RESEARCH ARTICLE

A novel mechanism underlying phytate-mediated biological action-phytate hydrolysates induce intracellular calcium signaling by a Gαq protein-coupled receptor and phospholipase C-dependent mechanism in colorectal cancer cells

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Phytate (inositol hexa-phosphate, IP6) possesses multiple biological functions including anticancer activity. IP6 is converted to inositol di-, tri-, and tetra-phosphates (IP2, IP3, and IP4) by phytase in large intestinal microbes; however, their contribution to the IP6-mediated functions has not been investigated. We have developed the preparations of IP2–4 and IP3-rich phytate hydrolysate (IP3-RPH) by IP6 digestion using microbial phytase, and examined the induction of intracellular Ca^{2+} signaling in response to the preparations in colorectal cancer cells. IP2–4, but not inositol (IP0) and IP6, induced increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in Caco-2 cells with the following rank order: IP3 > IP2 = IP4. Inositol tri-phosphate (IP3)-RPH induced increases in $[Ca^{2+}]_i$ in both undifferentiated Caco-2 and HT-29 cells, but not in differentiated Caco-2. The IP3-RPH-induced $[Ca^{2+}]_i$ increase was resistant to extracellular Ca^{2+} depletion, however, it was impaired by inhibitors of phospholipase C, inositol 1, 4, 5 tri-phosphate receptor, ryanodine receptor, and Gαq protein. These results show that the putative G protein-coupled receptor on the plasma membrane senses the IP6 hydrolysates and activates phospholipase Cβ, resulting in Ca^{2+} mobilization through Ca^{2+} channels coupled with the inositol 1, 4, 5 tri-phosphate and ryanodine receptors on the sarco-endoplasmic reticulum Ca^{2+} store in colorectal cancer cells.

Keywords:
Calcium signaling / Colorectal cancer cells / G protein-coupled receptor / Phospholipase C / Phytate hydrolysates

1 Introduction

Phytate, myo-inositol 1,2,3,4,5,6 hexa-phosphate (IP6), is ubiquitously distributed throughout the plant kingdom. Matured plant seeds are especially rich in IP6, particularly cereal grains, nuts, and legume seeds in which concentrations range from 0.4 to 6.4% [1]; therefore, the daily intake of IP6 generally depends on the consumption of cereals and a wide variation in daily intake is reported [2–4]. A significant body of evidence indicates that diets rich in cereals reduce the risk of large intestinal cancer [5, 6]. IP6 has been considered to contribute to this cereal-mediated protective effect, although dietary fiber is another possible constituent. Indeed, many animal studies have shown that dietary supplementation with IP6 provides substantial protection against experimentally induced colonic cancer [7–9]. However, the precise mechanisms underlying the IP6-mediated protective effect against colonic cancer remains poorly understood.

In order to elucidate the molecular mechanisms of the IP6-mediated effect, numerous *in vitro* studies using cultured cancer cells have been conducted with IP6 itself

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Abbreviations: **2-APB,** 2-aminoethoxydipheyl borate; **$[Ca^{2+}]_i$,** intracellular Ca^{2+} concentration; **GPCR,** G protein-coupled receptor; **IP0,** inositol; **IP2,** inositol di-phosphate; **IP3,** inositol tri-phosphate; **IP3 (1, 4, 5),** inositol 1, 4, 5 tri-phosphate; **IP3R,** inositol 1, 4, 5 tri-phosphate receptor; **IP3-RPH,** inositol tri-phosphate-rich phytate hydrolysate; **IP4,** inositol tetra-phosphate; **IP6,** inositol hexa-phosphate or phytate; **PLC,** phospholipase C; **RYR,** ryanodine receptor; **SER,** sarco-endoplasmic reticulum

[10–12]. IP6 is reported to suppress the growth of colonic cancer cells through G1/G0 arrest and S-phase inhibition [10], and induce apoptosis through inhibition of the Akt/NFkappaB pathway [11]. IP6 is poorly digested by the gastrointestinal digestive enzymes [13], although intrinsic phytase naturally present in cereals can partly hydrolyze IP6 during the digestive process [14]. Therefore, a significant part of dietary phytate reaches to the large intestinal lumen in single-stomached animals such as humans and rats. Once there, large intestinal microbes possessing high levels of phytase activity convert IP6 to inositol di-, tri-, tetra-, and penta-phosphates (IP2–5). It has been reported that phosphorus of IP6 in oatmeal was almost completely removed through the intestine of adult man [15] and that 56% of dietary IP6 was hydrolyzed in conventional rats [16]. Although IP6 itself can exert biological effects prior to degradation by intrinsic and microbial phytase, the contribution of IP6 hydrolysates to the IP6-mediated biological effect should be considered. However, there is no information to date on the biological activities of IP6 hydrolysates.

Calcium (Ca^{2+}) is an important and versatile intracellular signal transduction messenger that is involved in the regulation of almost all cellular processes, including proliferation, apoptosis, and gene transcription [17]. Cancer growth is reliant on an increase in proliferation and decreases in apoptosis and differentiation. Therefore, the homeostasis of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) has become a major focus in current cancer and cancer therapy research [18, 19]. An anticancer agent, cisplatin, has been reported to induce apoptosis by increasing $[Ca^{2+}]_i$ in enucleated human melanoma and colon cancer cells [20]. A phenolic compound derived from ginger, 6-gingerol, possesses antitumorigenic and pro-apoptotic activities and induces increased $[Ca^{2+}]_i$ in Madin–Darby Canine Kidney cells [21].

In this study, we first developed the preparations of IP2, IP3, and IP4 from IP6 using microbial phytase and HPLC. We then investigated the induction of intracellular Ca^{2+} mobilization by each IP6 hydrolysate in human colorectal cancer cells (Caco-2 and HT-29), and the signaling pathway leading to Ca^{2+} mobilization.

2 Materials and methods

2.1 Chemicals

Reagents and supplies for cell culture as well as fura-2AM for intracellular Ca^{2+} measurement were purchased from Invitrogen (San Francisco, CA). U73122 (a phospholipase C (PLC) inhibitor), 2-aminoethoxydipheyl borate (2-APB) (an inositol 1, 4, 5 tri-phosphate receptor (IP3R) antagonist), dantrolene (a ryanodine receptor (RYR) antagonist), and genistein (a tyrosine kinase inhibitor) were purchased from Calbiochem (San Diego, CA). YM-254890 (a Gαq protein inhibitor) was kindly donated by Astellas Pharma (Tokyo, Japan). All other chemicals were obtained from Wako Pure Chemical Industries.

2.2 Preparation of IP2, IP3 and IP4

Forty grams of myo-inositol hexakis(dihydrogen phosphate) (IP6) and 6 mg of phytase from *Aspergillus ficcum* (Sigma, Saint Louis) were suspended in 200 mL of 50 mM sodium acetate, pH 5.5, and incubated at 37°C for 2–6 h. The resulting hydrolysate was boiled for 20 min to inactivate the phytase and cooled on ice. The hydrolysate was subjected to anion-exchange chromatography to isolate each IP0 phosphate after filtration using a 0.2 μm filter. Separation of inositol and each inositol phosphate was performed using an HPLC system equipped with a packed column with an anion-exchange resin, Dowex 1-X8 (32 mm inner diameter, 1450 mm long; The Dow Chemical, Midland, MI). The column was eluted by a linear gradient of HCl and post-column derivitization was achieved with a solution of 1 g/L $Fe(NO_3)_3$ in 0.33 M $HClO_4$ followed by detection of A_{295} [22]. The fractions corresponding to IP2, IP3 and IP4 were collected. The representative chromatogram is shown in Fig. 1A.

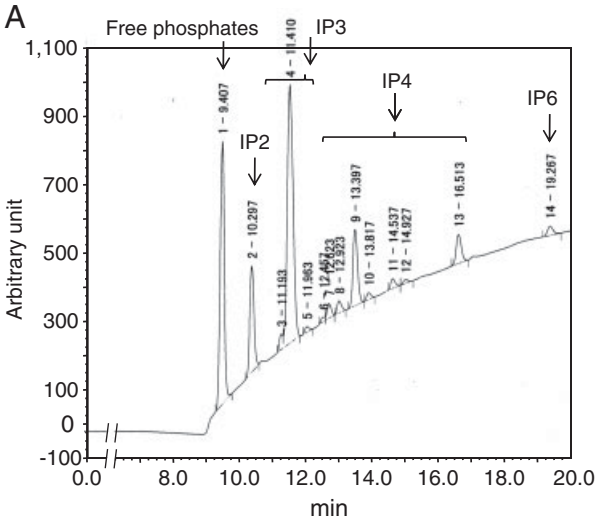
The alignment of phosphate groups in the isolated IP2, IP3 and IP4 were determined by NMR analysis as described previously [23]. NMR analysis revealed that IP2 and IP3 carry phosphate groups on positions 1 and 2, and positions 1, 2, and 6 of the inositol ring (IP2, IP3). IP4 was found to be a mixture of IP4 carrying four phosphates on positions 1, 2, *X*, and 6 ($X = 3$, 4, or 5). The chemical structures of the inositol phosphates used in the study are shown in Fig. 1B.

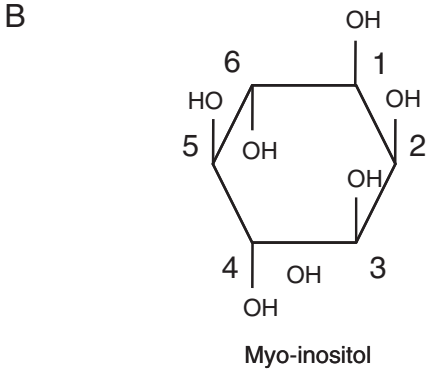
2.3 Preparation of IP3-rich phytate hydrolysate

IP6 hydrolysate was prepared by digestion with the phytase for 4 h as described above. The hydrolysate was desalted using a packed column with a strongly acidic cation-exchange resin, Diaion PK216 (55 mm inner diameter, 550 mm long; Mitsubishi Chemical, Tokyo, Japan). The eluate obtained after concentration by rotary evaporator is referred to as IP3-rich phytate hydrolysate (IP3-RPH). The molar ratio of IP2:IP3:IP4 in the IP3-RPH was found to be 1:8.6:3.6 according to the HPLC analysis as described above. The representative chromatogram is shown in Fig. 2.

2.4 Cell culture

Human colorectal cancer cells, Caco-2 (HTB-37; American Type Culture Collection, Rockville, MD) and HT-29 (HTB-38, American Type Culture Collection), were grown in





	Positions of phosphate groups
IP0 (inositol)	--
IP2	1, 2
IP3	1, 2, 6
IP4	1, 2, x, 6 (x=3, 4 or 5)
IP6 (phytate)	1, 2, 3, 4, 5, 6

Figure 1. HPLC analysis and the chemical structures of inositol phosphates used in the study. (A) Phytate was hydrolyzed by phytase for 2 h and then subjected to the HPLC analysis. Peaks in the profiles corresponding to each of the free phosphates and IP2–6 are indicated with labels and arrows. (B) Chemical structure of myo-inositol and the positions of the phosphate groups in each inositol phosphate identified by NMR analysis are shown.

DMEM supplemented with 100 mL/L decomplemented fetal bovine serum, 44 mM sodium bicarbonate, 1 mM sodium pyruvate, 50 000 U/L penicillin, and 50 mg/L streptomycin in a humidified 5% CO_2 atmosphere at 37°C. A rat basophilic leukemia cell, RBL-2H3 (CRL-2256, American Type Culture Collection), was grown in Eagle's minimal essential medium supplemented with 100 mL/L decomplemented fetal bovine serum, 44 mM sodium bicarbonate, and the antibiotics described above. Caco-2, HT-29, and RBL-2H3 cells were used between passage 35 and 60, 160 and 180, and 5 and 10, respectively.

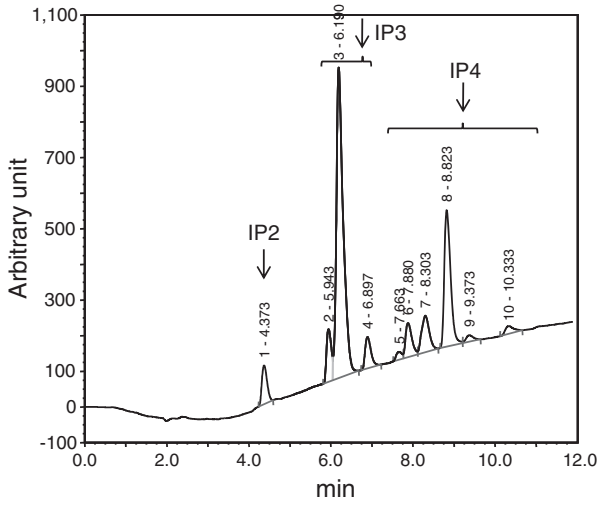


Figure 2. HPLC analysis of IP3-RPH. Phytate was hydrolyzed by phytase for 4 h. Peaks in the profiles corresponding to each form of IP2–4 are indicated with labels and arrows.

2.5 Intracellular Ca^{2+} measurement

$[Ca^{2+}]_i$ was measured according to a general protocol on the basis of changes in the excitation spectrum of the fluorescent probe fura-2, a calcium-sensitive ratiometric dye. Caco-2, HT-29, and RBL-2H3 cells were grown on collagen-coated cover slips and loaded with 10 μmol/L fura-2AM and 0.2 g/L cremophor EL for 20 min at 37°C. The cover slips together with the cells were mounted in a quartz cuvette containing 2 mL Hanks' Balanced Salt Solution (134 NaCl, 4.2 $NaHCO_3$, 0.34 Na_2HPO_4, 5.4 KCl, 0.44 KH_2PO_4, 1.25 $CaCl_2$, 0.49 $MgCl_2$, 0.41 $MgSO_4$, 5.6 D-glucose, 4.0 L-glutamine, and 10 HEPES (mM), pH 7.4) after washing with the solution, and introduced into the sample chamber of a CAF-110 spectrofluorometer (JASCO International, Tokyo, Japan) at 25°C with stirring at 400 rpm. After stabilization of the fluorescence, cells were exposed to the test agents. Fura-2 fluorescence intensity was monitored at an emission wavelength of 510 nm by alternating the excitation wavelength between 340 and 380 nm with a dual excitation monochromator. The ratio of the signals at 340 and 380 nm was calculated, and the maximal and minimal fluorescence ratios were obtained by the addition of 0.2% Triton X-100 and 20 mM EGTA in 18 mM Tris, respectively (final concentrations). The transformation of fluorescence signals into $[Ca^{2+}]_i$ was performed by the method of Grynkiewicz *et al.* [24]. Data are expressed as changes in $[Ca^{2+}]_i$ from basal levels before exposure to the test agents (0 min). All values are expressed as means with their SEM.

Inositol, IP2, IP3, IP4, and IP6 at 3 mM and IP3-RPH at 1.5–10 mM (final concentration) were used for the $[Ca^{2+}]_i$ measurements in Caco-2 cells on day 2 post-seeding. Measurement was also performed with 3 mM IP3-RPH in Caco-2 cells on days 2–21 post-seeding. In order to specify the signaling pathways involved in Ca^{2+} mobilization by IP3-RPH, $[Ca^{2+}]_i$ measurements were performed in HT-29 cells under Ca^{2+}-free conditions and loaded with the following five signaling inhibitors for 20 min along with fura-2AM; U73122 (a PLC inhibitor), 2-APB (an IP3R antagonist), dantrolene (an RYR antagonist), genistein (a tyrosine kinase inhibitor), and YM-254890 (a Gαq protein inhibitor). HT-29 cells were treated with pronase for 10 min at 37°C to digest the extracellular domain of the transmembrane proteins and with neuraminidase for 2 h at 37°C to cleave the glycosidic linkages of neuraminic acid on the membrane surface.

2.6 Evaluations of alkaline phosphatase and sucrase activities

Caco-2 cells grown in a 6-well plate were lysed with 0.5 mL of 50 mM Tris buffer containing 1% Triton X-100 and protease inhibitors (5 μg/mL aprotinin, 3 μg/mL leupeptin hemisulfate, 5 mmol/L benzamidine hydrochloride, and 1 mmol/L PMSF, pH 7.4) on days 2, 4, 14, and 21 post-seeding. Alkaline phosphatase activity and protein concentration of the cell lysis were measured using commercially available kits (ALP-K Test Wako; Wako Pure Chemical Industries, and BCA protein assay reagent; Pierce Biotechnology, Rockford, IL, respectively). Sucrase activity was determined as described previously [25]. All values are expressed as means with their SEM. Statistical analyses were performed by 1-way ANOVA followed by Tukey–Kramer multiple range test using the general linear models procedure of the Statistical Analysis Systems program (version 6.07; SAS Institute Cary, NC). A difference with $p < 0.05$ was considered significantly.

3 Results

3.1 Induction of Ca^{2+} signaling by inositol and each inositol phosphate in Caco-2 cells

Increases in $[Ca^{2+}]_i$ were transiently induced by 3 mM IP2, IP3, and IP4, but not by inositol (IP0) or phytate (IP6), in Caco-2 cells on day 2 (Fig. 3A). IP3 induced larger increases (70–100 nM) in $[Ca^{2+}]_i$ than did either IP2 or IP4, which showed similar increases (30–60 nM).

Caco-2 cells were then exposed to various concentrations of IP3-RPH (1.5–10 mM; Fig. 3B). The dose-dependent increases in $[Ca^{2+}]_i$ were induced by IP3-RPH at concentrations of up to 3 mM. 5 mM IP3-RPH induced an increase in $[Ca^{2+}]_i$ of similar intensity to that induced by 3 mM, which was much higher than that induced by 10 mM. The shape and intensity of the increases in $[Ca^{2+}]_i$ were similar for IP3-RPH and IP3 at 3 mM.

The induction of Ca^{2+} signaling by IP3-RPH was examined in Caco-2 cells cultured for the various periods after seeding (2–21 days; Fig. 3C). Desensitization to IP3-RPH was observed in Caco-2 cells with increased culture duration. The increases in $[Ca^{2+}]_i$ induced by IP3-RPH was much lower on day 5 than on day 2 and no substantial increase was observed on day 7, 14, or 21.

3.2 Cellular differentiation of Caco-2 cells

The activities of intestinal alkaline phosphatase and sucrase in Caco-2 cells increased with increases in the duration of culture. Compared with that observation on day 2 (0.63 ± 0.09 IU/g protein), alkaline phosphatase activity was approximately three-fold higher on day 4 (1.90 ± 0.30), six-fold higher on day 14 (3.74 ± 0.26), and ten-fold higher on day 21 (6.16 ± 0.12) whereas, sucrase activity (193 ± 2 IU/g protein on day 2) was approximately 2.5-fold higher on day 4 (466 ± 4), 8.5-fold

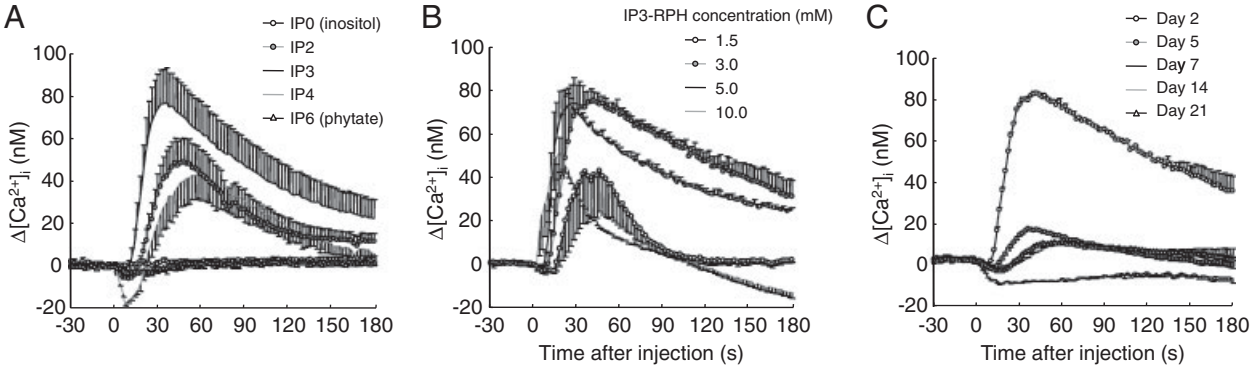


Figure 3. $[Ca^{2+}]_i$ in Caco-2 cells in response to inositol phosphates. (A) Caco-2 cells were exposed to 3 mM IP0, IP2, IP3, IP4, and IP6. (B) Caco-2 cells were exposed to IP3-RPH at various concentrations (1.5–10 mM). (C) Caco-2 cells were exposed to 3 mM IP3-RPH on days 2–21 post-seeding. Values are means ± SEM, $n = 4$.

higher on day 14 (1648 ± 87), and 12-fold higher on day 21 (2291 ± 100).

3.3 Induction of Ca^{2+} signaling by IP3-RPH in Caco-2, HT-29, and RBL-3H2 cells

The induction of Ca^{2+} signaling by IP3-RPH was examined in the Caco-2, HT-29, and RBL-3H2 cells (Fig. 4A). HT-29 cells showed higher increases in $[Ca^{2+}]_i$ (90–110 nM) by IP3-RPH than did Caco-2 cells (70–80 nM). IP3-RPH did not induce Ca^{2+} signaling in RBL-3H2 cells, though the $[Ca^{2+}]_i$ was gradually and slightly shifted to around 20 nM during the measurement period.

The induction of Ca^{2+} signaling was also examined at various concentrations of IP3-RPH (1.5–10 mM) in HT-29 cells (Fig. 4B). The maximal increase in $[Ca^{2+}]_i$ was observed in the cells exposed to 3 mM IP3-RPH, and the increases in $[Ca^{2+}]_i$ fell with increases in concentration.

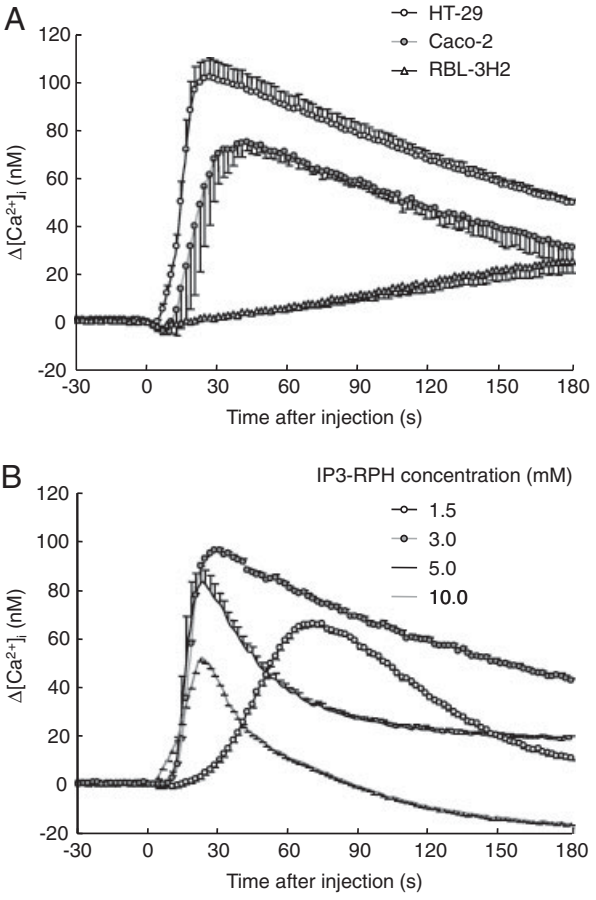


Figure 4. Intracellular Ca^{2+} concentrations in different cells in response to IP3-RPH. (A) HT-29, Caco-2, and RBL-3H2 cells were exposed to 3 mM IP3-RPH. (B) HT-29 cells were exposed to IP3-RPH at various concentrations (1.5–10 mM). Values are means ± SEM, $n = 4$.

3.4 PLC-dependent Ca^{2+} mobilization from sarco-endoplasmic reticulum

HT-29 cells were equilibrated with HBSS in the absence of Ca^{2+} and then exposed to 3 mM IP3-RPH (Fig. 5A). Elimination of extracellular Ca^{2+} did not influence the IP3-RPH-mediated Ca^{2+} signaling in the HT-29 cells. HT-29 cells were pre-treated with thapsigargin (a sarco-endoplasmic reticulum calcium ATPase (SERCA) inhibitor) and then exposed to IP3-RPH (Fig. 5B). Thapsigargin completely abolished the increases in $[Ca^{2+}]_i$ in response to IP3-RPH.

Pre-treatment of HT-29 cells with 2-APB (an antagonist of IP3R on the membrane of the sarco-endoplasmic reticulum (SER)) and dantrolene (an RYR antagonist) partially and entirely blocked the induction of Ca^{2+} signaling by IP3-RPH, respectively (Figs. 5C and D).

HT-29 cells were pre-treated with 10 and 20 μM U73122 (a PLC inhibitor; Fig. 5E). The increases in $[Ca^{2+}]_i$ by IP3-RPH fell with increases in the concentration of U73122 and was completely abolished by pre-treatment with 20 μM U73122.

3.5 The involvement of a putative G protein-coupled receptor in sensing IP3-RPH

HT-29 cells were pre-treated with pronase and neuraminidase to examine the involvement of transmembrane proteins and saccharides on the cell surface membranes in the IP3-RPH-mediated increases in $[Ca^{2+}]_i$ (Figs. 6A and B). Pre-treatment with pronase, but not neuraminidase, desensitized the response of HT-29 cells to IP3-RPH and reduced the increases in $[Ca^{2+}]_i$ induced by IP3-RPH in a dose-dependent manner.

An inhibitor of the Gαq protein-coupled receptor, YM-254890, completely abolished the increases in $[Ca^{2+}]_i$ induced by IP3-RPH (Fig. 6C). A non-specific tyrosine kinase inhibitor, genistein, did not have any effect on the IP3-RPH-mediated Ca^{2+} signaling (Fig. 6D).

4 Discussion

This study demonstrates that IP2, IP3 and IP4 induce increases in $[Ca^{2+}]_i$ in Caco-2 and HT-29 colorectal cancer cells, and that IP3 is the most potent in inducing these increases. The $[Ca^{2+}]_i$ mobilization in response to IP3-RPH is mediated by PLC, which is associated with the putative Gαq protein-coupled receptor. IP0 and IP6 both lack this activity; therefore, our results indicate a novel mechanism underlying the IP6-mediated biological action in colonic cancer cells.

We first explored the induction of Ca^{2+} signaling by IP0 and each inositol phosphate (IP2, IP3, IP4, and IP6) in Caco-2 cells and found the rank order of potency to be: IP3 > IP2 = IP4. Neither IP0 nor IP6 showed any effects on $[Ca^{2+}]_i$. These results clearly show that the Ca^{2+} mobilization does not simply

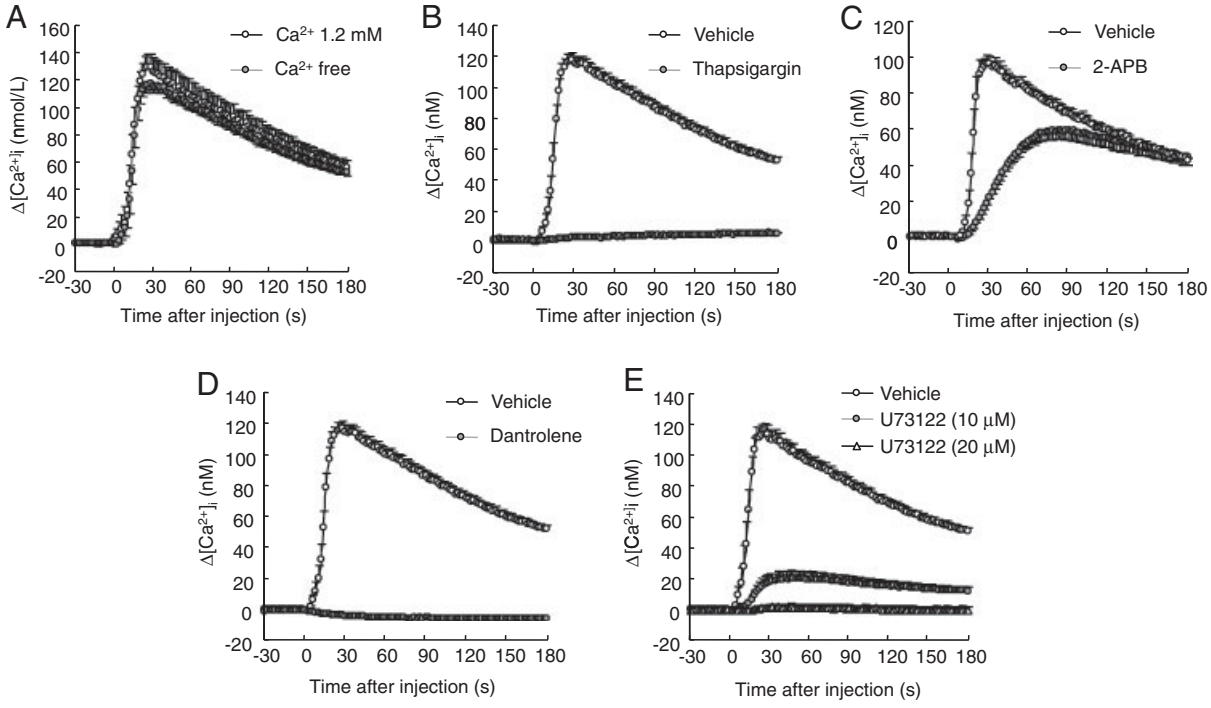


Figure 5. Intracellular Ca^{2+} concentrations in HT-29 cells pre-treated with signal inhibitors in response to IP3-RPH. (A) HT-29 cells were exposed to 3 mM IP3-RPH in the presence and absence of Ca^{2+}. (B, C, D, E) HT-29 cells were pre-treated with thapsigargin (B), 2-APB (C), dantrolene (D) and U73122 (E), and then exposed to 3 mM IP3-RPH. Values are means ± SEM, $n = 4$.

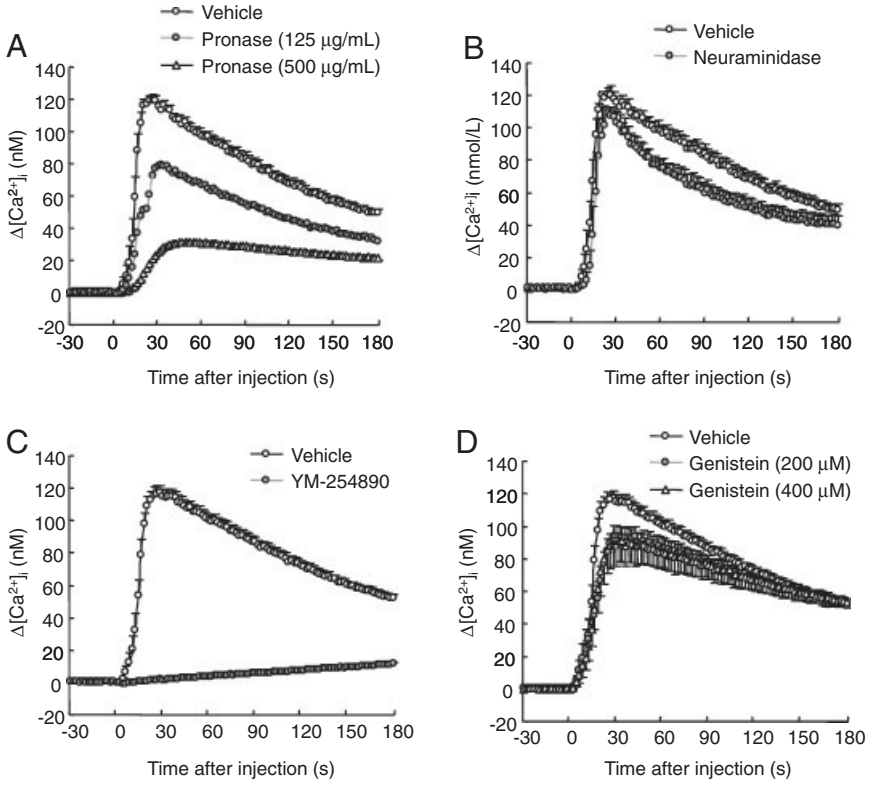


Figure 6. Intracellular Ca^{2+} concentrations in HT-29 cells pre-treated with pronase, neuraminidase, and signal inhibitors in response to IP3-RPH. (A, B, C, D) HT-29 cells were pre-treated with pronase (A), neuraminidase (B), YM-254890 (C), and genistein (D), and then exposed to 3 mM IP3-RPH. Values are means ± SEM, $n = 4$.

depend on the presence of an inositol ring or the number of phosphate groups in the inositol phosphates, and is associated with the combination and configuration of phosphate groups in the molecules. It seems reasonable that the phosphate groups in positions 1 and 2 of the inositol ring shared by IP2, IP3, and IP4, which all induce the Ca^{2+} mobilization, have an important role in the Ca^{2+} mobilization. In addition, the phosphate group in position 6, which is present in IP3, but not in IP2, plays a role in the amplification of Ca^{2+} mobilization. Meanwhile, the phosphate groups in positions 3, 4, and 5 are likely to negatively influence the inositol phosphate-mediated Ca^{2+} mobilization, as IP6 lacks this activity and IP4 has less effect than IP3.

We found that the increases in $[Ca^{2+}]_i$ induced by IP3-RPH were higher in HT-29 than Caco-2 cells and that RBL-3H2, which is extensively used as a mast cell model, shows no response to IP3-RPH. Further, the responses to IP3-RPH in Caco-2 cells were decreased by extending the culture period. Caco-2 cells are known to differentiate to intestinal epithelial cells during prolonged culture [26], indicated by the higher activities of sucrase and alkaline phosphatase on the latter days in our study. These results indicate that IP3-RPH and inositol phosphates (IP2–4) selectively exert biological activity in undifferentiated colonic cancer cells rather than in normal differentiated epithelial cells or mast cells. This is supported by many *in vivo* studies showing no substantial effects on the functions of the intestines or other tissues in animals fed IP6 [9, 14, 16].

Based on the evidence that IP3-RPH-mediated increases in $[Ca^{2+}]_i$ are resistant to extracellular Ca^{2+} depletion and are blocked by thapsigargin (an SER calcium ATPase inhibitor), it is thought that Ca^{2+} is mobilized by IP3-RPH from the SER. Furthermore, our results demonstrated that both the IP3R- and RYR-coupled Ca^{2+} channels on the SER contribute to the Ca^{2+} mobilization in response to IP3-RPH. It is known that Ca^{2+} release *via* the RYR that occurs in response to increases in $[Ca^{2+}]_i$ *via* the IP3R [17], and that inositol 1, 4, 5 tri-phosphate (IP3 (1, 4, 5)) is cleaved from phophatidylinositol 4, 5 bisphosphate distributed in the plasma membrane by PLC [27]. The PLC inhibitor (U73122) dose-dependently reduced the Ca^{2+} mobilization by IP3-RPH and completely blocked it at higher doses in our results. However, an IP3R antagonist (2-APB) showed only partial inhibition and an RYR antagonist completely blocked the Ca^{2+} mobilization. The latter may be explained by the previous work showing that the RYR antagonist blocks IP3 (1, 4, 5)-mediated Ca^{2+} increases as well in guinea-pig colonic smooth muscle cells [28], although the former remains unexplainable. Taken together, these results indicate that the PLC-dependent increase in $[Ca^{2+}]_i$ *via* the IP3R on the SER by IP3-RPH triggers Ca^{2+} release from the SER *via* the RYR (Fig. 7).

We examined the involvement of the G protein-coupled receptor (GPCR) and tyrosine kinase coupled receptor in the IP3-RPH-mediated Ca^{2+} mobilization, because PLCβ and PLCγ are known to be associated with GPCR and tyrosine kinase coupled receptor, respectively [27]. The evidence that YM-254890 (a Gαq protein inhibitor), but not genistein (a non-specific tyrosine kinase inhibitor), impaired the Ca^{2+} mobilization by IP3-RPH obviously demonstrates that the putative Gαq protein-coupled receptor senses extracellular IP3-RPH, resulting in PLCβ activation. The existence of the putative GPCR on the cell surface membrane for IP3-RPH is supported by the inhibition of Ca^{2+} mobilization in the cells treated with pronase. The number of GPCRs has been estimated at nearly 800 in both human and mouse genomes by bioinformatic analyses [29]; however, no GPCRs recognizing inositol phosphates as a ligand that have been identified. Further investigations are required to identify the GPCR that senses IP3-RPH.

It is clear that IP3-RPH did not induce the Ca^{2+} mobilization by direct stimulation of the IP3R on the SER after permeation into cells because the Ca mobilization was completely abolished by the PLC and Gαq protein inhibitors as mentioned above. IP3 (inositol 1, 2, 6 tri-phosphate) is the IP3-RPH component most responsible for the Ca^{2+} mobilization; however, it is a position isomer of IP3 (1, 4, 5), an intracellular second messenger, and is unlikely to be recognized by the IP3R. Meanwhile, DeWald *et al.* [30] demonstrated that the intracellular deliveries of some inositol phosphates achieved by their conjugations with the hydrophobic carriers directly induce the Ca^{2+} mobilization from SER. IP3-RPH, however, is highly hydrophilic and is not thought to easily permeate the plasma membranes.

In vivo studies using animal models have demonstrated that the dietary intake of IP6 has a protective effect on colonic cancer at the early and late stages of carcinogenesis. It has been reported that an IP6-containing diet protects against early biomarkers of colon cancer by reducing the

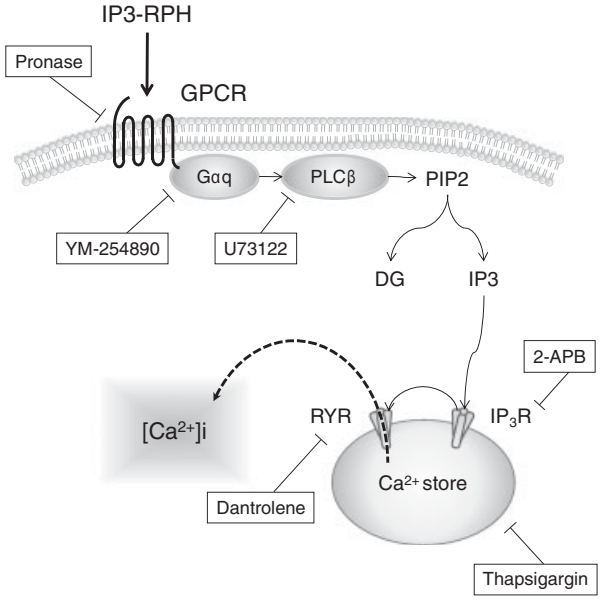


Figure 7. A diagram showing signaling pathway leading to Ca^{2+} mobilization by IP3-RPH.

proliferating cell nuclear antigen-labeling index of cell proliferation [31]. Supplemental feeding of IP6 decreases the incidence of aberrant crypts, an intermediate biomarker of colon cancer [9]. Meanwhile, *in vitro* studies using colorectal cancer cells have been conducted to understand the molecular mechanisms of the IP6-mediated protective effect [10, 12, 32]. IP6 has been consistently shown to exert anticancer effects, such as decreasing cell proliferation [10] and inducing cell differentiation [12] and apoptosis [11]. However, all of the previous studies have dealt only with intact IP6, although many studies have shown that IP6 is cleaved into IP2–5 and phosphates by phytase in the bacteria of the large intestine and that IP2–5 is distributed throughout the large intestinal lumen [14–16]. Therefore, it is important to investigate the effects of IP2–5 as well as those of intact IP6 to gain an understanding of the molecular mechanisms of IP6-mediated biological activity. In this study we found that IP2–4, but not IP0 or IP6, induce Ca^{2+} signaling. Intracellular Ca^{2+} impacts nearly every aspect of cellular function, including the proliferation, differentiation, and apoptosis of cells, which jointly determine cancer cell growth [17–19]. The anticancer activities by some agents and food factors such as cisplatin and 6-gingerol are reportedly involved with the induction of Ca^{2+} signaling [20, 21]. Our results suggest that the IP6 hydrolysates possibly participate in the IP6-mediated biological activity, although we did not clarify the cellular functions associated the $[Ca^{2+}]_i$ increase induced by IP3-RPH.

In summary IP6 is hydrolyzed by phytase and induces intracellular Ca^{2+} signaling in human colonic cancer cells, HT-29 and Caco-2. The putative GPCR on the plasma membrane senses the IP6 hydrolysates and activates PLCβ, resulting in Ca^{2+} mobilization from the SER through Ca^{2+} channels coupled with the IP3 (1, 4, 5) and RYRs on the SER.

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