Cyanidin-3-glucoside Inhibits ATP-induced Intracellular Free Ca^{2+} Concentration, ROS Formation and Mitochondrial Depolarization in PC12 Cells

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Flavonoids have an ability to suppress various ion channels. We determined whether one of flavonoids, cyanidin-3-glucoside, affects adenosine 5’-triphosphate (ATP)-induced calcium signaling using digital imaging methods for intracellular free Ca^{2+} concentration ([Ca^{2+}]_{i}), reactive oxygen species (ROS) and mitochondrial membrane potential in PC12 cells. Treatment with ATP (100 μM) for 90 sec induced [Ca^{2+}]_{i}, increases in PC12 cells. Pretreatment with cyanidin-3-glucoside (1 μg/ml to 100 μg/ml) for 30 min inhibited the ATP-induced [Ca^{2+}]_{i}, increases in a concentration-dependent manner (IC_{50}=15.3 μg/ml). Pretreatment with cyanidin-3-glucoside (15 μg/ml) for 30 min significantly inhibited the ATP-induced [Ca^{2+}]_{i} responses following removal of extracellular Ca^{2+} or depletions of intracellular [Ca^{2+}]_{i} stores. Cyanidin-3-glucoside also significantly inhibited the relatively specific P2X2 receptor agonist 2-MeSATP-induced [Ca^{2+}]_{i} responses. Cyanidin-3-glucoside significantly inhibited the thapsigargin or ATP-induced store-operated calcium entry. Cyanidin-3-glucoside significantly inhibited the ATP-induced [Ca^{2+}]_{i} responses in the presence of nimodipine and ω-conotoxin. Cyanidin-3-glucoside also significantly inhibited KCl (50 mM)-induced [Ca^{2+}]_{i} increases. Cyanidin-3-glucoside significantly inhibited ATP-induced mitochondrial depolarization. The intracellular Ca^{2+} chelator BAPTA-AM or the mitochondrial Ca^{2+} uniporter inhibitor RU360 blocked the ATP-induced mitochondrial depolarization in the presence of cyanidin-3-glucoside. Cyanidin-3-glucoside blocked ATP-induced formation of ROS. BAPTA-AM further decreased the formation of ROS in the presence of cyanidin-3-glucoside. All these results suggest that cyanidin-3-glucoside inhibits ATP-induced calcium signaling in PC12 cells by inhibiting multiple pathways which are the influx of extracellular Ca^{2+} through the nimodipine and ω-conotoxin-sensitive and -insensitive pathways and the release of Ca^{2+} from intracellular stores. In addition, cyanidin-3-glucoside inhibits ATP-induced formation of ROS by inhibiting Ca^{2+}-induced mitochondrial depolarization.

Key Words: ATP, Calcium, Cyanidin-3-glucoside, Mitochondrial membrane potential, Reactive oxygen species

INTRODUCTION

Adenosine 5’-triphosphate (ATP) induces an intracellular free Ca^{2+} concentration ([Ca^{2+}]_{i}) increase by release of Ca^{2+} from inositol-1,4,5-trisphosphate (IP₃)-sensitive Ca^{2+} stores through activation of P2Y-receptor-mediated phospholipase C (PLC) and influx of extracellular Ca^{2+} through activation of P2X-receptor [1]. Increases in [Ca^{2+}]_{i}, can induce mitochondrial depolarization [2] and mitochondrial matrix Ca^{2+} overload can lead to enhanced formation of reactive oxygen species (ROS) [3]. Moreover, ATP-induced [Ca^{2+}]_{i} increase in PC12 cells may be involved in the cell death [4], the differentiation [5] and the release of catecholamine [6].

Flavonoids show anti-oxidant effects, anti-inflammatory

ABBREVIATIONS: AM, acetoxymethylester; AMPA, α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ATP, adenosine 5’-triphosphate; DMEM, Dulbecco’s modified Eagle’s medium; EGTA, ethylene glycol tetra acetic acid; ER, endoplasmic reticulum; FBS, fetal bovine serum; HDCFDA, dichlorodihydrofluorescein diacetate; HEPES-HBSS, HEPES-buffered Hank’s balanced salt; HS, horse serum; IP₃, inositol-1,4,5-trisphosphate; MeSATP, 2-Methylthiadenosine triphosphate tetrasodium salt; NMDA, N-methyl-D-aspartate; PC12, pheochromocytoma; PKC, protein kinase C; PLC, phospholipase C; ROS, reactive oxygen species; SOC, store-operated calcium channels; SOCE, store-operated calcium entry.
activity, anti-carcinogenic effects, anti-viral effects, anti-aging effects [7-9]. Flavonoids also have an ability to suppress various ion channels including Ca\(^{2+}\) channels [10,11]. In addition, flavonoids have been reported to inhibit agonist-induced [Ca\(^{2+}\)], increase [12-14] and inhibit cell death [8,13].

Cyanidin-3-glucoside is a major anthocyanin which belongs to the flavonoid family [9]. Cyanidin-3-glucoside has been reported to prevent neuronal cell death [15-17]. However, the effects of cyanidin-3-glucoside on ATP-induced [Ca\(^{2+}\)], increase, ROS formation, and mitochondrial depolarization in PC12 cells have not been explored yet.

In this study, we evaluated how cyanidin-3-glucoside can ameliorate ATP-induced [Ca\(^{2+}\)], increase, mitochondrial depolarization and formation of ROS. Our results indicate that cyanidin-3-glucoside inhibits ATP-induced calcium signaling in PC12 cells by inhibiting multiple pathways which are the influx of extracellular Ca\(^{2+}\) through nifedipine and ω-conotoxin-sensitive and-insensitive pathways and the release of Ca\(^{2+}\) from intracellular stores. In addition, cyanidin-3-glucoside inhibits ATP-induced formation of ROS by inhibiting Ca\(^{2+}\)-induced mitochondrial depolarization.

**METHODS**

**Materials**

Materials were purchased as follows: fura-2 acetoxymethyl ester (AM), 2,7-dichlorodihydrofluorescein diacetate (H2DCFDA) and rhodamine 123 from Molecular Probes (Eugene, OR, USA); Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS, heat-inactivated) and horse serum (HS, heat-inactivated) from Invitrogen (Carlsbad, CA, USA); ATP disodium salt, and all other reagents from Sigma (St. Louis, MO, USA).

**Preparation of cyanidin-3-glucoside**

Cyanidin-3-glucoside fraction from black soybean [cv. Cheongja 3, Glycine max (L.) Merr.] which was grown at Cheongja 3, Glycine max (L.) Merr., which was grown at Cando, Jeju Island, South Korea, was prepared as described by Ahn et al [13]. Cyanidin-3-glucoside was stored at -70°C until use.

**Cell culture**

We purchased rat pheochromocytoma (PC12) cells from ATCC Company (Manassas, VA 20108, USA). PC12 cells from passage 6-14 were grown in 100 mm dish in DMEM, supplemented with 10% FBS and 10% HS at 37°C in a humidified atmosphere of 5% CO\(_2\) and 90% O\(_2\) air. Cells from the stock culture were plated in six-well culture plates at a density of 3×10\(^5\) cells per well, onto 25 mm round glass coverslips starting from 200 ng/ml and washed with H\(_2\)O. Cells were used for experiment 2-3 days after plating.

**Digital calcium imaging**

Digital calcium imaging was performed as described by Ahn et al [13]. Cells were loaded at 37°C for 45 min in 10 μM fura-2 AM in HEPES-buffered Hank’s balanced salt solution (HEPES-HBSS:20 mM HEPES, 137 mM NaCl, 1.3 mm CaCl\(_2\), 0.4 mM MgSO\(_4\), 0.5 mM MgCl\(_2\), 0.4 mM KH\(_2\)PO\(_4\), 0.6 mM Na\(_2\)HPO\(_4\), 3.0 mM NaHCO\(_3\), and 5.6 mM glucose) containing 0.5% bovine serum albumin. The fura-2 loaded cells were alternately excited at 340±10 and 380±10 nm by rapidly switching optical filters mounted on a computer-controlled wheel (Lambda DG-4, Sutter Instruments, Novato, CA, USA), which was placed between a 200 W Xe arc lamp and the epifluorescence port of the microscope. Excitation light reflected from a dichroic mirror (400 nm) through a 20X objective (Nikon TE300, Tokyo, Japan) was collected with a cooled charge-coupled device camera cascade 512B (512×512 binned to 256×256 pixels, Photometrics, Tucson, AZ, USA) controlled by a computer. Image pairs were collected every 3-460 sec using an Axon Imaging Work Bench 6.0 (INDEC Biosystems, Santa Clara, CA, USA); exposure to excitation light was 120 ms per image. Background images were collected at the beginning of each experiment after removing cells from the coverslips. [Ca\(^{2+}\)]\(_i\) was calculated from the ratio of the background-subtracted digital images. Ratio values were converted to free [Ca\(^{2+}\)]\(_i\) by the equation [Ca\(^{2+}\)]\(_i\)=K\(_d\)·(R−R\(_{\text{min}}\))/(R\(_{\text{max}}\)−R), in which R is the 340/380 nm fluorescence emission ratio, and K\(_d\)=224 nM is the dissociation constant for fura-2. The maximum ratio (R\(_{\text{max}}\)=9.326), the minimum ratio (R\(_{\text{min}}\)=0.302), and the constant K\(_d\) (the ratio of the fluorescence measured at 380 nm in Ca\(^{2+}\)-free and saturating solutions) were determined by treating cells with 10 μM ionomycin in Ca\(^{2+}\)-free (1 mM EGTA) and saturating (5 mM Ca\(^{2+}\)) solutions.

**Measurement of mitochondrial membrane potential**

Rhodamine 123 was used for measurements of mitochondrial membrane potential. PC12 cells were loaded with 10 μM rhodamine 123 for 20 min. The fluorescence of rhodamine 123 was detected by using the same method as the calcium imaging. Images were collected every 60 sec for first 10 min of treatment and for the following 20 min images were collected every 5 min interval. The fluorescence of rhodamine was detected at 530±30 nm following excitation of cells at 485±22 nm.

**Measurement of ROS production**

To monitor intracellular accumulation of ROS, the fluorescent probe H2DCFDA was used. Cells were loaded with 10 μM H2DCFDA for 30 min. The fluorescence of H2DCFDA was detected by using the same system as in calcium imaging. The fluorescence was detected at 530±30 nm following excitation of cells at 485±22 nm 30 min after treatment of ATP or vehicle.

**Statistical analyses**

Data are expressed as mean±SEM for all cells from several independent experiments. Significance was determined with an ANOVA followed by a Bonferroni’s t-test and a non-paired or paired Student’s t-test. The IC\(_{50}\) of cyanidin-3-glucoside was calculated using a non-linear least-square fit of the Hill equation to the concentration-response data.

**RESULTS**

Treatment with ATP (100 μM) for 90 sec transiently in-
ATP induces [Ca^{2+}], increase in PC12 cells. Reproducible [Ca^{2+}] response could be elicited by subsequent treatment with ATP (100 μM) for 90 sec at a 35 min interval (relative to peak 1=101.0±2.1% n=28) (Fig. 1A). In the preliminary study, the inhibitory effects of cyanidin-3-glucoside on the ATP-induced [Ca^{2+}], responses reached a maximum at 30 min duration when cells were pretreated with cyanidin-3-glucoside (10 μg/ml) for various durations (10 to 40 min)(data not shown). Therefore, we used an exposure time of 30 min to investigate how cyanidin-3-glucoside affects the ATP-induced [Ca^{2+}] responses. Treatment with cyanidin-3-glucoside (1 μg/ml) for 30 min did not affect the ATP-induced [Ca^{2+}], increase (Fig. 1B), whereas treatment with increasing concentrations of cyanidin-3-glucoside (3 μg/ml to 100 μg/ml) inhibited ATP-induced responses in a concentration-dependent manner (Fig. 1C~F). A non-linear least-square fit of the Hill equation to the concentration-response data yielded an IC_{50} of 15.3±0.1 μg/ml for cyanidin-3-glucoside (Fig. 1G). However, treatment with various concentrations of cyanidin-3-glucoside (1 μg/ml to 100 μg/ml, Fig. 1B~F) for 30 min did not significantly affect the basal level of [Ca^{2+}], (data not shown). We therefore used 15 μg/ml as a concentration of cyanidin-3-glucoside in the following experiments to investigate the inhibitory mechanisms of cyanidin-3-glucoside.

ATP induces [Ca^{2+}], increases by a release of Ca^{2+} from IP3-sensitive stores through activation of P2Y-receptor-mediated PLC and an influx of Ca^{2+} from the extracellular space through by activating P2X-receptor [1]. We tested whether cyanidin-3-glucoside affects the ATP-induced [Ca^{2+}], responses following removal of extracellular Ca^{2+} or depletion of intracellular [Ca^{2+}], stores (Fig. 2). The removal of Ca^{2+} by pretreatment with the Ca^{2+}-free HEPES-HBSS containing 100 μM EGTA for 2 min significantly inhibited the subsequent ATP-induced [Ca^{2+}], increases, but ATP still induced the [Ca^{2+}], responses (relative to peak 1=34.4±5.2%, n=22). Pretreatment with cyanidin-3-glucoside (15 μg/ml) for 30 min further inhibited the ATP-induced responses in the presence of the Ca^{2+}-free solution for 2 min (relative to peak 1=26.1±3.3%, n=22, p<0.01) (Fig. 2B & D). These results suggest that cyanidin-3-glucoside inhibits ATP-induced [Ca^{2+}], increases by inhibiting a release of Ca^{2+} from intracellular stores through activation of P2Y-receptor-mediated PLC.

Pretreatment with an endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitor thapsigargin, which depletes and irreversibly prevents the refilling of intracellular stores [19], decreased the subsequent ATP-induced [Ca^{2+}], increases (relative to peak 1=77.5±2.8%, n=19). Treatment with cyanidin-3-glucoside for 30 min also inhibited the ATP-induced responses in thapsigargin-treated cells (relative to peak 1=40.9±3.4%, n=19, p<0.01) (Fig. 2C & D). These result suggest that cyanidin-3-glucoside inhibits ATP-induced [Ca^{2+}], increases by inhibiting an influx of Ca^{2+} from the extracellular space.

It has been reported that P2X2 receptors are expressed in the undifferentiated PC12 cells, which we used in this study [20]. Since there is no specific P2X2 receptor agonist, we used the relatively specific P2X2 receptor agonist 2-MeSATP to induce [Ca^{2+}], increases in PC12 cells [21]. Reproducible [Ca^{2+}], responses could be elicited by subsequent treatment with 2-MeSATP (100 μM) for 90 sec at 35 min interval (relative to peak 1=98.9±3.5%, n=31). Pretreatment with cyanidin-3-glucoside (15 μg/ml) for 30 min significantly inhibited the 2-MeSATP-induced [Ca^{2+}], responses (relative to peak 1=26.7±4.2% n=37, p<0.01) containing 100 μM EGTA for 2 min significantly inhibited the subsequent ATP-induced [Ca^{2+}], increases, but ATP still induced the [Ca^{2+}], responses (relative to peak 1=34.4±5.2%, n=22). Pretreatment with cyanidin-3-glucoside (15 μg/ml) for 30 min further inhibited the ATP-induced responses in the presence of the Ca^{2+}-free solution for 2 min (relative to peak 1=26.1±3.3%, n=22, p<0.01) (Fig. 2B & D). These results suggest that cyanidin-3-glucoside inhibits ATP-induced [Ca^{2+}], increases by inhibiting a release of Ca^{2+} from intracellular stores through activation of P2Y-receptor-mediated PLC.

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Fig. 2. Inhibitory effects of cyanidin-3-glucoside on ATP-induced a release of Ca\textsuperscript{2+} from intracellular stores and an influx of Ca\textsuperscript{2+} from the extracellular space. (A) Reproducible [Ca\textsuperscript{2+}]\textsubscript{i} increases were elicited by superfusion with 100 μM ATP for 90 sec at 35 min intervals. (B) Removal of extracellular Ca\textsuperscript{2+} with the 100 μM EGTA-containing Ca\textsuperscript{2+}-free HEPES-HBSS for 2 min inhibited the ATP-induced responses. Pretreatment with cyanidin-3-glucoside (15 μg/ml) for 30 min inhibited the ATP-induced responses in the presence of the Ca\textsuperscript{2+}-free HEPES-HBSS. (C) Pretreatment with thapsigargin (1 μM) for 25 min inhibited the ATP-induced responses. Pretreatment with thapsigargin and cyanidin-3-glucoside significantly inhibited the ATP-induced responses by 57.8% and 88.0%, respectively. Data are mean±SEM. *p<0.01 relative to respective control (one-way ANOVA with Bonferroni’s test).

Fig. 3. Inhibitory effects of cyanidin-3-glucoside on thapsigargin or ATP-induced store-operated calcium entry (SOCE). Treatment with thapsigargin (1 μM) (A) or ATP (100 μM) (B)-containing Ca\textsuperscript{2+}-free HEPES-HBSS (100 μM EGTA) for 10 min induced a release of Ca\textsuperscript{2+} from intracellular stores in the absence or presence of cyanidin-3-glucoside. Subsequent treatment with 1.25 mM Ca\textsuperscript{2+}-containing HEPES-HBSS induced SOCE-induced [Ca\textsuperscript{2+}]\textsubscript{i} increases. Data are mean±SEM. *p<0.01 relative to respective vehicle (non-paired Student’s t-test).

(Data not shown). These results suggest that cyanidin-3-glucoside inhibits P2X2 receptor-induced [Ca\textsuperscript{2+}]\textsubscript{i} increases in PC12 cells.

It has been reported that there are store-operated calcium channels (SOC) in PC12 cells [22]. We tested whether cyanidin-3-glucoside affects the store-operated calcium entry (SOC) following treatment with thapsigargin or ATP in the Ca\textsuperscript{2+}-free condition (Fig. 3). Treatment with thapsigargin (1 μM) or ATP (100 μM)-containing Ca\textsuperscript{2+}-free HEPES-HBSS (100 μM EGTA) for 10 min induced a Ca\textsuperscript{2+} release from intracellular stores. Subsequent treatment with 1.25 mM Ca\textsuperscript{2+}-containing HEPES-HBSS induced SOCE-induced [Ca\textsuperscript{2+}]\textsubscript{i} increases. Pretreatment with cyanidin-3-glucoside (15 μg/ml) for 30 min significantly inhibited the thapsigargin-induced SOCE responses by 57.8% and the ATP-induced responses by 88.0%. It has been reported that curcumin, a non-flavonoid polyphenol [23], inhibits SOCE in Jurkat-T cells [24].

ATP depolarizes a cell membrane through the P2X-receptor-mediated influx of Na\textsuperscript{+} and Ca\textsuperscript{2+} [25], and secondarily activates voltage-gated Ca\textsuperscript{2+} channels in PC12 cells [1]. Since the L-type and N-type Ca\textsuperscript{2+} channels are expressed in the PC12 cells [26], we tested whether cyanidin-3-glucoside affects the ATP-induced secondary Ca\textsuperscript{2+} influx through voltage-gated L-type and N-type Ca\textsuperscript{2+} channels (Fig. 4). Pretreatment with both the L-type Ca\textsuperscript{2+} channel antagonist nimodipine (3 μM) and the N-type Ca\textsuperscript{2+} channels blocker ω-conotoxin (3 μM) for 5 min significantly inhibited the ATP-induced [Ca\textsuperscript{2+}]\textsubscript{i} responses (relative to peak 1=62.0±8.4%, n=27), indicating that nimodipine and ω-conotoxin-sensitive and -insensitive pathways are involved in the
Inhibitory effects of cyanidin-3-glucoside on ATP or high KCl-induced Ca\(^{2+}\) responses. Moreover, treatment for 30 min with cyanidin-3-glucoside (15 μg/ml) further inhibited the ATP-induced responses in the presence of both nimodipine and ω-conotoxin (relative to peak 1=20.1±2.5%, n=23, p<0.01). To confirm the inhibitory effects of cyanidin-3-glucoside on the secondary Ca\(^{2+}\) influx through ATP-induced activation of voltage-gated Ca\(^{2+}\) channels, we tested whether cyanidin-3-glucoside inhibits depolarization-induced Ca\(^{2+}\) increase using high KCl-containing solution (Fig. 4B). Reproducible [Ca\(^{2+}\)] increases were induced by treatment for 60 sec with 50 mM KCl-containing HEPES-HBSS at 35 min interval. Pretreatment for 30 min with cyanidin-3-glucoside (15 μg/ml) significantly inhibited the subsequent high KCl-induced [Ca\(^{2+}\)] responses (relative to peak 1=55.1±7.2%, n=26, p<0.01). However, the inhibitory effects were not completely recovered after the washout (recovery for control: 98.0±4.8%, n=27; recovery for C3G: 79.9±3.4%, n=26, p<0.01). These results suggest that cyanidin-3-glucoside inhibits ATP-induced [Ca\(^{2+}\)] responses by increasing multiple pathways which are an influx of Ca\(^{2+}\) through the nimodipine and ω-conotoxin-sensitive and -insensitive pathways and a release of Ca\(^{2+}\) from intracellular stores.

Phenolic compounds have been reported to inhibit several kinases involved in signal transduction, mainly tyrosine
kinase and protein kinase C [27,28]. We tested whether cyanidin-3-glucoside inhibits ATP-induced [Ca$^{2+}$], increases through an inhibition of tyrosine kinase or protein kinase C (Fig. 5). Each pretreatment for 30 min with a non-specific protein kinase C inhibitor staurosporine (100 nM), a specific protein kinase C inhibitor GF109203X (300 nM) and a tyrosine kinase inhibitor genistein (50 μM) did not affect the inhibitory effects of cyanidin-3-glucoside on ATP-induced [Ca$^{2+}$], increases. These results indicate that cyanidin-3-glucoside inhibits ATP-induced calcium signaling in a protein kinase-independent manner.

In this study, cyanidin-3-glucoside inhibited ATP-induced [Ca$^{2+}$] increase. Increases in [Ca$^{2+}$], have been reported to induce a mitochondrial depolarization [2]. We tested whether cyanidin-3-glucoside affects ATP-induced mitochondrial depolarization through calcium signaling using rhodamine 123 (Fig. 6). Treatment with 100 μM ATP for 30 min induced a mitochondrial depolarization. Treatment for 30 min with 15 μg/ml ATP for 30 min increased the mitochondrial membrane potential, but it significantly inhibited the ATP-induced mitochondrial depolarization (p < 0.05, n=26). Treatment for 30 min with the intracellular Ca$^{2+}$ chelator BAPTA-AM (10 μM) further blocked the ATP-induced mitochondrial depolarization in the presence of cyanidin-3-glucoside (p < 0.01, n=27). These results suggest that cyanidin-3-glucoside inhibits the ATP-induced mitochondrial depolarization by inhibiting [Ca$^{2+}$] increases. In the following experiment, we determined whether cyanidin-3-glucoside inhibits the ATP-induced depolarization by inhibiting a Ca$^{2+}$ influx from the cytosol into the mitochondria. Treatment for 30 min with the mitochondrial Ca$^{2+}$ unipporter inhibitor RU360 (10 μM) alone did not affect the mitochondrial membrane potential (RU360, n=23), but it also further blocked the ATP-induced mitochondrial depolarization in the presence of ROS [29]. In this study, cyanidin-3-glucoside inhibited ATP-induced mitochondrial depolarization. We checked whether cyanidin-3-glucoside affects ATP-induced formation of ROS through calcium signaling using H$_2$DCFDA (Fig. 7). Treatment with 100 μM ATP for 30 min increased
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formation of ROS. Treatment for 30 min with cyanidin-3-glucoside (15 μg/ml) significantly inhibited the formation of ROS. Treatment with BAPTA-AM (10 μM) for 30 min further decreased the ATP-induced formation of ROS below the basal levels in the absence or presence of cyanidin-3-glucoside. These results suggest that cyanidin-3-glucoside inhibits ATP-induced formation of ROS by inhibiting Ca\(^{2+}\)-induced mitochondrial depolarization.

DISCUSSION

The present study suggests that cyanidin-3-glucoside clearly inhibits ATP-induced [Ca\(^{2+}\)], increases in PC12 cells by inhibiting multiple pathways which are an influx of extracellular Ca\(^{2+}\) and a release of Ca\(^{2+}\) from intracellular stores. Cyanidin-3-glucoside inhibits ATP-induced formation of ROS through Ca\(^{2+}\)-induced mitochondrial depolarization.

In this study, cyanidin-3-glucoside inhibited ATP-induced [Ca\(^{2+}\)], increases in PC12 cells by inhibiting a release of Ca\(^{2+}\) from intracellular stores through activation of P2Y-receptor-mediated PLC and an influx of extracellular Ca\(^{2+}\), which are the nimodipine and ω-conotoxin-sensitive and -insensitive pathways and ionotropic P2X2 receptors. Cyanidin-3-glucoside also inhibited the SOCE-induced Ca\(^{2+}\); increase induced by ATP. The multiple inhibitory effects of phenolic compounds on agonist-induced calcium signaling in neuronal cells have been reported in our previous studies. A simple phenolic compound octyl gallate has been reported to inhibit ATP-induced calcium signaling in PC12 cells by inhibiting a release of Ca\(^{2+}\) from intracellular stores and an influx of Ca\(^{2+}\) from the extracellular space through P2X receptor non-selective cation channels and voltage-gated Ca\(^{2+}\) channels [30]. In addition, apigenin [12] and phenolic compound-containing acorn extract [31] have been reported to inhibit glutamate-induced calcium signaling by inhibiting a release of Ca\(^{2+}\) from intracellular stores and an influx of Ca\(^{2+}\) from the extracellular space through ionotropic glutamate receptors and voltage-gated Ca\(^{2+}\) channels in cultured rat hippocampal neurons. Another flavonoid proanthocyanidin has been reported to inhibit the glutamate-induced calcium signaling by inhibiting a release of Ca\(^{2+}\) from intracellular stores and an influx of Ca\(^{2+}\) through ionotropic glutamate receptors without affecting the voltage-gated Ca\(^{2+}\) channels in cultured rat hippocampal neurons [13]. The inhibitory effects of polyphenols such as curcumin [24] and Hawthorn extract WS ®1442 [32] on SOCE have been also reported.

Flavonoids have been reported to interact with the membrane lipid bilayer to exert their biological functions [33]. The location of interaction between the flavonoids and the membrane has been reported to be either at the surface of cell membrane or in the hydrophobic core of membrane based on their chemical properties [34]. The membrane lipid can affect ion channel structure and function [35]. Therefore, it is possible that cyanidin-3-glucoside inhibit ATP-induced Ca\(^{2+}\) signaling by interacting directly with multiple ion channels in the cell membrane and indirectly through the membrane lipid. In a future study, the details of how cyanidin-3-glucoside inhibits ATP-induced [Ca\(^{2+}\)] increases at the molecular level should be investigated.

Protein phosphorylation such as tyrosine phosphorylation and serine-threonine phosphorylation can induce an influx of extracellular Ca\(^{2+}\) [36,37]. ATP was found to activate PLC in PC12 cells [1], which can activate PKC. ATP was also found to activate tyrosine kinase in PC12 cells [38]. Each pretreatment with a tyrosine kinase inhibitor genistein, a non-specific protein kinase C inhibitor staurosporine, and a specific protein kinase C inhibitor GF109203X did not affect the inhibitory effects of cyanidin-3-glucoside on ATP-induced [Ca\(^{2+}\)] increases. These results indicate that cyanidin-3-glucoside inhibits ATP-induced calcium signaling in a protein kinase-independent manner. A simple phenolic compound octyl gallate has been also reported to have the same protein kinase-insensitive inhibitory effects on ATP-induced calcium signaling in PC12 cells [30]. In this study, cyanidin-3-glucoside inhibited ATP-induced [Ca\(^{2+}\)] increase. Increases in [Ca\(^{2+}\)] have been reported to induce a mitochondrial depolarization [2] and a formation of ROS [29]. The mitochondrial matrix Ca\(^{2+}\) overload can also enhance a formation of ROS [3]. In this study, cyanidin-3-glucoside inhibited the mitochondrial depolarization and the formation of ROS. The intracellular Ca\(^{2+}\) chelator BAPTA-AM or the mitochondrial Ca\(^{2+}\) uniporter inhibitor RU360 further blocked the ATP-induced mitochondrial depolarization in the presence of cyanidin-3-glucoside. In addition, BAPTA-AM also further decreased the ATP-induced formation of ROS below the basal levels in the presence of cyanidin-3-glucoside. The black soybean cyanidin-3-glucoside has been reported to inhibit glutamate-induced excessive formation of ROS and mitochondrial depolarization in rat cultured cortical neurons [18]. All these results suggest that cyanidin-3-glucoside inhibits ATP-induced formation of ROS by inhibiting Ca\(^{2+}\)-induced mitochondrial depolarization. Moreover, cyanidin-3-glucoside has been reported to have a neuroprotective effect against neuronal cell death [15-17,39]. All these reports suggest a possibility that cyanidin-3-glucoside can induce protection against agonist-induced neuronal cell death through inhibiting Ca\(^{2+}\).
signals, oxidative stress, and mitochondrial depolarization.

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