

# Fucoidan inhibits $\text{Ca}^{2+}$ responses induced by a wide spectrum of agonists for G-protein-coupled receptors

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**Abstract.** Fucoidan, a sulfated polysaccharide extracted from brown seaweed, has been used in traditional Chinese herbal medicine to treat thyroid tumors for many years. Although a number of its cellular effects have been investigated, the role of fucoidan in molecular signaling, particularly in  $\text{Ca}^{2+}$  signaling, remains largely unknown. In the present study, the effects of fucoidan on  $\text{Ca}^{2+}$  responses in HeLa cells, human umbilical vein endothelial cells and astrocytes were investigated using a wide range of receptor agonists. Fucoidan inhibited the increase in intracellular free calcium concentration that was induced by histamine, ATP, compound 48/80 and acetylcholine. The responses induced by the same agonists in the absence of extracellular  $\text{Ca}^{2+}$  were also markedly suppressed by fucoidan. Reverse transcription-polymerase chain reaction demonstrated that 0.5 and 1.0 mg/ml fucoidan treatment for 3 h decreased histamine receptor 1 expression in HeLa cells. Similarly, the expressions of purinergic receptor P2Y, G-protein coupled (P2YR)1, P2YR2 and P2YR11 were significantly downregulated within cells pretreated with 1.0 mg/ml fucoidan for 3 h, and 0.5 mg/ml fucoidan significantly inhibited P2YR1 and P2YR11 expression. The results demonstrated that fucoidan may exert a wide spectrum of inhibitory effects on  $\text{Ca}^{2+}$  responses and that fucoidan may inhibit a number of different G-protein coupled receptors associated with  $\text{Ca}^{2+}$  dynamics.

## Introduction

Fucoidan is a sulfated polysaccharide extracted from brown seaweed. It has a chain structure similar to that of heparin (1,2). It exerts various biological activities including anticoagulation and inhibition of cell proliferation (3-7). Fucoidan may have antitumor activity against a variety of tumors, such as leukemia, sarcoma-180, non-small-cell human bronchopulmonary carcinoma (NSCLC-N6), and breast cancer (8-12). A double-blind randomized controlled trial showed low-molecular-weight fucoidan combined with chemical agents significantly improved the disease control rate in metastatic colorectal cancer patients (13). However, little is known about the mechanism underlying these biological activities. Intracellular calcium ions are important second messengers that control a variety of cellular responses (14). Several newly identified T-type calcium channel blockers have been shown to be able to inhibit the growth of human cancer cells by blocking  $\text{Ca}^{2+}$  influx into the cells (15), a process that affects cell cycle progression and cell proliferation. These blockers are potential therapeutic agents for the tumors that depend on T-type calcium channel to grow (16). These studies suggest that calcium signaling may participate in tumorigenesis and targeting their signaling could have therapeutic values.

However, details about the effect of fucoidan on  $\text{Ca}^{2+}$  signaling are largely unknown. In Sertoli cells, fucoidan could activate the  $\text{Ca}^{2+}$  influx regulated by the L-type voltage-operated  $\text{Ca}^{2+}$  channels by serving as an L-selectin ligand (17). However, the opposite effect of fucoidan on  $\text{Ca}^{2+}$  signaling has also been reported. In polymorphonuclear leukocytes, fucoidan could partly inhibit the oxLDL-induced increase in intracellular calcium concentration  $[\text{Ca}^{2+}]_i$  by serving as a scavenger receptor ligand (18). Despite the fact that different cell types were used in these studies, and that opposite effects of fucoidan on  $\text{Ca}^{2+}$  signaling were found, these studies commonly demonstrated that both L-selectin receptors and scavenger receptors were sensitive to fucoidan. Also, many tumor cells that do not express L-selectin receptors or the scavenger receptors still exhibit  $\text{Ca}^{2+}$  responses (19). In HeLa cells, for example,  $\text{Ca}^{2+}$  responses are induced through other receptors (20-23). These findings indicate that a wide variety of cell surface receptors may involve in the  $\text{Ca}^{2+}$

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response, and also suggest that fucoidan may participate in the  $\text{Ca}^{2+}$  signaling through these cell surface receptors.

In the present study, we investigated the effects of fucoidan on intracellular  $\text{Ca}^{2+}$  responses by using a variety of stimulants, including histamine, ATP, compound 48/80, and acetylcholine (ACh). We found that fucoidan inhibited the increase in  $[\text{Ca}^{2+}]_i$  induced by histamine, ATP, compound 48/80, and acetylcholine effectively. To further verify the effects of fucoidan on various G-protein coupled receptors, RT-PCR was applied to identify the expression of metabotropic histamine receptors and the purinergic P2Y receptors after fucoidan treatment. Consistently with the effect of fucoidan on the inhibition of  $[\text{Ca}^{2+}]_i$ , both H1R and subtypes of P2YRs (P2YR1, P2YR2 and P2YR11) expressions were significantly suppressed after fucoidan treatment. Taken together, our results demonstrate that fucoidan exerts a wide spectrum of inhibitory effects on  $\text{Ca}^{2+}$  responses and inhibits various G-protein coupled receptors related to  $\text{Ca}^{2+}$  dynamics, suggesting that fucoidan inhibits  $\text{Ca}^{2+}$  signaling by directly inhibiting G-protein coupled receptors.

## Materials and methods

**Chemicals.** Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, and 0.25% trypsin-EGTA (ethylene glycol tetraacetic acid) were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Fura-2 acetoxymethyl ester (fura-2/AM) was purchased from Dojindo Laboratories (Kumamoto, Japan). Acetylcholine chloride (ACh) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Fucoidan (MW=c.a. 20 kDa), adenosine-5'-triphosphate (ATP), compound 48/80, and histamine were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Heparin was obtained from Ajinomoto Co., Inc. (Tokyo, Japan).

**Passage cultures of HeLa cells, astrocytes, and HUVECs.** HeLa cells and astrocytes (a gift from Dr R Susuki, Photon Medical Research Center, Hamamatsu University School of Medicine) were cultured at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide ( $\text{CO}_2$ ) in DMEM supplemented with 10% FBS and 100 units/ml penicillin-streptomycin. These cells were maintained in 60-mm dishes (Corning Incorporated, Corning, NY, USA). For studies of  $\text{Ca}^{2+}$  response, the cells were subcultured in 35-mm glass-bottom dishes (Iwaki Glass, Chiba, Japan) at approximately  $0.5 \times 10^5$  cells per dish.

Human umbilical vein endothelial cells (HUVECs), purchased from Health Science Research Resources Bank (HSRRB; Osaka, Japan), were maintained in E-STIM Endothelial Cell Culture Medium (BD Biosciences, Franklin Lakes, NJ, USA) with 10% FBS, 10 ng/ml EGF, and 200  $\mu\text{g}/\text{ml}$  endothelial cell growth supplements (ECGS) on 75-mm flasks. They were cultured in a humidified incubator containing 5%  $\text{CO}_2$  and 95% air at 37°C. The HUVECs were removed from the substrate with 0.25% trypsin and 0.02% EDTA (BD Biosciences) and passaged on coverslips in 35-mm-diameter culture dishes at a density of  $1 \times 10^4$  cells/ml. The subcultures were used for  $[\text{Ca}^{2+}]_i$  measurements on day 2 after passage.

**Dye loading and medium.** The  $[\text{Ca}^{2+}]_i$  was measured using the fluorescent  $\text{Ca}^{2+}$  indicator dye fura-2. For staining, HeLa

cells, HUVECs, and astrocytes were incubated in a medium containing fura-2/AM (2.5  $\mu\text{M}$ ) for 20 min at 37°C. The cells were then rinsed twice with the medium prepared for calcium imaging. The medium, here referred to as recording medium, contained (in mM): NaCl, 140; KCl, 5;  $\text{CaCl}_2$ , 2;  $\text{MgCl}_2$ , 1.2; glucose, 2; and HEPES (pH 7.4), 10. A calcium-free recording medium was also used in which  $\text{CaCl}_2$  was removed,  $\text{MgCl}_2$  was increased to 3.2 mM, and 1 mM EGTA was added (pH 7.4).

**Calcium imaging.** Fluorescence imaging for  $[\text{Ca}^{2+}]_i$  was performed 24 h after subculture for HeLa cells and astrocytes, and 48 h after subculture for HUVECs. The cells in each culture dish loaded with fura-2 AM were placed on the stage of an inverted microscope (IX 70; Olympus, Tokyo, Japan). They were superfused continuously using silicone tubing warmed to 37°C and connected to a reservoir syringe held at a height of 70 cm. The cells were illuminated at wavelengths of 340 and 380 nm, alternating every 3 sec. Time-lapse images of the fluorescence emitted were obtained at 510 nm with a 40x objective lens (UApo 40x/340, NA 0.9; Olympus). An intensified charge-coupled device (CCD) camera (C4742-95; Hamamatsu Photonics K.K., Hamamatsu, Japan) was used to capture the images. Approximately 10-20 cells were observed in a microscopic field, and the changes in  $[\text{Ca}^{2+}]_i$  in individual cells were determined using the fluorescence ratio of 340/380 nm and an image-analysis software (Aquacosmos, Hamamatsu Photonics). The changes in  $[\text{Ca}^{2+}]_i$ , here referred to as  $\text{Ca}^{2+}$  responses, were quantified by calculating the areas under the response curve recorded in each cell image, which are expressed as the integrated amplitude of  $\text{Ca}^{2+}$  responses.

**Reverse transcription-polymerase chain reaction (RT-PCR).** In order to examine the effects of fucoidan on expressions of specific G-protein coupled receptors, RT-PCR was used to identify the mRNA expressions of H1R and P2YRs. HeLa cells were serum-starved for 24 h and treated with 0.5 and 1.0 mg/ml fucoidan respectively, for 3 h. The cells were harvested and extracted with RNA. Total RNA pellet was resolved in 10  $\mu\text{l}$  of diethylpyrocarbonate-treated water, and 1  $\mu\text{g}$  of each RNA sample was used for the RT reaction. RNA samples were reverse-transcribed to cDNA by RT EasyTM II kit (First-strand cDNA for Real-Time PCR) (Foregene Co., Ltd., Chengdu, China). RT-qPCR was conducted by Real Time PCR EasyTM-SYBR-Green I kit (Foregene Co., Ltd.). TaqMan primers and the probe for H1R were listed in Table I. Amplicon size and reaction specificity were confirmed by agarose gel electrophoresis. Identities of the PCR products were verified by sequencing using a genetic analysis system. Semi-quantitative RT-PCR was used to test the P2YRs expressions. cDNA was amplified by PCR using 2X Taq PCR StarMix (GenStar Biosolutions Co., Ltd., Beijing, China) according to the manufacturer's instructions. The conditions consisted of an initial denaturation step at 95°C for 2 min, followed by 40 cycles of 30 sec denaturation step at 95°C, 30 sec annealing step at 53.5°C to 56.5°C, and 1 min extension step at 72°C. A final extension step of 5 min at 72°C was also performed. PCR products were separated by electrophoresis on a 1% agarose gel. All PCR results were derived with cycle number producing a signal in the linear portion of the amplification curve. The primers were designed as presented in Table II. The

Table I. Primers for reverse transcription-quantitative polymerase chain reaction.

Primer	Sequence (5'-3')
H1R	F: CAGAGGATCAGATGTTAGGTGATAGC R: AGCGGAGCCTCTTCCAAGTAA
TaqMan probe	FAM-CTTCTCTCGAACGGACTCAGATAC CACC-TAMRA
GAPDH	F: CGGATTTGGTCGTATTGGG R: CGGATTTGGTCGTATTGGG

F, forward; R, reverse; H1R, histamine receptor 1.

GAPDH gene was used as the internal standard, and data were expressed as the ratio of H1R mRNA to GAPDH mRNA or P2YRs mRNA to GAPDH mRNA, respectively.

**Statistical analysis.** For the quantification of  $\text{Ca}^{2+}$  responses, 10-20 cells were analyzed in each observation. All observations were repeated using 3-5 culture dishes. The integrated amplitude of the  $\text{Ca}^{2+}$  response was calculated using an extended baseline for subtraction. Statistical significance was determined by one-way ANOVA followed by Student's t-test, and differences were considered significant at  $P < 0.05$ .

## Results

**Effects of fucoidan on  $\text{Ca}^{2+}$  responses induced by histamine in HeLa cells.** Histamine ( $2.5 \mu\text{M}$ ) was applied three times to induce  $\text{Ca}^{2+}$  responses. The first challenge induced a rapid rise in  $[\text{Ca}^{2+}]_i$  followed by a sustained plateau in HeLa cells in the presence of extracellular  $\text{Ca}^{2+}$  (Fig. 1A). When the cells were superfused with the recording medium containing fucoidan ( $0.5 \text{ mg/ml}$ ), the second histamine challenge (in the same medium) was followed, and only a small response was induced, i.e., a small peak with a small or no plateau preceded by a long delay time (Fig. 1A). The third challenge, which took place after the removal of fucoidan, induced a quite large response (Fig. 1A), suggesting that the suppressive effect of fucoidan was reversible. The recovery of the responses was observed within 2 min after removal of fucoidan.

A dose-response curve for the suppression of histamine-induced responses was obtained by using different concentrations of fucoidan (Fig. 1B). In the presence of extracellular  $\text{Ca}^{2+}$  (filled circles), the  $\text{Ca}^{2+}$  responses were gradually suppressed by fucoidan in a dose-dependent manner. The response was completely suppressed at a concentration of  $1.5 \text{ mg/ml}$ . Similarly, in the absence of extracellular  $\text{Ca}^{2+}$  (EGTA  $1 \text{ mM}$  present) (filled squares), fucoidan suppressed  $\text{Ca}^{2+}$  responses gradually as its concentration increased. In both the presence and absence of extracellular  $\text{Ca}^{2+}$ ,  $1.5 \text{ mg/ml}$  of fucoidan was sufficient to completely abolish histamine-induced  $\text{Ca}^{2+}$  responses.

In the presence of extracellular  $\text{Ca}^{2+}$ , challenges with histamine induced  $\text{Ca}^{2+}$  responses of a similar amplitude each time. However, in the absence of extracellular  $\text{Ca}^{2+}$ , stimulation with

Table II. Primers for semi-quantitative polymerase chain reaction.

Primer	Sequence (5'-3')
P2YR1	F: GACTTCTTGTACGTGCTGACTCT R: GACCTCTTGTACCTGATACGTG
P2YR2	F: CTCTACTTTGTACACCACCAG R: TTCTGCTCCTACAGCCGAAT
P2YR11	F: GAGGCCTGCATCAAGTGTCTG R: ACGTTGAGCACCCGCATGATG
GAPDH	F: ATTCCATGGCACCCTCAAGGCT R: TCAGGTCCACCACTGACACGTT

F, forward; R, reverse; P2YR, purinergic receptor P2Y, G-protein coupled.

an agonist, such as histamine, depleted intracellular  $\text{Ca}^{2+}$  pool, and thus the second or the third challenge in the same cell always induced a very small or no response at all. Therefore, we compared these responses statistically in the absence or presence of extracellular  $\text{Ca}^{2+}$  by integrating the amplitudes of the first responses induced by the first 3-min challenge only. For the control, we used 3-min applications of agonists after recording the baseline for 3 min. For the test, fucoidan was added to the recording medium during the same baseline period (3 min). Then, without removal of fucoidan, the subsequent doses of agonists were further added to the medium (Fig. 2A and C). In both presence and absence of extracellular  $\text{Ca}^{2+}$ , fucoidan at a concentration of  $0.5 \text{ mg/ml}$  suppressed the  $\text{Ca}^{2+}$  responses induced by histamine very effectively (Fig. 2B and D) as compared to the control.

**Effects of fucoidan on  $\text{Ca}^{2+}$  responses induced by ATP in HeLa cells.** Application of  $5 \mu\text{M}$  ATP to HeLa cells rapidly induced oscillating  $\text{Ca}^{2+}$  responses, as visualized by fura-2 imaging in the presence and absence of extracellular  $\text{Ca}^{2+}$ . The amplitudes of such responses were as large as those induced by histamine. We measured  $\text{Ca}^{2+}$  responses by varying the concentration of fucoidan in the presence and absence of extracellular  $\text{Ca}^{2+}$ . In the absence of calcium ions (EGTA,  $1 \text{ mM}$ ) (filled squares, Fig. 1C),  $\text{Ca}^{2+}$  responses were gradually suppressed as fucoidan concentration increased from  $0.005$  to  $5.0 \text{ mg/ml}$ . Responses were almost completely suppressed at a fucoidan concentration of  $5.0 \text{ mg/ml}$ . The suppression of ATP-induced  $\text{Ca}^{2+}$  responses by fucoidan appeared to be dose-dependent. Although the dose-dependent suppression curve observed in the presence of external  $\text{Ca}^{2+}$  (filled circles) was roughly parallel to that observed in the absence of extracellular  $\text{Ca}^{2+}$  (plus EGTA), the curve obtained with  $2 \text{ mM}$  extracellular  $\text{Ca}^{2+}$  did not reach zero. Even at a fucoidan concentration of  $5.0 \text{ mg/ml}$ , responses could not be suppressed completely in the presence of extracellular  $\text{Ca}^{2+}$ .

**Effects of fucoidan on  $\text{Ca}^{2+}$  responses induced by compound 48/80 in HeLa cells.** Compound 48/80 is an oligomeric mixture of the condensation products of p-methoxyphenethylamine

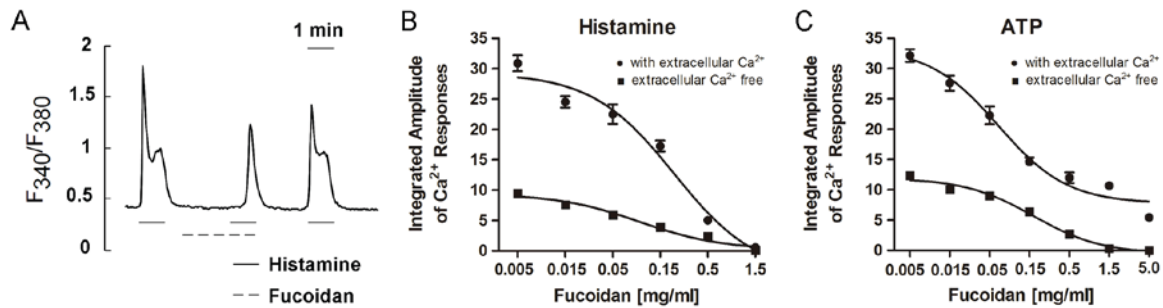


Figure 1. Dose-dependent effects of fucoidan on Ca<sup>2+</sup> responses induced by ATP and histamine in HeLa cells. (A) The trace of Ca<sup>2+</sup> responses induced by histamine (2.5  $\mu$ M). Presented is a representative trace of Ca<sup>2+</sup> responses in a single cell. An increase in fucoidan concentration in the second challenge suppressed the response observed elsewhere. Ca<sup>2+</sup> responses induced by (B) histamine (2.5  $\mu$ M) and (C) ATP (5  $\mu$ M) in the presence of fucoidan. Ca<sup>2+</sup> responses were measured in the presence (circles) and absence (squares) of extracellular Ca<sup>2+</sup> (1 mM EGTA added) in the superfusion medium. Data are presented as the mean  $\pm$  standard error of the mean. ATP, adenosine triphosphate; EGTA, ethylene glycol-bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid.

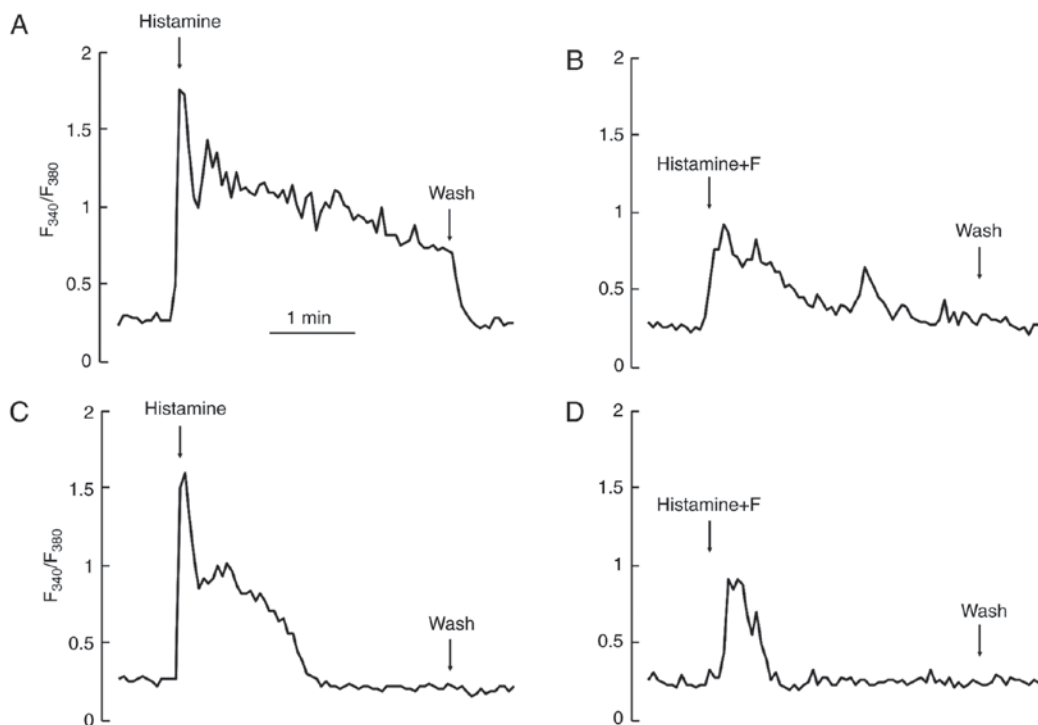


Figure 2. Ca<sup>2+</sup> responses induced by histamine in cultured HeLa cells. Ca<sup>2+</sup> responses traced from time-lapse images obtained via the application of 2.5  $\mu$ M histamine to cells superfused with a medium containing 2 mM calcium, recording in (A) the absence and (B) the presence of 0.5 mg/ml fucoidan. Ca<sup>2+</sup> responses elicited by the application of 2.5  $\mu$ M histamine to cells superfused with calcium-free medium were also analyzed, recording in the (C) absence and (D) the presence of fucoidan (0.5 mg/ml). F, fucoidan.

and formaldehyde. It activates G proteins by a mechanism analogous to that of mastoparan, which interacts directly with G proteins to mimic the role of the intracellular loop of G-protein-coupled receptors (24,25). We measured the Ca<sup>2+</sup> responses induced by compound 48/80 in the absence of extracellular Ca<sup>2+</sup>. Compound 48/80 induced rapid Ca<sup>2+</sup> responses in HeLa cells (Fig. 3A), and it was suppressed significantly by fucoidan at a concentration of 0.5 mg/ml (Fig. 3A). The responses induced by compound 48/80 were very sensitive to fucoidan.

*Effects of fucoidan on Ca<sup>2+</sup> responses induced by cholinergic agonists in HeLa cells.* Acetylcholine (ACh) has been shown to act through two major types of receptors, nicotinic and

muscarinic receptors. Nicotinic receptors are ligand-gated ion channels (nAChR, also known as ionotropic cholinergic receptors), while muscarinic receptors belong to the G-protein coupled receptor superfamily of seven transmembrane domain proteins (mAChR, also known as metabotropic cholinergic receptors). Our fura-2 Ca<sup>2+</sup> imaging showed that nAChR and mAChR agonists ACh (10  $\mu$ M) and carbachol (CCh, 10  $\mu$ M) increased the [Ca<sup>2+</sup>]<sub>i</sub> in HeLa cells. Both of these Ca<sup>2+</sup> transients were completely blocked by addition of atropine (10  $\mu$ M) (Fig. 3B), a muscarinic antagonist. When fucoidan was applied at 0.5 mg/ml, the ACh- or CCh-induced Ca<sup>2+</sup> responses were suppressed to an extent similar to that of inhibition by atropine (10  $\mu$ M) (Fig. 3B). This suppressive effect was reversed immediately after fucoidan was removed from the medium.



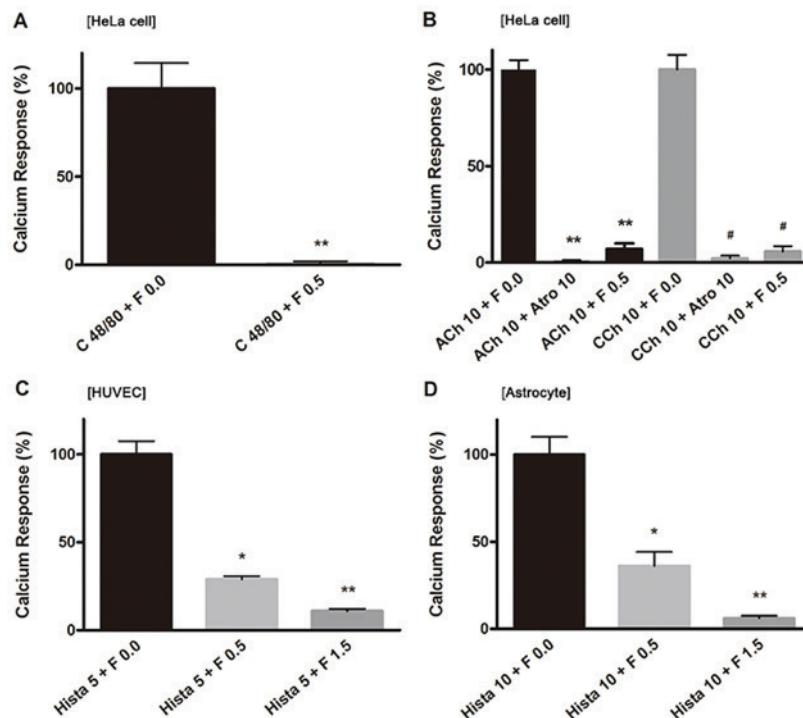


Figure 3.  $\text{Ca}^{2+}$  responses induced by various chemicals in different cells. The percentage of  $\text{Ca}^{2+}$  responses were analyzed in the following treatment groups: (A) Compound 48/80 (100  $\mu\text{M}$ ) and fucoidan (0.5 mg/ml) in HeLa cells; (B) 10  $\mu\text{M}$  ACh or 10  $\mu\text{M}$  CCh, atropine (10  $\mu\text{M}$ ) and fucoidan (0.5 mg/ml) in HeLa cells; (C) histamine (5  $\mu\text{M}$ ) and fucoidan (0.5 and 1.5 mg/ml) in HUVECs; and (D) histamine (10  $\mu\text{M}$ ) and fucoidan (0.5 and 1.5 mg/ml) in astrocytes. In each measurement, 10-20 cells were selected arbitrarily for analysis. The ordinate is expressed in reference to the control value. All data points indicate an average of 3-5 independent measurements (dishes), and data are presented as the mean  $\pm$  standard error of the mean. \* $P < 0.01$ , \*\* $P < 0.05$  and \*\*\* $P < 0.01$  vs. the corresponding 0 mg/ml fucoidan group. C 48/80, compound 48/80; F, fucoidan; ACh, acetylcholine; Atro, atropine; CCh, carbachol; Hist, histamine; HUVEC, human umbilical vein endothelial cells.

**Effects of fucoidan on  $\text{Ca}^{2+}$  responses induced by histamine in HUVECs and astrocytes.** In order to examine the cell specificity of the effects of fucoidan on  $\text{Ca}^{2+}$  responses, we verified the sensitivity of  $\text{Ca}^{2+}$  responses induced by an agonist common to different cell types, specifically HUVECs and astrocytes. Histamine induced  $\text{Ca}^{2+}$  response in HUVECs and astrocytes similar to those induced in HeLa cells. In HUVECs, the  $\text{Ca}^{2+}$  responses induced by histamine (5  $\mu\text{M}$ ) were suppressed to approximately 30% the magnitude of the first uninhibited application by the addition of fucoidan at 0.5 mg/ml and to approximately 10% by 1.5 mg/ml (Fig. 3C). In astrocytes,  $\text{Ca}^{2+}$  responses induced by histamine (10  $\mu\text{M}$ ) were reduced to approximately 36 and 6% the magnitude of the first uninhibited application when the cells were given fucoidan at 0.5 and 1.5 mg/ml, respectively (Fig. 3D), suggesting that histamine receptors expressed both in HUVECs and astrocytes were sensitive to fucoidan in a manner similar to that of HeLa cells.

**Effect of heparin on  $\text{Ca}^{2+}$  responses induced by histamine in HeLa cells.** Heparin is also a sulfated polysaccharide with a structure similar to that of fucoidan. It has been shown that heparin via micropipette-injection into cells inhibited  $\text{Ca}^{2+}$  responses by inhibiting the inositol 1,4,5-trisphosphate ( $\text{InsP}_3$ ) receptor (26,27). In our study, heparin was applied extracellularly. Fig. 4 shows a representative single cell trace of  $[\text{Ca}^{2+}]_i$  during a heparin test. In a series of trials, HeLa cells were stimulated first with histamine (2.5  $\mu\text{M}$ ) and then superfused with a recording medium containing 40 unit/ml of heparin. Without removal of heparin, the same concentration

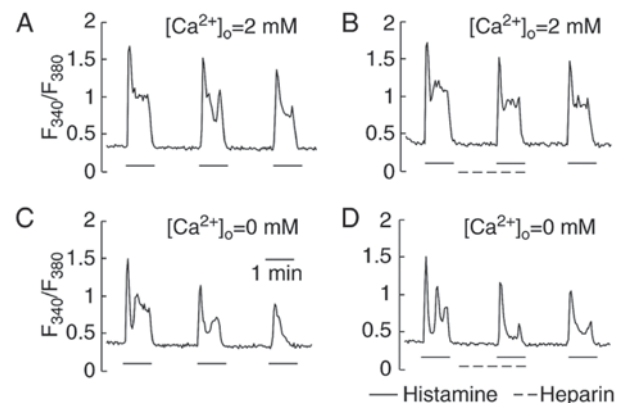


Figure 4. Effects of heparin on histamine-induced  $\text{Ca}^{2+}$  responses in cultured HeLa cells.  $\text{Ca}^{2+}$  responses traced from time-lapse images obtained via the application of 2.5  $\mu\text{M}$  histamine to cells superfused with a medium containing 2 mM calcium, recording in the (A) absence and (B) presence of heparin.  $\text{Ca}^{2+}$  responses elicited by the application of 2.5  $\mu\text{M}$  histamine to cells superfused with calcium-free medium were also analyzed, recording in the (C) absence and (D) presence of heparin.  $[\text{Ca}^{2+}]_o$ , extracellular calcium.

of histamine (2.5  $\mu\text{M}$ ) was re-administered to evaluate the effects of heparin on the  $\text{Ca}^{2+}$  response. By comparing the peak  $\text{Ca}^{2+}$  response induced by histamine in the presence of extracellular  $\text{Ca}^{2+}$  (Fig. 4A and B) with that in the absence (Fig. 4C and D), heparin was found to neither facilitate nor suppress the generation of  $\text{Ca}^{2+}$  responses. The removal of heparin did not increase the peak of  $\text{Ca}^{2+}$  responses induced by the next histamine stimulation. The  $\text{Ca}^{2+}$  responses

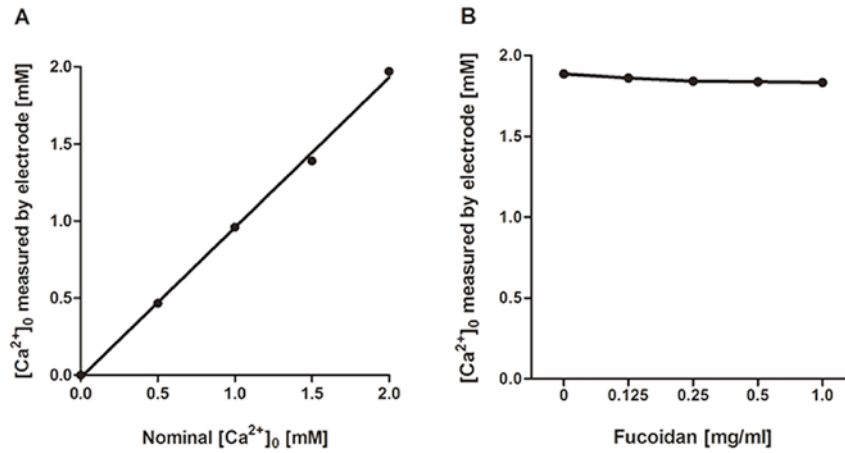


Figure 5. Calcium ions in media were not chelated by fucoidan. (A) Calibration curve for a calcium-sensitive electrode generated by increasing the nominal  $Ca^{2+}$  concentration in the recording medium. (B) Calcium ion concentrations in the fucoidan-containing recording medium were measured using the electrode. The dose of fucoidan varied from 0.0 to 1.0 mg/ml. One-way analysis of variance identified no statistically significant differences between the calcium ion concentrations of fucoidan-containing medium and the recording medium ( $P > 0.05$ ).  $[Ca^{2+}]_0$ , extracellular calcium.

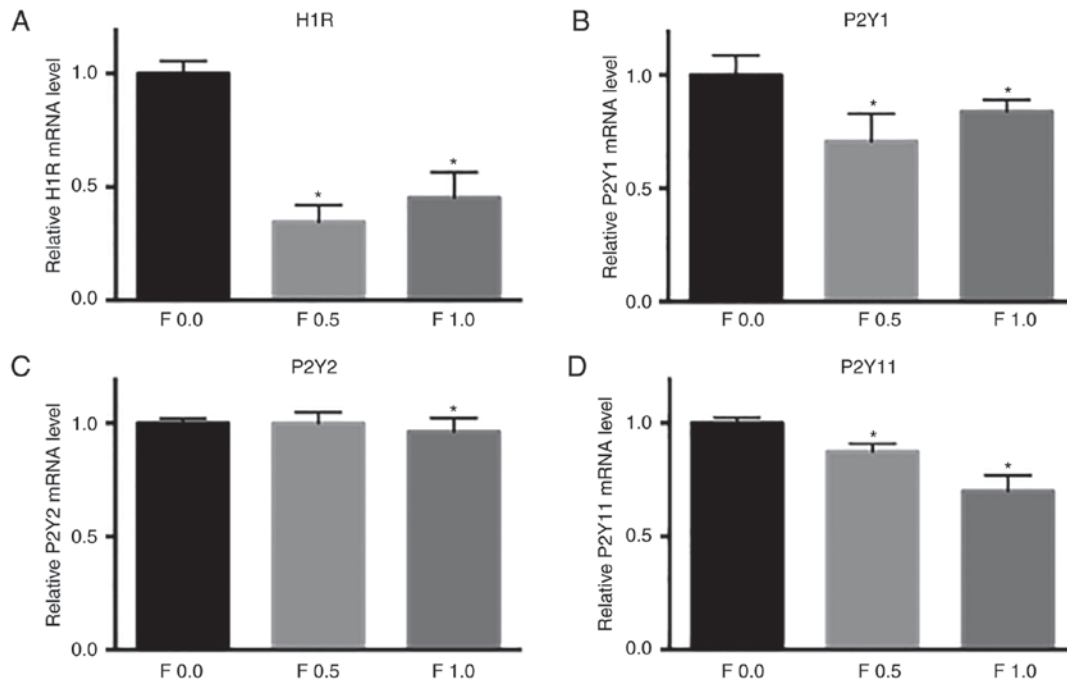


Figure 6. Effects of fucoidan on the expressions of H1R and the P2YRs in HeLa cells. GAPDH was used as the internal standard. (A) The ratio of H1R mRNA to GAPDH mRNA in the untreated cells, or cells treated with either 0.5 or 1.0 mg/ml fucoidan, as indicated. The relative mRNA expressions of (B) P2YR1, (C) P2YR2 and (D) P2YR11, in the untreated cells, or cells treated with either 0.5 mg/ml or 1.0 mg/ml fucoidan, as indicated, were also analyzed. \* $P < 0.05$  vs. 0 mg/ml fucoidan (control). H1R, histamine receptor 1; P2YR, purinergic receptor P2Y, G-protein coupled; F, fucoidan.

induced by ATP (5  $\mu$ M) were also insensitive to heparin (data not shown).

**Effects of fucoidan on calcium ions in the medium.** As described above, fucoidan showed inhibitory effects on  $Ca^{2+}$  responses linked to a wide range of receptors. We suspected that the inhibition of  $Ca^{2+}$  responses by fucoidan would be attributable to one of its physical properties related to interaction with calcium ions. Fucoidan is a sulfated polysaccharide and has many negatively charged sulfate groups. These may chelate positively charged  $Ca^{2+}$  in the extracellular medium. Assuming that this is the case, the shortage of external  $Ca^{2+}$

would lead to a reduction or complete loss of  $Ca^{2+}$  response. Our data, obtained using a  $Ca^{2+}$ -sensitive electrode (Fig. 5), show that fucoidan has only a very small effect on the effective concentration of calcium ions in solution. This excluded the possibility that the suppression of  $Ca^{2+}$  response by fucoidan was due to its effect on  $Ca^{2+}$  chelating. The biological effects of fucoidan can then be solely attributed to its interactions with membrane proteins.

**Effects of fucoidan on expressions of H1R and P2YRs in HeLa cells.** To identify the histamine receptors and P2YRs expressed in HeLa cells and the effect of fucoidan on expressions of H1R

Table III. Spectrum of effects induced by fucoidan.

Membrane receptor	HeLa cells					HUVECs	Astrocytes
	Hist	ATP	ACh	48/80	BK	Hist	Hist
GPCR	+	+	+	+	/	+	+
Ion channel	/	-	/	/	/	/	/

HUVEC, human umbilical vein endothelial cells; GPCR, G-protein coupled receptor; Hist, histamine; ATP, adenosine triphosphate; ACh, acetylcholine; 48/80, compound 48/80; BK, Bay K8644; +, inhibitory effect; -, no effect; /, no match.

and P2YRs, RT-PCR of H1R, P2YR1, P2YR2, and P2YR11 were performed. H1R expression in HeLa cells treated by 0.5 or 1.0 mg/ml fucoidan for 3 h was significantly lower than that of untreated cells (Fig. 6A). Consistently, the expressions of P2YR1 and P2YR11 in HeLa cells induced by either 0.5 or 1.0 mg/ml for 3 h were decreased significantly compared with untreated cells. And the expression of P2YR2 was also inhibited by 1.0 mg/ml fucoidan treatment for 3 h compared with that of the untreated cells (Fig. 6B, C and D). These results suggested that fucoidan suppressed the histamine-induced  $\text{Ca}^{2+}$  response by interacting with the H1 type metabotropic histamine receptors, and inhibited ATP-evoked  $[\text{Ca}^{2+}]_i$  by inhibiting the purinergic G-protein-coupled P2Y receptors.

## Discussion

In this report, we described the effects of fucoidan on intracellular  $\text{Ca}^{2+}$  responses induced by the ligands for various receptors to explore the potentials of direct interactions of fucoidan with these receptors. We showed that irrespective of cell types, fucoidan had an inhibitory effect on increases in  $[\text{Ca}^{2+}]_i$  induced by ligands for different receptors in a dose-dependent manner. Our results demonstrated a dose-dependent pattern of fucoidan suppression on histamine- and ATP-induced  $\text{Ca}^{2+}$  responses. Furthermore, we showed that such inhibitory effects of fucoidan were not affected by the external calcium ions. It has been found that fucoidan interacts with a wide variety of receptors, and more importantly, fucoidan has a large number of sulfate residues, it is unlikely that such molecules could diffuse into the cells. In addition, RT-PCR assay identified that fucoidan inhibits mRNA expressions of various G-protein-coupled receptors H1R and P2YRs. Taken together, our results strongly suggest that fucoidan inhibition of  $\text{Ca}^{2+}$  responses may be due to a direct inhibition of different receptors in different cell types.

Histamine receptors are a class of G-protein-coupled receptors (GPCR) with histamine as their endogenous ligand (28). HeLa cells display well characterized  $\text{Ca}^{2+}$  responses upon histamine stimulation.  $\text{Ca}^{2+}$  responses should be attributed to activation of metabotropic histamine receptors. Our results showed that the integrated amplitude of  $\text{Ca}^{2+}$  responses was approximately three times larger in the presence of extracellular  $\text{Ca}^{2+}$  than in its absence (Fig. 1B). Previous studies have shown that histamine evokes an increase in  $[\text{Ca}^{2+}]_i$ , of which the initial transient increase is independent of extracellular  $\text{Ca}^{2+}$ . This is followed by a sustained increase that can be

abolished by the removal of extracellular  $\text{Ca}^{2+}$  (29,30). Previous studies have also demonstrated that all histamine-induced  $\text{Ca}^{2+}$  responses are completely inhibited by the H1 receptor antagonist pyrilamine but not by cimetidine, an inhibitor of  $\text{H}_2$  receptors. It has been suggested that the histamine H1-receptor mediates these responses in HeLa cells (31,32).

Histamine liberation from mast cells can be induced by compound 48/80 (33). This stimulates the metabotropic receptor family. This receptor is also expressed on the plasma membranes of HeLa cells and is very sensitive to fucoidan (Fig. 3A). Furthermore, mRNA of histamine was significantly inhibited by fucoidan suggesting that fucoidan probably suppressed the histamine-induced  $\text{Ca}^{2+}$  response (Fig. 1B) by interacting with the H1 type of metabotropic histamine receptors (Fig. 6A).

Purinergic receptors include ligand-gated P2X receptors and G-protein-coupled P2Y receptors. ATP is a stimulant of both P2X receptors and P2Y receptors (34,35). In the presence of extracellular  $\text{Ca}^{2+}$ ,  $\text{Ca}^{2+}$  responses resulted from both P2X and P2Y receptors. Fucoidan showed dose-dependence in inhibiting  $\text{Ca}^{2+}$  responses induced by ATP (5  $\mu\text{M}$ ) in the presence of extracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_o=2\text{ mM}$ ) (Fig. 1C). However, this inhibition is partial, suggesting that fucoidan must affect either the P2X or P2Y purinergic receptors but not both. In the absence of extracellular  $\text{Ca}^{2+}$  (1 mM EGTA added), P2X receptors lost their functions, and only P2Y receptors contributed to the increase in  $[\text{Ca}^{2+}]_i$ . Fucoidan at 5.0 mg/ml completely inhibited the increase in  $[\text{Ca}^{2+}]_i$  in the absence of extracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_o=0\text{ mM}$ ) (Fig. 1C). Fig. 1C shows that fucoidan suppresses ATP-induced responses by inhibiting the metabotropic P2Y but not ionotropic P2X receptors. To further identify whether fucoidan impacts on the metabotropic P2Y receptors or not, RT-PCR was used to verify the effect of fucoidan on the expressions of different subtypes of P2YRs. Consistently with the previous results, both treatments of 0.5 and 1.0 mg/ml fucoidan for 3 h inhibit the expression of P2YR1 and P2YR11 significantly, and 1.0 mg/ml fucoidan treatment for 3 h suppresses the expression of P2YR2, which suggests that fucoidan suppresses the P2YRs expression directly, by which inhibits the ATP-evoked  $\text{Ca}^{2+}$  response (Fig. 6B-D).

Two main classes of ACh receptors are nAChRs and mAChRs: the former are stimulated by nicotine and ACh, and the latter are stimulated by muscarine and ACh. ACh-induced  $\text{Ca}^{2+}$  responses can be completely blocked by atropine in HeLa cells. Atropine is a competitive antagonist only against

the mAChR, suggesting that no nAChR was expressed in the HeLa cells. Fucoidan inhibited the ACh- or CCh-induced  $\text{Ca}^{2+}$  responses to an extent similar to that of atropine. This demonstrated that fucoidan can block  $\text{Ca}^{2+}$  responses induced by mAChRs in HeLa cells.

We examined the inhibitory effects of fucoidan on  $\text{Ca}^{2+}$  responses both in the presence and absence of extracellular  $\text{Ca}^{2+}$ . In the absence of extracellular  $\text{Ca}^{2+}$  (chelated by EGTA), the  $\text{Ca}^{2+}$  channel does not contribute to  $\text{Ca}^{2+}$  response. The  $\text{Ca}^{2+}$  response in HeLa cells could not be induced by Bay K8644, an L-type  $\text{Ca}^{2+}$  channel activator, suggesting that the major  $\text{Ca}^{2+}$  channel (L-type) is not expressed in HeLa cells (data not shown). This is similar to the results reported by Gavazzo *et al* (36).

We conclude that, in the case of HeLa cells, HUVECs, and astrocytes,  $\text{Ca}^{2+}$  responses induced by histamine, ATP, compound 48/80, and ACh can be abolished by fucoidan through an inhibition of G-protein-coupled receptors. Specifically, the downstream of signal transduction is inhibited at the individual sites of the membrane receptors.

In summary, a broad range of membrane receptors, including metabotropic receptors, are strongly sensitive to fucoidan (Table III). The following points indicate that fucoidan interacts with the cell membrane by a direct extracellular approach only: i) Fucoidan is a large molecule (approximately 20 kDa). ii) Fucoidan is a negatively charged molecule with many sulfate residues, making diffusion through the cell membrane difficult. iii) The effects of fucoidan appear immediately after application and disappear soon after removal. iv) Fucoidan suppresses endocytosis dramatically, so it could not enter the cell in this way (37). It is quite reasonable to assume that receptors can be internalized when they are occupied by their own ligand (the cell membrane itself is endocytosed). This is visualized by other researchers in histamine receptors and others. So, probably, there is a feedback mechanism in the cell when the density of receptor proteins in intracellular vesicles or in Golgi apparatus become high, expression of mRNA is reduced.

It is known that heparin is an inhibitor of  $\text{InsP}_3$  receptors. Because fucoidan has a structure similar to that of heparin, a function similar to that of heparin (inhibition of intracellular  $\text{InsP}_3$  receptor pathway) can be expected to underlie its inhibitory effect. Heparin was tested by direct injection into the cytoplasm to inhibit the release of  $\text{Ca}^{2+}$  from the endoplasmic reticulum (26,27). In the present study, we applied heparin extracellularly to HeLa cells without any membrane treatment. Heparin did not show any inhibitory effect on the  $\text{Ca}^{2+}$  responses induced by histamine either in the presence or absence of external  $\text{Ca}^{2+}$  (Fig. 4). Therefore, the fucoidan effect is very difficult to be explained by assuming that the molecule binds to the  $\text{InsP}_3$  receptors located on the endoplasmic reticulum. Fucoidan exerts its inhibitory effects on  $\text{Ca}^{2+}$  responses very fast after its application, and these inhibitory effects are reversed within 3 min of its removal from the medium. These findings also support the idea that fucoidan inhibits receptor proteins to suppress  $\text{Ca}^{2+}$  responses.

The present work demonstrates that fucoidan has a wide spectrum of effects, most of them somehow connected to inhibit a  $\text{Ca}^{2+}$  response induced by diverse types of agonists and that these effects occur in a dose-dependent manner.

Inhibition was found to be associated with the inhibitory effects on the activities of G-protein-coupled receptors irrespective of cell types. HeLa cells, HUVECs, and astrocytes showed the similar results. The clinical use of fucoidan must be considered with a great care because it has immediate, strong effects on receptor activity, endocytosis and delayed effects on cell proliferation. The degradation of fucoidan into smaller units can be presumed to reduce these effects (although we did not examine this). When administered orally (and degraded by digestive acids), fucoidan might be less effective. While clinical use by injection or local spray inside the body may yield significant results, but which can be quite unpredictable because of the large number of fucoidan targets.

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