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A New Teleocidin Analog from *Streptomyces* sp. MM216-87F4 Induces Substance P Release from Rat Dorsal Root Ganglion Neurons

Koichi Nakae, Nobuo Hosokawa, Ryuichi Sawa, Yumiko Kubota, Tohru Masuda, Syunichi Ohba, Masayuki Igarashi, Naoko Nakagawa, Yoshio Nishimura, Yuzuru Akamatsu

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Abstract A new teleocidin analog was isolated from the fermentation medium of *Streptomyces* sp. MM216-87F4 and its structure was elucidated as 14-*O*-(*N*-acetylglucosaminyl) teleocidin A (GlcNAc-TA). GlcNAc-TA induces the translocation of protein kinases Cα and θ fused with enhanced green fluorescent protein (PKCα-EGFP and PKCθ-EGFP) to the plasma membrane in stable transfectants, and reduces intracellular calcium mobilization induced by agonists of G-protein coupled receptors in various cell lines without causing irritation of the mouse ear. Further, GlcNAc-TA sensitizes the release of excitatory neuropeptides substance P induced by capsaicin from primary-cultured dorsal root ganglion (DRG) neurons of the rat and GlcNAc-TA alone also triggers substance P release in a dose-dependent manner. This study provides the first observation that a teleocidin analog without a free hydroxyl group at C-14 acts as a PKC activator and directly induces the release of excitatory neuropeptide.

Keywords DRG neurons, substance P, teleocidins, PKC activator, Ca^{2+} mobilization.

Introduction

The signaling pathway following various G-protein coupled receptors (GPCRs) involved in intracellular calcium mobilization and its inhibitor might contribute to the analyses of this mechanism as valuable biological tools. Our screening program for inhibitory compounds of agonists-induced Ca^{2+} mobilization from microbial metabolites revealed a new teleocidin analog derived from the culture medium of *Streptomyces* sp. MM216-87F4. This finding was of interest to us since teleocidins are well known as protein kinase C (PKC) activators and PKC plays crucial roles in the signal transduction pathways affecting physiological activities. In this paper, we describe the isolation, the physico-chemical properties and the biological activities of 14-*O*-(*N*-acetylglucosaminyl) teleocidin A (GlcNAc-TA).

K. Nakae (Corresponding author)**, N. Hosokawa, R. Sawa, Y. Kubota, T. Masuda, S. Ohba, M. Igarashi, N. Nakagawa, Y. Nishimura, Y. Akamatsu:** Microbial Chemistry Research Center, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141-0021, Japan, E-mail: nakaek@bikaken.or.jp

Materials and Methods

Taxonomy

The strain MM216-87F4 was isolated from a soil sample collected at Fujisawa-shi, Kanagawa prefecture, Japan. The type of diaminopimelic acid (DAP) isomers was determined by the method of Staneck and Roberts [1]. The 16S rDNA (16S ribosomal RNA gene, 1453 bp, positions 19-1504, *Escherichia coli* numbering system [2]) was amplified by the polymerase chain reaction using genomic DNA of the strain and sequenced. The most related sequences were searched using the BLAST algorithm in the DNA Data Bank of Japan (DDBJ).

Transfection of Plasmid into EP_1-CHO Cells

Chinese hamster ovary cells stably expressing mouse

prostaglandin EP_1 receptor (EP_1-CHO) were generously provided by Professor Ichikawa [3]. Plasmid pPKCα-EGFP and pPKCθ-EGFP were purchased from Clontech, U.S.A. Transfection was carried out with TransFast transfection reagent (Promega, U.S.A.). After 24 hours, cells were diluted into plates containing 96 wells, by the limiting dilution-culture method and selected by culture medium containing 1 mg/ml of G418. Then, the stable single clone expressing either PKCα-EGFP or PKCθ-EGFP was established.

Visualization of PKCα-EGFP and PKCθ-EGFP Translocation by Confocal Microscopy

Cells (7000 cells/well) were plated in 96-well glass bottom plates (Iwaki, Japan) and washed with assay buffer (Hank's balanced salt solution, 17 mM HEPES pH 7.4, 0.1% BSA). Fluorescence images were obtained with the 488 nm excitation using laser-scanning confocal microscopy (LSM510, Zeiss); emission was collected through a pass filter of 505～550 nm band. Cells were imaged on the stage of an inverted microscopy (Axiovert 100 M) with a 40X Zeiss Plan-Neofluar objective.

Ca^{2+} Mobilization Assay

Ca^{2+} loading buffer was prepared by mixing 2 μM Fluo-3AM (Dojindo, Japan) and 0.02% Pluronic F-127 (Molecular Probes, U.S.A) in the Ca^{2+} assay buffer (17 mM HEPES pH 7.4, 0.1% BSA, 1 mM probenecid, Hank's balanced salt solution). Cells were incubated in the Ca^{2+} loading buffer at 37°C for 60 minutes, and then washed with Ca^{2+} assay buffer. The drug concentrations were 100 nM PGE_2 (Cayman Chemical, U.S.A) in mouse EP_1-CHO cells, 200 nM sulprostone (Cayman Chemical, U.S.A) in K562 cells, 2 μM ATP, 30 nM Neuropeptide Y (NPY) (Peptide Institute Inc., Japan) and 40 nM [Pyr^1]-Apelin-13 (Peptide Institute Inc., Japan) in HEL cells, respectively. Fluorescence emission at 480 nm induced by ligands were measured using a FDSS6000 fluorimeter (Hamamatsu Photonics, Japan).

Irritation Test

Chemicals were exposed by applying 10 μl of methanol with appropriate chemical concentrations to the inside of ears in mice (female mice of ICR strain, aged 5 weeks (Charles River, Japan)). The extent of irritation on the ear was observed after 24 hours.

DRG Neuron Preparation

The primary DRG neurons were isolated from newborn rats (SD strain). Single cells were obtained by mechanical dispersion in 1 mg/ml of collagenase A solution (Hank's balanced salt solution, pH 7.4) at 37°C for 60 minutes. The resulting DRG neurons were cultured in HAM's F-12 medium (GIBCO BRL, UK) supplemented with 80 ng/ml of nerve growth factor (Sigma, U.S.A), 0.1 mM of 5-fluorouracil (Sigma, U.S.A), 7.5 mg/ml of L-ascorbic acid and 10% of FCS in collagen type I-coated 96-well plates (Becton Dickinson, U.S.A.).

Substance P Release Assay

DRG neurons were washed with assay buffer (Hank's balanced salt solution, 17 mM HEPES pH 7.4, 0.1% BSA), and treated with various concentrations of GlcNAc-TA and Phorbol 12-myristate 13-acetate (PMA) at 25°C for 10 minutes. Then, the release of substance P into assay buffer induced with or without 30 nM of capsaicin at 37°C for 30 minutes was determined using an EIA kit (Cayman Chemical).

General

Optical rotation was measured by a JASCO P-1030 polarimeter. UV spectrum was obtained on a Hitachi U2800 spectrometer. IR spectra were recorded using a Horiba FT-210 Fourier transform infrared spectrometer. NMR data were recorded on a JEOL JNM-ECA600 spectrometer. Mass spectra were obtained with a JEOL JMS-T100LC spectrometer.

Results

Taxonomy

The isomer of DAP in whole-cell hydrolysates of the strain MM216-87F4 was determined to be the LL-form. The 16S rDNA sequence of the strain showed high identity with that of *Streptomyces* such as *Streptomyces scabrisporus* (1435/1453, 98%), suggesting that the strain MM216-87F4 belongs to the genus *Streptomyces*. Therefore, the strain was designed as *Streptomyces* sp. MM216-87F4.

Fermentation and Isolation

Streptomyces sp. MM216-87F4 was cultured in a wheat medium (810 g) under static condition for 14 days at 30°C. The wheat medium was then treated with acetone (2.2 liters). The solution was concentrated, and the resulting aqueous solution was extracted with EtOAc (3.0 liters). The extract was dried (anhydrous Na_2SO_4) and evaporated to give yellow oil (270 mg). The oil was dissolved in a small volume of MeOH and applied on HPLC column (Capcell Pak C18 column, 30×250 mm) using 63% aqueous acetonitrile containing 0.01% TFA as mobile phase. Active fractions were combined and concentrated *in vacuo*

Table 1 Physico-chemical properties of GlcNAc-TA

Appearance	white powder
$[\alpha]_D^{25}$	+130.2° (*c* 0.15, MeOH)
HRESI-MS (positive, *m*/*z*)	
Found	663.3748 $(M+Na)^+$
Calcd.	663.3734 (as $C_{35}H_{52}N_4O_7Na$)
Molecular formula	$C_{35}H_{52}N_4O_7$
UV λ_{max}^{MeOH} nm (ε)	229 (19000), 279 (7416), 297 (sh, 7202)
IR ν_{max} (KBr) cm^{-1}	3346, 3305, 2927, 1640, 1508, 1076

Table 2 ^{13}C and ^{1}H NMR data of GlcNAc-TA in CD_3OD

Position	Conformer-1		Conformer-2	
	^{13}C	^{1}H multiplicity (*J* Hz)	^{13}C	^{1}H multiplicity (*J* Hz)
2	123.13 d	6.90 s	127.65 d	7.09 s
3	114.02 s		107.54 s	
3a	120.10 s		130.07 s	
4	147.39 s		144.96 s	
5	106.97 d	6.47 d (8.1)	123.06 d	6.94 d (8.0)
6	121.49 d	6.91 d (8.1)	121.23 d	7.02 s (8.0)
7	123.02 s		129.59 s	
7a	138.19 s		138.25 s	
8	35.19 t	3.09, 3.18 br d (17.3) dd (3.8, 17.3)	28.55 t	2.82, 2.94 dd (1.6, 14.4) dd (4.4, 14.4)
9	55.15 d	4.34 m	53.88 d	4.37 m
11	175.57 s		174.21 s	
12	72.20 d	4.39 d (10.2)	77.45 d	3.07 d (11.0)
14	72.55 t	3.57, 3.91 dd (7.5, 10.4) dd (3.8, 10.4)	68.82 t	3.31, 3.47 m m
15	29.68 d	2.52	25.56 d	2.30 m
16	20.18 q	0.61 d (6.5)	19.91 q	0.89 d (6.6)
17	22.07 q	0.88 d (6.5)	19.99 q	1.25 d (6.6)
18	33.45 q	2.87 s	36.42 q	2.70 s
19	44.67 s		44.93 s	
20	25.93 q	1.44 s	25.49 q	1.49 s
21	148.77 d	6.13 dd (10.8, 17.6)	147.83 d	6.15 dd (10.8, 17.6)
22	112.85 t	5.03, 5.12 dd (1.4, 17.6) dd (1.4, 10.8)	113.36 t	5.11, 5.19 dd (1.1, 17.6) dd (1.1, 10.8)
23	39.77 t	1.74, 2.05 ddd (5.0, 12.1, 12.7) ddd (4.5, 10.0, 12.7)	40.31 t	1.81, 1.87 m, m
24	24.37 t	1.47, 1.80 m, m	24.37 t	1.69, 1.92 m, m
25	125.88 d	5.04 m	125.76 d	5.08 m
26	131.96 s		132.25 s	
27	17.40 q	1.29 br s	17.68 q	1.45 br s
28	25.83 q	1.58 s	25.90 q	1.63 s
1′	102.82 d	4.48 d (8.5)	102.47 d	4.41 d (8.3)
2′	57.18 d	3.68 m	57.54 d	3.66 m
3′	75.93 d	3.46 m	76.00 d	3.46 m
4′	72.08 d	3.30 m	72.38 d	3.30 m
5′	78.30 d	3.30 m	78.09 d	3.25 ddd (2.3, 6.0, 9.7)
6′	62.76 t	3.68, 3.87 m, m	62.85 t	3.68, 3.87 m, m
NHAc Me	23.10 q	2.01 s	23.18 q	1.99 s
NHAc CO	173.97 s		173.87 s	

Chemical shifts in ppm with TMS as an internal standard.

(9.0 mg). Further purification was carried out by preparative TLC (3 : 1 $CHCl_3$: MeOH) to give GlcNAc-TA (3.0 mg).

Physico-chemical Properties and Structural Elucidation

The physico-chemical properties of GlcNAc-TA are summarized in Table 1. GlcNAc-TA was obtained as a white powder. The molecular formula of GlcNAc-TA was determined to be $C_{35}H_{52}N_4O_7$ by HRESI-MS. The characteristic absorption maxima at 279 and 297 nm in the UV spectrum suggested the presence of an indol moiety. GlcNAc-TA showed positive and negative color reactions with phosphomolybdic acid/sulfuric acid and ninhydrin, respectively. The ^1H and ^{13}C assignments of GlcNAc-TA are summarized in Table 2. The ^1H and ^{13}C NMR spectra of GlcNAc-TA showed a mixture of conformers in the ratio of 2 to 1 (conformer-1 : conformer-2) as observed in other teleocidins [4] and the existence of *N*-acetylglucosamine. The long-range coupling from an anomeric proton (H-1′) to a methylene carbon bearing oxygen (C-14) in the HMBC spectrum indicated the connectivity between *N*-acetylglucosamine and teleocidin A. All of these data, together with various NMR analyses, suggested that GlcNAc-TA is 14-*O*-(*N*-acetylglucosaminyl) teleocidin A (Fig. 1). The details of the structure elucidation will be published elsewhere.

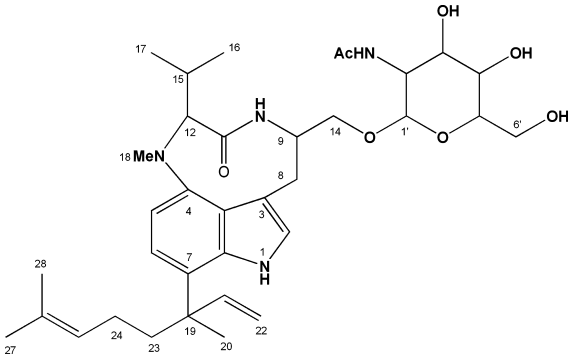

Fig. 1 Structure of GlcNAc-TA.

Biological Activities

The Effect of GlcNAc-TA on the Translocation of PKCα-EGFP and PKCθ-EGFP

Teleocidin derivatives are known to activate PKC and translocate PKC to the plasma membrane. For studying the biological function of each PKC isozymes in response to various stimuli, the PKC-EGFP translocation assay was performed to observe directly the protein response in living cells [5]. To investigate the biological activity of GlcNAc-TA, we established a EP_1-CHO cell line stably expressing PKCα-EGFP or PKCθ-EGFP and observed their translocation to the plasma membrane. We also examined the effect of other PKC activators, PMA, teleocidin A and cytoblastin [6] on PKCα-EGFP or PKCθ-EGFP translocation (Fig. 2). In the absence of stimuli, PKCα-EGFP was mainly present in the cytoplasm. The induction of 30 μM GlcNAc-TA caused the translocation of PKCα-EGFP to the plasma membrane within 30 minutes and a similar observation was made for PKCθ-EGFP. These results confirmed GlcNAc-TA act as a PKC activator.

The Effect of Ca^{2+} Mobilization Induced by Agonists in Various Cell Lines

Phorbol esters such as PMA and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) are reported to alter Ca^{2+} mobilization induced by agonists [7, 8]. We examined whether teleocidin derivatives, including GlcNAc-TA, wound also have inhibitory activity against Ca^{2+} mobilization induced by agonists in various cell lines. As shown in Fig. 3a, GlcNAc-TA and the other teleocidin derivatives inhibited dose-dependently Ca^{2+} mobilization induced by PGE_2 in EP_1-CHO cell and the similar effects were also observed in sulprostone-induced Ca^{2+} mobilization. The effects of compounds are summarized in Table 3. All of these PKC activators inhibited Ca^{2+} mobilization, and IC_{50} values of GlcNAc-TA were not affected by varieties of ligands and cell lines.

Table 3 The inhibitory effect of compounds (IC_{50} (nM)) on Ca^{2+} mobilization with various stimulus

Ligand	Cell	GlcNAc-TA	PMA	Teleocidin A	Cytoblastin
PGE_2	mEP_1-CHO	2100	4.8	37	6000
Sulprostone	K562	2500	3.9	44	10000
ATP	HEL	4300	4.0	8.6	
NPY	HEL	4300	5.6	9.6	
[Pyr^1]-Apelin-13	HEL	3800	3.3	7.7	

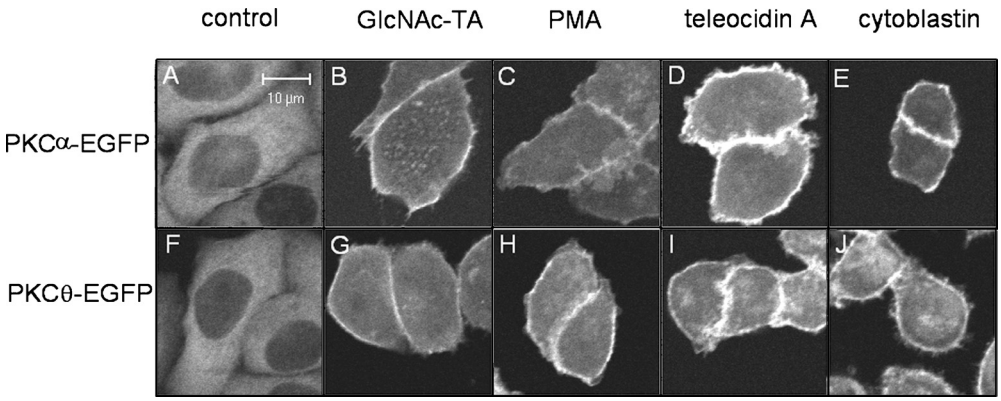

Fig. 2 Confocal images of EP_1-CHO expressing PKCα-EGFP and PKCθ-EGFP treated with various stimuli.

(A∼E) Translocation of PKCα-EGFP to the plasma membrane. (F∼J) Translocation of PKCθ-EGFP to the plasma membrane. (A and F) Control. (B and G) 30 μM of GlcNAc-TA were treated. (C and H) 100 nM of PMA were treated. (D and I) 100 nM of teleocidin A were treated. (E and J) 100 μM of cytoblastin were treated. Acquired at the end of experiment, t=15∼30 minutes

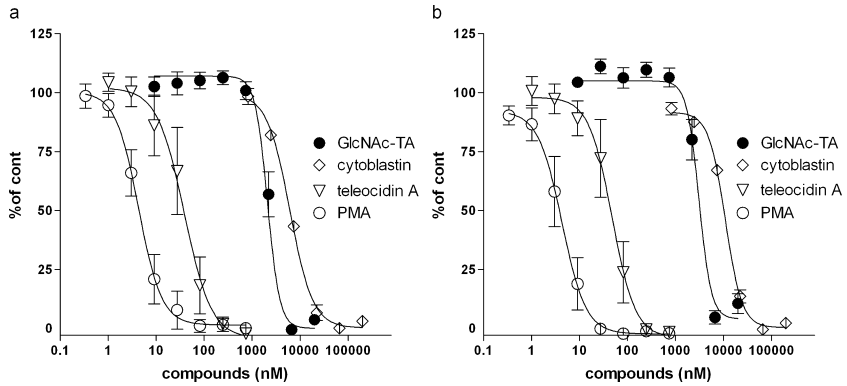

Fig. 3 The effects of various compounds on agonist-induced Ca^{2+} mobilization in cells.

(a) Effects of various compounds on 100 nM of PGE_2-induced Ca^{2+} mobilization in EP_1 transfectants. Data represent means±S.E.M. of 3∼6 independent experiments. (b) Effects of various compounds on 200 nM of sulprostone-induced Ca^{2+} mobilization in K562. Data represent means±S.E.M. of 3∼6 independent experiments.

The Effect of GlcNAc-TA on Irritation of Mouse Ear

Teleocidin derivatives were known to have irritant activity of mouse ear and potent tumor-promoting activity [9]. Therefore, the effect of GlcNAc-TA on the mice ear was examined (Fig. 4). Teleocidin derivative teleocidin B caused irritation at least 0.4 μg/ear in the mouse whereas GlcNAc-TA showed no irritation at 600 μg/ear. Irritant activity of GlcNAc-TA was distinct from other teleocidin derivatives although GlcNAc-TA acted as PKC activators.

The Effect of GlcNAc-TA and PMA on Substance P Release from DRG Neurons

Capsaicin is a ligand of transient receptor potential vanilloid 1 (TRPV1) and induces the release of substance P from DRG neurons [10]. It is reported that PKC regulates the currents of DRG neurons induced by capsaicin in rats [11]. Little is known about the effect of PKC activator on the release of substance P, however. We were interested in the effects of GlcNAc-TA and PMA on the release of substance P caused by capsaicin from DRG neurons. As shown in Fig. 5, GlcNAc-TA alone triggered the release of substance P in a dose-dependent manner. Moreover, GlcNAc-TA sensitized the release of substance P induced by capsaicin in a dose-dependent manner. Similar phenomena were also observed by the treatment with PMA. These results suggested that PKC activators sensitized and induced the release of substance P.

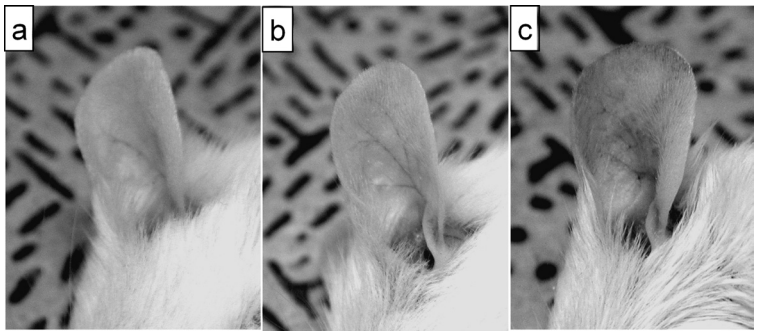

Fig. 4 The effect of GlcNAc-TA and teleocidin B on irritation of mice ear.

Chemicals were exposed on inside of mice ear (a) control, (b) 600 μg/ear of GlcNAc-TA, (c) 0.4 μg/ear of teleocidin B.

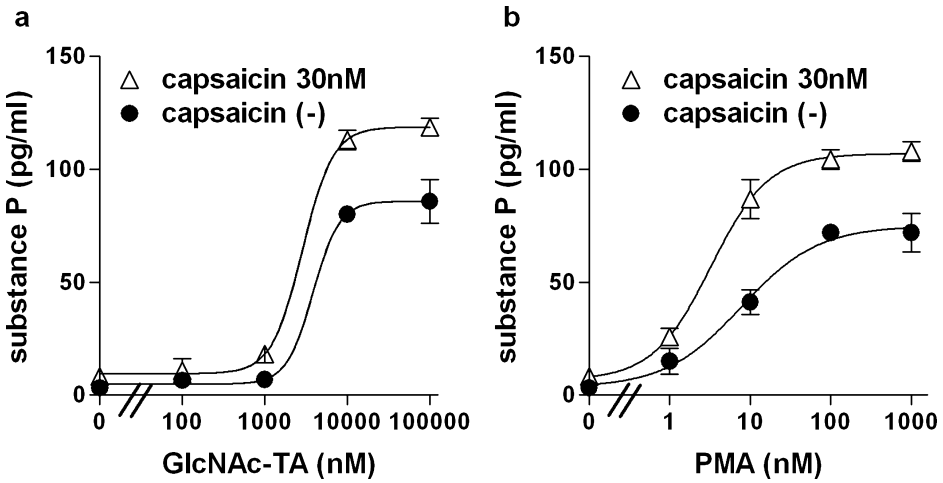

Fig. 5 The effect of GlcNAc-TA and PMA on substance P release from DRG neurons.

(a) Concentration-response of substance P release from DRG neurons induced by GlcNAc-TA with or without 30 nM capsaicin for 40 minutes. (b) Concentration-response of substance P release from DRG neurons induced by PMA with or without 30 nM capsaicin for 40 minutes. Data represent mean±S.D. of one representative experiment.

Discussion and Summury

Studies on teleocidin derivatives have clarified that a free hydroxyl group at C-14 is required for biological activities such as the affinity to PKC so far [12]. Our findings indicate that glucosylated GlcNAc-TA retains activities although it is less potent than teleocidin A. We established that GlcNAc-TA translocats PKCα-EGFP of the conventional PKCs and PKCθ-EGFP of novel PKCs to the plasma membrane and confirmed that GlcNAc-TA also inhibits Ca^{2+} mobilization induced by five agonists of GPCRs at level of 2.1～4.3 μM as well as other activators of PKC which are reported to inhibit the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) and Ca^{2+} mobilization by agonists of GPCRs like as endothelin-1, bradykinin and carbachol [7, 13, 14]. These activities are consistent with the known biological properties of teleocidins. However, GlcNAc-TA causes no irritation at levels as high as 600 μg/ear while teleocidin B causes irritation at 0.4 μg/ear in the mouse model. Teleocidin B with a free hydroxyl group at C-14 is reported to show tumor-promoting activity similar to teleocidin A [9]. At this stage, it is still unclear whether a free hydroxyl group at C-14 plays an important role in exhibiting irritant activities. However, our results reveal that the irritation activity is not proportional to the inhibitory activity of intracellular Ca^{2+} mobilization.

In DRG neuron, the release of excitatory neuropeptide substance P is induced by capsaicin, bradykinin and ATP [10], and capsaicin-induced currents are enhanced by the activation of PKC [11]. Moreover, it was reported that PKC signaling is involved in the mechanism of sensitization and also that TRPV1 is directly phosphorylated by PKC [15].

We clarified that GlcNAc-TA synergistically sensitizes the release of substance P induced by capsaicin from DRG neurons. In addition, GlcNAc-TA alone induces the release of substance P. The similar effects are also observed in PMA. In view of these facts, it seems that GlcNAc-TA affects the release of substance P *via* PKC pathway although the mechanism of the direct release of substance P is still not clear. This is the first observation that PKC activation directly triggers and sensitizes the release of substance P from DRG neurons in a dose-dependent manner.

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