Octyl Gallate Inhibits ATP-induced Intracellular Calcium Increase in PC12 Cells by Inhibiting Multiple Pathways

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INTRODUCTION

ATP is a neurotransmitter or neuromodulator in many areas of the peripheral and central nervous system [1,2]. ATP induces an increase in intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}$]) in PC12 cells by activating both P2X-receptor-regulated non-selective cation channels [3] and P2Y-receptor-mediated phospholipase C (PLC) [4,5]. In addition, the ATP-induced activation of non-selective cation channels induces an increase in [Ca$^{2+}$], through the depolarization-induced activation of voltage-gated L-type Ca$^{2+}$ channels [5].

Fruits, vegetables, beverages, plants, and some herbs are enriched with powerful antioxidant polyphenols. Phenolic compounds are attracting increasing interest from consumers and manufacturers because numerous epidemiological studies have suggested associations between the consumption of polyphenol-rich foods or beverages and the prevention of certain chronic diseases such as cancers and cardiovascular diseases [6,7]. Moreover, these compounds have been reported to be able to protect neuronal cells in various in vitro and in vivo models through different intracellular targets [8-13]. Phenolic compounds affect the function of voltage-gated ion channels [14-17] including voltage-gated Ca$^{2+}$ channels [18]. Phenolic compounds have ability to affect agonist-induced [Ca$^{2+}$], increase in neuronal cells [19-22]. Octyl gallate is a simple phenol compound that potently inhibits the flux of Ca$^{2+}$ into rat pituitary GH4C1 cells [23].

ATP-induced [Ca$^{2+}$], increase in PC12 cells may be involved in the release of catecholamine in PC12 cells [24-26] and in cell death [27]. Although octyl gallate has been reported to have inhibitory effects on Ca$^{2+}$ flux into rat pituitary GH4C1 cells, there are no reports on the effect of octyl gallate against ATP-induced Ca$^{2+}$ signaling in cultured...
PC12 cells. The present study examined whether octyl gallate inhibits ATP-induced \([Ca^{2+}]_i\), increases in PC12 cells using fura-2-based digital Ca\(^{2+}\) imaging and whole-cell patch clamping.

**METHODS**

**Materials**

Fura-2 acetoxyxymethylester (AM) was purchased 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) were obtained from Invitrogen (Carlsbad, CA, USA). ATP (disodium salt), bovine serum albumin, octyl gallate and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Cell culture**

PC12 rat medulla pheochromocytoma cells were grown in DMEM supplemented with 10% FBS and 10% HS at 37°C in a humidified atmosphere of 10% CO\(_2\) and 90% O\(_2\). To measure \([Ca^{2+}]_i\), cells from a stock culture were plated in wells of six-well culture plates at a density of 3×10\(^5\) cells/well; each well contained a 25 mm-diameter coverslip (Fisher Scientific, Pittsburgh, PA, USA). Cells were used for experiment 2–3 days after plating.

**Ca\(^{2+}\) imaging**

Digital imaging of Ca\(^{2+}\) was performed as described previously [28]. Cells were loaded with 12 μM fura-2 AM in HEPES-buffered Hank’s solution (HEPES-HBSS; 20 mM HEPES, 137 mM NaCl, 1.26 mM CaCl\(_2\), 0.4 mM MgSO\(_4\), 0.5 mM MgCl\(_2\), 5 mM KCl, 0.4 mM KH\(_2\)PO\(_4\), 0.6 mM Na\(_2\)HPO\(_4\), 3 mM NaHCO\(_3\), and 5 mM glucose) containing 0.5% bovine serum albumin for 45 min at 37°C. CaCl\(_2\) was removed to nominally Ca\(^{2+}\)-free HEPES HBSS. To elicit depolarization-induced activation of the voltage-gated Ca\(^{2+}\) channels, we used 50 mM KCl HEPES HBSS, in which 137 mM NaCl and 5 mM KCl were replaced with 92.3 mM NaCl and 50 mM KCl, respectively. The loading was terminated by washing with HEPES-HBSS for 15 min before starting the experiment. The coverslip was mounted in a flow through chamber, which was superfused at 2 μl/min. Solutions were selected with a multi-port valve coupled to several reservoirs. The chamber containing the fura-2-loaded cells was mounted on the stage of an inverted microscope and alternately excited at 340 or 380 nm by rapidly switching optical filters (10 nm band pass) mounted on a computer-controlled wheel placed between a 100 W Xe arc lamp and the epi-fluorescence port of the microscope. Excitation light was reflected from a dichroic mirror (400 nm) through a 20x objective (Nikon; N.A. 0.5). Digital fluorescence images (510 nm, 40 nm band-pass) were 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 3–60 s using an Imaging Work Bench 6.0 (INDEC BioSystems, Santa Clara, CA, USA) exposure to excitation light was 120 ms per image. Cells were delimited by producing a mask that contained pixel values above a threshold applied to the 380 nm image. Background images were collected at the beginning of each experiment after removing cells from another area to the coverslip. Autofluorescence from cells not loaded with the dye was less than 5% and so was not corrected.

**Whole-cell patch clamping**

Whole-cell currents were recorded at a holding potential of –70 mV with an Axopatch ID patch-clamp amplifier (Axon Instruments). The external solution was composed of the following: 140 mM NaCl; 5 mM KCl; 1.3 mM CaCl\(_2\); 1 mM MgCl\(_2\), 20 mM HEPES; and 10 mM glucose, and the pH was adjusted to 7.3 with NaOH. Pipettes were filled with buffer consisting of the following: 140 mM CsCl; 1 mM MgCl\(_2\); 1 mM CaCl\(_2\); 10 mM EGTA; and 10 mM HEPES, adjusted pH 7.3 with CsOH. All experimental parameters were controlled using pClamp 6.03 software (Molecular Devices, Sunnyvale, CA, USA). Drugs were dissolved in the external solution and delivered with a linear array of 0.32 mm inner diameter micropipillary tubes. The tips of the drug application pipettes were placed within 100 μm of the cells [29].

**Statistical analyses**

Data are expressed as means±SEM. Significance was determined with a one-way ANOVA followed by a Bonferroni’s test and non-paired or paired Student’s t-test.

**RESULTS**

**Effect of octyl gallate on ATP-induced \([Ca^{2+}]_i\); increase in cultured PC12 cells**

Treatment with ATP (100 μM) for 90 s transiently induced increased \([Ca^{2+}]_i\), in PC12 cells. Reproducible responses could be elicited by applying ATP (100 μM) for 90 s at 30 min intervals (relative to peak 1=103.0±3.0%, n=99) (Fig. 1A). To determine whether octyl gallate specifically affects the ATP-induced \([Ca^{2+}]_i\); increase, cells were pretreated for 10 min with 100 nM, 300 nM, 1 μM, 3 μM, 10 μM, 20 μM octyl gallate. Treatment with 100 nM octyl gallate for 10 min did not significantly affect the ATP-induced \([Ca^{2+}]_i\) increase (relative to peak 1=109.0±7.4%, n=35) (Fig. 1B), whereas treatment with the increasing concentrations of octyl gallate (300 nM to 20 μM) significantly inhibited ATP-induced responses in a concentration-dependent manner (relative to peak 1=84.8±2.7%, n=26 at 300 nM; 74.8±3.1%, n=30 at 1 μM; 56.9±4.0%, n=39 at 3 μM; 20.5±4.0%, n=21 at 10 μM; 14.7±3.1%, n=15 at 20 μM; Figs. 1C–H). A non-linear least-squares fit by the prism 5.0 to the concentration-response data yielded an IC\(_{50}\) of 2.84±0.18 μM for octyl gallate (Fig. 1H). Thereafter, 3.0 μM octyl gallate was used to quantify and confirm the inhibitory effects of octyl gallate on ATP-induced \([Ca^{2+}]_i\); response.

**Inhibitory effects of octyl gallate on ATP-induced \([Ca^{2+}]_i\) release from intracellular stores and \([Ca^{2+}]_i\) influx from the extracellular space**

ATP induces releases Ca\(^{2+}\) from intracellular stores or \([Ca^{2+}]_i\) influx from the extracellular space in PC12 cells. The nature of the octyl gallate-mediated inhibition of ATP-in-
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Fig. 1. Concentration-dependent inhibitory effects of octyl gallate on the ATP-induced \([\text{Ca}^{2+}]_{i}\) increase in PC12 cells. (A–G) After pretreating cells with various concentration of octyl gallate, subsequent ATP-induced \([\text{Ca}^{2+}]_{i}\) response was observed. Image pairs were collected at 3–60 s intervals. ATP and octyl gallate were applied as indicated by the horizontal bars. (H) Summary of concentration-response data. The ATP-induced response amplitude is presented as a percentage of the initial control (relative to peak 1) (n=99, 35, 30, 39, 21, 15 at 0, 100 nM, 300 nM, 1 μM, 3 μM, 10 μM, 20 μM, respectively). A non-linear least-squares fit by the prism software 5.0 to the concentration-response data yielded an IC\(_{50}\) of 2.84 μM for octyl gallate. Data represent mean±SEM.

duced \([\text{Ca}^{2+}]_{i}\) increase was investigated. Specifically, we tested whether the removal of extracellular Ca\(^{2+}\) by treatment with nominally Ca\(^{2+}\)-free HEPES HBSS or the depletion of intracellular Ca\(^{2+}\) stores by treatment with thapsigargin which depletes and irreversibly prevents the refilling of intracellular stores [30], affected ATP-induced \([\text{Ca}^{2+}]_{i}\) increase in the absence or presence of octyl gallate.

Reproducible responses were elicited by applying ATP (100 μM) for 90 s at 30 min intervals (103.0±3.0% of the control responses, n=99) (Fig. 2A, D). Removal of Ca\(^{2+}\) for 2 min markedly inhibited the subsequent ATP-induced \([\text{Ca}^{2+}]_{i}\) response, but ATP still induced a small response (relative to peak 1=17.2±5.6%, n=33). These results suggest that the ATP-induced \([\text{Ca}^{2+}]_{i}\) increase in PC12 cells is largely mediated by Ca\(^{2+}\) influx from the extracellular space. Pretreatment with octyl gallate (3 μM) for 10 min significantly inhibited the ATP-induced response (relative to peak 1=12.4±5.4%, n=33) (Fig. 2B, D). Pretreatment with thapsigargin significantly decreased the subsequent ATP-induced response (relative to peak 1=73.9±2.7%, n=59). Treatment with octyl gallate for 10 min significantly inhibited the ATP-induced responses in thapsigargin-treated cells (relative to peak 1=48±2.8%, n=59) (Fig. 2C, D).

Inhibitory effects of octyl gallate on ATP-induced secondary Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels

ATP activates secondarily voltage-gated L-type Ca\(^{2+}\) channels in PC12 cells through the P2X receptor-mediated depolarization by influx of Ca\(^{2+}\) and Na\(^{+}\) [5]. We tested whether octyl gallate affects the ATP-induced secondary activation of voltage gated L-type Ca\(^{2+}\) channels (Fig. 3). Pretreatment with nimodipine (1 μM) for 10 min significantly inhibited the subsequent ATP-induced \([\text{Ca}^{2+}]_{i}\) response (relative to peak 1=58.8±2.7%, n=25) (Fig. 3A1). Treatment with octyl gallate (3 μM) for 10 min also significantly inhibited the subsequent ATP-induced \([\text{Ca}^{2+}]_{i}\) re-
Inhibitory effects of octyl gallate on the ATP-induced release of Ca\(^{2+}\) from intracellular stores and Ca\(^{2+}\) influx from the extracellular space. (A) Reproducible [Ca\(^{2+}\)]\(i\) increases were elicited by super fusion with 100 μM ATP for 90 s at 30 min intervals. ATP-induced [Ca\(^{2+}\)]\(i\) were recorded after treatment with nominally Ca\(^{2+}\)-free HEPES-HBSS for 2 min (B) or with thapsigargin (1 μM) for 15 min (C); then 20 min later the ATP-induced responses were recorded in the presence of octyl gallate (3 μM) for 10 min. (D) Summary of the effects of octyl gallate on ATP-induced [Ca\(^{2+}\)]\(i\) increase. The amplitude of the ATP-induced response is presented as a percentage of the initial control (relative to peak 1) for the control (n=99), nominally Ca\(^{2+}\)-free HEPES-HBSS-treated (0 Ca\(^{2+}\), n=33), 0 Ca\(^{2+}\) plus octyl gallate-treated (n=33), thapsigargin-treated (n=59), and thapsigargin plus octyl gallate-treated (n=59) cells. Data represent mean±SEM. *p<0.05 relative to respective control (paired Student’s \(t\)-test). **p<0.05 relative to respective non-octyl gallate-treated cells (paired Student’s \(t\)-test).

**Effects of protein kinase inhibitors on the ATP-induced [Ca\(^{2+}\)]\(i\) increases**

ATP induces activation of phospholipase C (PLC) in PC12 cells [5], which can activate protein kinase C (PKC) and tyrosine kinase. Phenolic compounds also potently inhibit several kinases involved in signal transduction, mainly PKC and tyrosine kinases [31], and tyrosine phosphorylation can induce Ca\(^{2+}\) influx [32]. Appropriately, an experiment was done to ascertain whether octyl gallate could...
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**Fig. 3.** Inhibitory effects of octyl gallate on the ATP-induced secondary Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels. Effects of nimodipine and octyl gallate on ATP (100 \(\mu\)M)-induced \([\text{Ca}^{2+}]_i\) increase in untreated cells (A1) and thapsigargin-treated cells (A2). Cells were pretreated with thapsigargin (1 \(\mu\)M) for 45 min during the fura-2 loading period, since thapsigargin-induced increase in \([\text{Ca}^{2+}]_i\) returns to near basal levels after a 15 min exposure to thapsigargin. The amplitude of the ATP-induced response after treatment of vehicle (control) (n=25, n=24), nimodipine (n=25, n=24), octyl gallate (n=25, n=24), nimodipine plus octyl gallate (n=25, n=24) is presented as a percentage of the initial control in untreated and thapsigargin-treated cells, respectively. (B) Effects of octyl gallate on KCl (50 mM K\(^+\) HEPES-HBSS)-induced \([\text{Ca}^{2+}]_i\) increase. The amplitude of the KCl-induced response after treatment of vehicle (n=31) or octyl gallate (n=26) is presented as a percentage of the initial control. Data represent mean±SEM. *p<0.05 relative to respective control or KCl-treated cell; †p<0.05 relative to respective nimodipine or octyl gallate-treated cells (one way ANOVA followed by Bonferroni’s test).

inhibit ATP-induced \([\text{Ca}^{2+}]_i\) increase via inhibition of PKC or tyrosine kinase (Fig. 4). Pretreatment for 10 min with the non-specific PKC inhibitor staurosporin (100 nM), the specific PKC inhibitor GF109203X (300 nM), or the tyrosine kinase inhibitor genistein (50 \(\mu\)M) did not affect the ATP-induced response. However, treatment with octyl gallate (3 \(\mu\)M) significantly inhibited the ATP-induced response in the presence of the non-specific or specific PKC inhibitors, and the tyrosine kinase inhibitor. Moreover, no differences were observed in the response induced by treatment with octyl gallate in the presence or absence of the kinase inhibitors.

**Fig. 4.** ATP-induced \([\text{Ca}^{2+}]_i\) increase were not inhibited by treatment with PKC inhibitors staurosporin, GF109203X or the tyrosine kinase inhibitor genistein. ATP-induced responses were induced in the presence of vehicle (control, n=59), octyl gallate (3 \(\mu\)M, n=39), staurosporin (100 nM, n=30), staurosporin (100 nM) plus octyl gallate (3 \(\mu\)M) (n=29), GF 109203X (300 nM, n=32), GF 109203X (300 nM) plus octyl gallate (3 \(\mu\)M) (n=29), genistein (50 \(\mu\)M, n=35), genistein (50 \(\mu\)M) plus octyl gallate (3 \(\mu\)M) (n=34) for 10 min following 90 s exposure to ATP and a 20 min wash. The amplitude of the ATP-induced response is presented as a percentage of the initial control responses. Data are expressed as mean±SEM. *p<0.05 relative to respective non-octyl gallate-treated cells (one way ANOVA followed by Bonferroni’s test).

**Fig. 5.** Inhibitory effects of octyl gallate on ATP-induced inward currents in PC12 cell. (A) Application of ATP (100 \(\mu\)M, 10 s) evoked inward currents (control). In the same cells, a second application of ATP induced inward current after 10 min washout (vehicle) (n=9). (B) Pretreatment with 3 \(\mu\)M octyl gallate for 10 min inhibited the ATP-induced inward current (octyl gallate, n=6). (C) Summary of the effect of octyl gallate on ATP-induced inward currents. The amplitude of second ATP-induced response (I\(_{p2}\)) is presented as a percentage of the initial control (I\(_{p1}\)) (I\(_{p2}\)/I\(_{p1}\)). Data are expressed as mean±SEM. *p<0.05 relative to control (non-paired student’s t-test).
**Inhibitory effects of octyl gallate on ATP-induced currents**

Activation of P2X receptors by ATP induces the influx of Na⁺ and Ca²⁺ across the cell membrane [33]. In this study, the removal of extracellular Ca²⁺ markedly inhibited the ATP-induced [Ca²⁺]i increase. The ATP-induced [Ca²⁺]i increase in thapsigargin-treated cells was also inhibited by treatment with octyl gallate (3 μM). These results suggest that ATP-induced cation currents are inhibited by octyl gallate. A whole-cell voltage clamping technique was next used to investigate whether treatment with octyl gallate could inhibit ATP-induced inward currents at a holding potential of −70 mV. Reproducible ATP-induced inward currents were elicited by treatment with ATP (100 μM) for 10 s at 10 min intervals (I_p2/Ip1=99.3±4.9%, n=9) (Fig. 5A). Pretreatment with octyl gallate (3 μM) for 10 min significantly inhibited the ATP-induced currents (I_p2/Ip1=52.9±7.3%, n=6) (Fig. 5B).

**DISCUSSION**

The present results demonstrate that octyl gallate clearly inhibits ATP-induced [Ca²⁺]i increases in PC12 cells, by inhibiting both non-selective P2X receptors from extracellular space and P2Y receptor-induced release of Ca²⁺ from intracellular stores. In addition, octyl gallate inhibited the ATP-induced currents and the depolarization-induced-[Ca²⁺]i increases partly in thapsigargin-treated and non-thapsigargin-treated cells, suggesting that the inhibition of Ca²⁺ influx by octyl gallate may be both nimodipine-sensitive and nimodipine-insensitive. ATP also induces [Ca²⁺]i increases partly by an influx of non-selective cations, such as Ca²⁺ and Na⁺, in PC12 cells [3]. In fact, using a whole-cell voltage-clamping technique in the present study, it was demonstrated that octyl gallate inhibits ATP-induced inward currents. These data suggest that octyl gallate inhibits ATP-induced Ca²⁺ influx by inhibiting non-selective cation channels.

Octyl gallate inhibits voltage-gated Ca²⁺ channels in rat pituitary GH₂C₁ cells [23]. In PC12 cells, ATP induces Ca²⁺ influx through the secondary activation of voltage-gated Ca²⁺ channels following depolarization of the membrane by the ATP-induced activation of non-selective cation channels [34]. In addition to L-type voltage-dependent Ca²⁺ channels, it has been reported that ATP can induce a [Ca²⁺]i increase in PC12 cells through dihydropyridine- and CTX-insensitive-voltage-dependent Ca²⁺ channels [34]. In this study, octyl gallate inhibited high KCl-induced [Ca²⁺]i increase. Treatment with octyl gallate or nimodipine inhibited ATP-induced Ca²⁺ influx in PC-12 cells. In addition, treatment with octyl gallate further inhibited the ATP-induced Ca²⁺ influx in the presence of nimodipine. Collectively, these data suggest that octyl gallate inhibits the ATP-induced Ca²⁺ influx through the secondary activation of voltage-gated L-type Ca²⁺ channels and dihydropyridine- and CTX-insensitive-voltage-dependant Ca²⁺ channels.

Phenolic compounds potently inhibit several kinases involved in signal transduction, mainly PKC and tyrosine kinases [31,35-37]. Protein phosphorylation such as tyrosine phosphorylation and serine-threonine phosphorylation can induce Ca²⁺ influx [32]. ATP induces PLC activation in PC12 cells [5], which can activate PKC. ATP was also found to induce the activation of tyrosine kinase in PC12 cells [38]. In the present study, the nonspecific PKC inhibitor staurosporin and the specific PKC inhibitor GF109203X, and the tyrosine kinase inhibitor genistein did not affect the inhibitory effects of octyl gallate on ATP-induced [Ca²⁺]i responses, although octyl gallate inhibited the ATP-induced responses in the presence of PKC inhibitors or tyrosine kinase inhibitors. These results indicate that octyl gallate inhibits ATP-induced [Ca²⁺]i increase in a protein kinase-independent manner.

In this study, octyl gallate inhibited the ATP-induced [Ca²⁺]i increases in concentration-dependent manner. Pretreatment with the Ca²⁺-ATPase antagonist thapsigargin decreased the ATP-induced [Ca²⁺]i responses. Pretreatment with octyl gallate inhibited ATP-induced Ca²⁺ release from intracellular stores following the removal of extracellular Ca²⁺. In the present study, however, we could not determine how octyl gallate inhibits the ATP-[Ca²⁺]i increase through the inhibition of P2Y receptor-mediated signaling.

The inhibitory effects of flavonoid on ion channels have been reported to be mediated by binding to ligand-gated channels, voltage-gated channels, and the membrane lipid bilayer. Apigenin has been reported to inhibit GABA-activated Cl⁻ currents through binding to the benzodiazepine site [39]. Genistein blocked the voltage-sensitive Na⁺ channels through a direct binding to the channels [40]. (-)-Epigallocatechin-3-gallate (EGCG) inhibits rKv1.5 channels [41]. In addition, polyphenols have also been reported to interact with the membrane lipid bilayer to exert their biological functions [42, 43]. Membrane lipid can affect ion channel structure and function [44]. Collectively, it is possible that octyl gallate in the present study exerted inhibition effects by directly binding to the P2X receptors and IP₃ receptor or L-type Ca²⁺ channels and then reducing allosteric inhibition of the channels. However, the present study has provided no further information on how octyl gallate might exert its inhibitory effects on channels at molecular level. ATP is a widely distributed neurotransmitter and neuromodulator in the peripheral and central nervous system [1,2]. ATP receptors regulate various functions including the release of neurotransmitters in the peripheral and central nervous systems [45-47]. ATP-induced [Ca²⁺]i increases in PC12 cells may be involved in the cell death [27]. The present study provides available information that octyl gallate inhibits ATP-induced [Ca²⁺]i increases by inhibiting multiple pathways. More research is needed to assess the alleged health benefits of octyl gallate including synaptic transmission and neuroprotective effect by modulating calcium homeostasis.

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