Horseradish peroxidase (HRP)-conjugated anti-mouse IgG were purchased from Sigma-Aldrich (St. Louis, MO). Gq inhibitor UBO-QIC was purchased from E. Kostenis (University of Bonn, Germany). BAPTA-AM, U73122, PP2 and gallein were obtained from TOCRIS (Bristol, U.K.). U0126, tyrphostin AG1478 and wortmannin were purchased from Calbiochem (Darmstadt, Germany). GM6001 was purchased from Merck (Darmstadt, Germany). EGTA (ethylene glycol-bis-N,N,N′,N′-tetraacetic acid) and RIPA (Radio-immunoprecipitation) lysis buffer were obtained from Beyotime (Haimen, China). Anti-phospho-ERK1/2 (Thr-202/Tyr-204) and anti-total ERK1/2 rabbit antibodies were purchased from Cell Signaling Technology (Danvers, MA). *B. mori* CAPA PVK-1 and -2 of 95% purity (HPLC area percentage) were synthesized by GL Biochem (Shanghai, China).

2.3. Molecular cloning and plasmid construction

Total RNA was isolated from the ovaries of three-day-old *B. mori* female pupae by using the RNAiso Plus reagent from Takara (Dalian, China). cDNA was synthesized using a PrimeScript 1st Strand cDNA Synthesis Kit from Takara (Dalian, China) according to the manufacturer's instructions. All primers were designed according to the published *B. mori* CAPA-PVK receptor 2 (BNGR-A27) gene sequences (GenBank accession No. AB330448) (Shen et al., 2017). The corresponding PCR products of BNGR-A27 were cloned into the sites of HindIII and BamHI of pCMV-FLAG and XhoI and HindIII of pEGFP-N1. pCMV-FLAG and pEGFP-N1 were used to construct recombinant plasmid for HEK293 cells; all of the targeted fragments were recombined by the Rapid DNA Ligation Kit (Beyotime, China), namely FLAG-A27 and A27-EGFP as previously described (Shen et al., 2017; Yang et al., 2013a,b,c). All of the constructs described above were sequenced by Hangzhou Tsingke Biology Company to verify sequences and orientations.

2.4. Cell culture and transfection

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone) supplemented with 10% FBS, 100 units/mL penicillin, 100 m g/mL streptomycin and 4 mM L-glutamine (Invitrogen) at 37 °C in a humidified incubator containing 5% CO2. The *Bom*-CAPA-PVK-R2 cDNA plasmid constructs were transfected into HEK293 cells by using Lipofectamine 2000 according to the manufacturer's instructions. Two days after transfection, stably expressing cells were selected by the addition of 800 mg/L G418.

2.5. Synthesis of small interfering RNAs and siRNA transfection

All small interfering RNAs (siRNAs) were chemically synthesized by Dharmacon RNA Technologies (Lafayette, CO). β-arrestin1/2 siRNAs were purchased as a SMART pool (Table. 1). Nonspecific control siRNA VII (5' -AAACUCUAUCUGCACGCUGAC-3′) was used as control for all siRNA experiments. The relative siRNAs were transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions (Luo et al., 2008). At 48 h after transfection, cells were split for the indicated assay to be conducted in the following

Table 1
β-arrestin1/2 siRNA sequence SMART pool.

β-arrestin1 siRNA	β-arrestin2 siRNA
5′-GAACUGCCCUUCACCCUAA-3′	5′-CGAACAAGAUGACCAGGUA-3′
5′-UGGAUAAGGAGAUCUAUUA-3′	5′-GAUGAAGGAUGACGACUAU-3′
5′-CGAGCACGCUUACCCUUUC-3′	5′-CGGCGUAGACUUUGAGAUU-3′
5′-CGAGCACGCUUACCCUUUC-3′	5′-CAACCUCAUUGAAUUUGAU-3′