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Short communications

Responsiveness of vomeronasal cells to a newt peptide pheromone, sodefrin as monitored by changes of intracellular calcium concentrations

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ABSTRACT

A peptide pheromone of the red-bellied male newt, sodefrin was tested for its ability to increase intracellular concentrations of Ca^{2+} ($[Ca^{2+}]_i$) in the dissociated vomeronasal (VN) cells of females by means of calcium imaging system. The pheromone elicited a marked elevation of $[Ca^{2+}]_i$ in a small population of VN cells from sexually developed females. The population of cells exhibiting sodefrin-induced elevation of $[Ca^{2+}]_i$ increased concentration-dependently. A pheromone of a different species was ineffective in this respect. The VN cells from non-reproductive females or from reproductive males scarcely responded to sodefrin in terms of elevating $[Ca^{2+}]_i$. In the cells from hypophysectomized and ovariectomized females, the sodefrin-inducible increase of $[Ca^{2+}]_i$ never occurred. The cells from the operated newts supplemented with prolactin and estradiol exhibited $[Ca^{2+}]_i$ responses to sodefrin with a high incidence. Thus, sex- and hormone-dependency as well as species-specificity of the responsiveness of the VN cells to sodefrin was evidenced at the cellular level. Subsequently, possibility of involvement of phospholipase C (PLC)-inositol 1,4,5-trisphosphate (IP_3) and/or PLC-diacylglycerol (DAG)-protein kinase C (PKC) pathways in increasing $[Ca^{2+}]_i$ in VN cells in response to sodefrin was explored using pharmacological approaches. The results indicated that PLC is involved in generating the Ca^{2+} signal in all sodefrin-responsive VN cells, whereas IP_3 in approximately 50% of the cells and DAG-PKC in the remaining cells. In the latter case, the increase of $[Ca^{2+}]_i$ was postulated to be induced by the influx of Ca^{2+} through the L-type channel. The significance of the finding is discussed.

1. Introduction

Sodefrin is the first peptide pheromone identified in a vertebrate [18]. It is secreted by the abdominal gland of the cloaca in the sexually mature male red-bellied newt, *Cynops pyrrhogaster* and exhibits a potent attracting activity to sexually mature conspecific females. This pheromone is generated from the precursor protein consisting of 189 amino acid residues [14], its synthesis being under the stimulatory control by prolactin and androgen [15,41]. The attraction of female newts is completely abolished by plugging the bilateral nostrils [34], indicating that sodefrin acts on the olfactory organs. Electro-olfactogram (EOG) response to sodefrin recorded in the ventral nasal epithelium of sexually mature female newts revealed that the pheromone markedly enhances the response on the epithelium of the lateral nasal sinus (LNS) region. In contrast, the epithelium of male newts or sexually undeveloped female newts scarcely responds to sodefrin [35,36]. The LNS is a diverticulum located in the lateral portion of the main chamber of the nasal cavity, which has been considered to correspond to the vomeronasal (VN) organ in other tetrapod animals [8]. The sensory epithelium of this region contains only microvillar cells [16]. It detects chemical signals including pheromones and general odorants [3].

Abbreviations: $[Ca^{2+}]_i$, intracellular concentrations of Ca^{2+}; CalC, calphostin C; DAG, diacylglycerol; E2, estradiol; EOG, electro-olfactogram; ER, endoplasmic reticulum; F340/F380, ratio of fluorescence intensity excited at 340 nm and 380 nm; HBSS, HEPES buffered standard solution; HX, hypophysectomized; ICCD, intensified charge-coupled device; IP_3, 1,4,5-trisphosphate; LNS, lateral nasal sinus; OVX, ovariectomized; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PRL, prolactin; THPG, thapsigargin; VN, vomeronasal.

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Recently, a wide variety of peptide or protein pheromones have been identified in vertebrate animals, see [2,33,37]. According to a study in mice, intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in the female VN cells are elevated, when subjected to peptides belonging to major histocompatibility class 1 that were derived from unfamiliar males [23]. ESP1, a peptide pheromone released into the tear fluids of male mice [19], has also been shown to elevate $[Ca^{2+}]_i$ in the VN cells of female mice [9].

Although the mechanisms underlying the increase of $[Ca^{2+}]_i$ in the VN cells seem to be still controversial [44], pheromonal stimulations are considered to activate mainly phospholipase C (PLC) pathway, resulting in the production of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) [11,13,20,39], leading to an elevation of $[Ca^{2+}]_i$ and excitation.

In the present experiments, we showed, employing an *in vitro* Ca^{2+} imaging technique, that the VN cells of the female newt respond to sodefrin to increase $[Ca^{2+}]_i$. Differences in the responsiveness to the pheromone in terms of elevating $[Ca^{2+}]_i$ in the VN sensory neurons between the female and male newts as well as between the reproductive and non-reproductive females were also analyzed. Additionally, the effects of hypophysectomy plus ovariectomy and of hormone supplementation on the responsiveness of VN cells to sodefrin were investigated. Then, as a step to analyze the signal transduction pathway for sodefrin in the newt VN sensory neurons, we focused on the PLC-IP_3 and PLC-DAG-protein kinase C (PKC) pathways and examined the effects of inhibitors of these signaling pathways on the sodefrin-inducible increase of $[Ca^{2+}]_i$.

2. Materials and methods

2.1. Experimental animals

The newts captured in the field were kept in a plastic tanks at 12 °C under a 12 h light/12 h dark cycle and fed *Tubifex* worms daily until use. Sexually developed female newts were used mainly. In some cases, sexually undeveloped females and sexually developed males were also used. In order to examine the hormonal effects on the responsiveness of VN cells, sexually developed females that were hypophysectomized (HX) and ovariectomized (OVX). Following the day of surgery, they were injected intraperitoneally with a combination of 5 μg estradiol (E2: Sigma–Aldrich, St. Louis, MO) and 1 IU prolactin (PRL: Sigma) in 50 μl of 0.65% saline or saline only every other day for 14 days prior to the experiment. Prior to the surgery or sacrifice, animals were anesthetized in 0.1% ethyl 3-aminobenzoate methanesulfonate salt (Sigma). All experimental procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Waseda University and Nippon Veterinary and Life Science University.

2.2. Pheromones and chemicals

The red-bellied newt (*C. pyrrhogaster*) pheromone, sodefrin (SIPSKDALLK) and sword-tailed newt (*C. ensicauda*) pheromone, silefrin (SILSKDAQLK) were purchased from Bachem AG (Bubendorf, Switzerland) and synthesized by Peptide Institute (Osaka, Japan), respectively. Inhibitors, namely U73122, thapsigargin (THPG), calphostin C (CalC), nimodipine, ω-conotoxin GIVA and mibefradil used in the Ca^{2+} imaging analysis were purchased from Sigma. These inhibitors were dissolved in dimethyl sulfoxide for stock solution. Before use, they were diluted in the HEPES buffered standard solution (HBSS) composed of 95 mM NaCl, 1 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 1 mM Na-pyruvate, 1.5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM $MgSO_4$, 1.8 mM $CaCl_2$, 16 mM glucose, 2 mM HEPES at pH 7.4.

2.3. Preparation of VN cells

The region of the LNS was opened surgically and the epithelia of the VN organ were excised under a dissection microscope. The mucosa were incubated for 7 min at 35 °C in the HBSS containing no Mg^{2+}/Ca^{2+} with 0.1 mg/ml collagenase (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and then physically dissociated by pipetting. The cell suspension was filtrated using 70 μm nylon membrane filter (BD falcon; Franklin Lakes, NJ, USA). After centrifugation at 700 rpm, collagenase solution was discarded and isolated cells were washed twice and stored in HEPES buffered medium 199 (M199; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan; in g/L: 7.62 M199, 0.22 glucose, 4.78 HEPES). Isolated VN cells were plated on cover slips coated with concanavalin A (Sigma) and maintained at 4 °C before use. The calcium imaging analysis started within 2 h after the isolation of VN cells. With this procedure, the cell viability and the responsiveness of the cells to sodefrin were confirmed to be stable.

2.4. Calcium imaging analysis

$[Ca^{2+}]_i$ was measured according to the method described previously [29,40] with a slight modification. In brief, the dissociated cells on coverslips were incubated with 4 μM fura-2 AM (Dojin Chemical, Kumamoto, Japan) in M199 containing 5% FBS (ICN Biomedicals Inc., Santa Ana, CA, USA) for 1 h at 4 °C. The cells were then mounted in a chamber, placed on the stage of TMD inverted microscope (Nikon, Tokyo, Japan), and perfused with HBSS by using a peristaltic pump (Watson Marlow, Falmouth, Cornwall, UK) at 0.5 ml/min. The fura-2 fluorescence from the cells due to excitation at 340 nm (F340) and that at 380 nm (F380) were detected every 5–8 s by an intensified charge-coupled device (ICCD) camera, and the ratio (F340/F380) was produced by the Argus-50 systems (Hamamatsu Photonics, Hamamatsu, Japan). Changes of the ratio in approximately 100 cells loaded with fura-2-AM within a low power-field (10× objective, 10× ocular) were monitored at one time. The monitoring was carried out 3–5 times per each of 4–5 samples from different individuals.

The pheromones and inhibitors were applied into the chamber by a perfusion system following the stabilizing period (>5 min) in HBSS. The switch time between the different solutions was less than 1 s. The baseline was recorded for 60 s prior to each stimulus. According to the criterion adopted by [12], the response was defined as positive when the peak value was at least twice the amplitude of the baseline fluctuation (mean + 2 SD). In most of the cases, the responsiveness of the cell to sodefrin was confirmed further prior to the cessation of each $[Ca^{2+}]_i$ recording. When necessary, the viability of the cell was also confirmed by subjecting the cell to the extracellular high-potassium stimulus (150 mM KCl).

2.5. Analyses of data

The ratio between the number of sodefrin-responsive cells and the total cells examined was expressed as percent responsive cells. To evaluate the effects of inhibitors, the ratio between the number of sodefrin-responsive cells that were blocked by a certain inhibitor and the number of cells responded to sodefrin prior to the application of the inhibitor was expressed as percent blocked cells. Statistical analyses were performed using Kruskal–Wallis one-way-ANOVA followed by Turkey's HSD test to evaluate the differences among the responsiveness of the VN cells to several doses of sodefrin and the differences among the responsiveness of the cells from reproductive and non-reproductive females and reproductive males. To evaluate the correlation between the peak amplitude of the fura-2 fluorescence ratio (defined as the peak value minus the baseline value) evoked by sodefrin and the baseline