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Effect of APGW-Amide on $[Ca^{2+}]_i$ in Rat Pheochromocytoma PC12 Cells

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ABSTRACT—In order to determine whether Ala-Pro-Gly-Try-NH_2 (APGW-amide) could affect mammalian excitable cells, we investigated the effect of APGW-amide in PC12 cells. APGW-amide caused a rapid $[Ca^{2+}]_i$ elevation, which was completely prevented by elimination of extracellular Ca^{2+} with EGTA and inhibited by two L-type Ca^{2+} channel blockers. $[Ca^{2+}]_i$ elevation was also blocked by a specific PKC inhibitor and prolonged pretreatment of cells with PMA. These results indicate that APGW-amide elevates $[Ca^{2+}]_i$ in PC12 cells, possibly by Ca^{2+} influx via L-type Ca^{2+} channel activated by PKC.

Keywords: APGW-amide, PC12 cell, L-type Ca^{2+} channel

Ala-Pro-Gly-Try-NH_2 (APGW-amide), a tetrapeptide discovered and isolated from the ganglia of a giant snail (*Achatina fulica Férussac*), has been considered as an inhibitory neurotransmitter of *Achatina* neurons. It has been shown that APGW-amide perfused at 3×10^{-6} M enhanced the outward current (I_o) induced by erythro-β-hydroxy-L-glutamic acid (erythro-L-BHGA) and inhibited I_o induced by dopamine in *Achatina* neurons (1). More recently, it was found that APGW-amide modulates the inward current (I_i) induced by *achatin*-I, but did not affect the I_i induced by oxytocin (2). These facts suggest that APGW-amide is acting not only as an inhibitory neurotransmitter but also as a neuromodulator in the *Achatina* central nervous system. As the next step, we attempted to determine if this molluscan tetrapeptide APGW-amide has some functions in mammalian excitable cells. For these studies, we selected PC12 cells as the representative of mammalian excitable cells that contain voltage-operated Ca^{2+} channels.

There are at least six types of voltage-operated Ca^{2+} channels, L-, N-, T-, P-, Q- and R-channels. Rat pheochromocytoma PC12 cells, a well-known excitable cell line, have often been used to study signal transduction and its mechanisms involving protein kinase C (PKC) (3), mitogen-activated protein (MAP) kinase and phospholipase D (PLD) (4). The voltage-operated Ca^{2+} channels in PC12 cells enable the cells to increase cytosolic Ca^{2+} levels dramatically. The extracellular Ca^{2+} influx induced by carbachol leads to stimulation of PLD and MAP kinase in PC12 cells (4). Although the contribution of L-type Ca^{2+} channel to cellular response in PC12 cells has been well characterized (5, 6), its relation to APGW-amide remains to be elucidated. In this study, we investigated the effect of APGW-amide on mobilization of Ca^{2+} in Fura-2/AM-loaded PC12 cells.

The PC12 cell line was a generous gift from Dr. Y. Sugimoto (Shirakawa Institute of Animal Genetics, Fukushima). Fura-2/AM was from Dojin Laboratories (Kumamoto). Fetal bovine serum was purchased from Nippon Bio-Supply Center (Tokyo). Dulbecco's modified Eagles' medium (DMEM) and horse serum were from Life Technologies (Grand Island, NY, USA). Nifedipine and verapamil were from Wako Pharmaceutic Co. (Osaka). Calphostin C was from Kyowa Medex Co. (Tokyo). APGW-amide and 4β-phorbol 12-myristate 13-acetate (PMA) were from Sigma (St. Louis, MO, USA). The former was dissolved in modified Krebs-Ringer buffer (KRB) containing 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 25 mM Hepes (pH 7.4), 0.1% BSA and 6 mM glucose. Flexiperm-Disc was from Heraeus Biotechnology (Hanau, Germany). The fluorescence image analyzer ARGUS-100/CA was from Hamamatsu Photonics Corp. (Hamamatsu). Other reagents were of analytic grade.

Monolayer cultures of PC12 cells were maintained in

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100-mm tissue-culture dishes on growth medium composed of DMEM medium supplemented with 10% (vol/vol) fetal bovine serum and 5% (vol/vol) horse serum. The cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. Stock cultures were subcultured routinely at a cell density of $2-3 \times 10^6$/dish at least once a week, and the medium was changed every 2–3 days (4).

PC12 cells were plated at a density of 5×10^4 cells/0.8 ml in each well of the Flexiperm-Disc in DMEM containing 10% FBS and cultured for 48–72 h. For the experiments, the cells were washed 2–3 times with KRB. After loading with 4 μM Fura-2/AM for 1.5 h at 37°C, the cells were rinsed with KRB to remove free extracellular dye, followed by postincubation for 10 min. Then the cells were stimulated with 30 μM APGW-amide (the ED_{50} value calculated from the dose-response curve of APGW-amide on the *Achatina* Neuron-RAPN was 6.2 μM) (7). Fluorescence intensity was measured for single cells at 37°C with excitation wavelengths of 340 and 360 nm on a fluorescence image analyzer, ARGUS-100/CA. From the obtained image data, the ratios of the fluorescence at 340 nm over that at 360 nm for each frame were calculated (8). Statistical analysis was conducted with one-way analysis of variance followed by Bonferroni/Dunn's multiple comparisons to detect differences among 4 groups (Fig. 2B) at 5% level, by using Stat View 5 (SAS Institute Inc., Cary, NC, USA).

As shown in Fig. 1A, APGW-amide (30 μM) caused a rapid rise of $[Ca^{2+}]_i$ in Fura-2/AM-loaded PC12 cells, which was followed by a slow decaying plateau. In order to determine if extracellular Ca^{2+} influx contributes to the induction of $[Ca^{2+}]_i$ increase, the extracellular Ca^{2+} (KRB contains 2 mM Ca^{2+}) was depleted by omission of added Ca^{2+} and then by chelating the residual Ca^{2+} with EGTA. Figure 1B shows that incubation of the cells with Ca^{2+}-free KRB for 30 min and with 1 mM EGTA for the final 5 min almost completely inhibited the $[Ca^{2+}]_i$ increase in response to APGW-amide. The data suggested that APGW-amide-elevated $[Ca^{2+}]_i$ was largely dependent on extracellular Ca^{2+} influx. The results were consistent with the data of involvement of Ca^{2+} influx in carbacol-induced transient increase of intracellular Ca^{2+} in PC12 cells (4).

There are at least six types of voltage-operated Ca^{2+} channels. Among them, L-type Ca^{2+} channel seems to be important for APGW-amide-induced $[Ca^{2+}]_i$ increase, because the involvement of L-type voltage-operated Ca^{2+} channel in agonist-induced $[Ca^{2+}]_i$ signal has been reported in PC12 cells (9). Thus we used nifedipine and verapamil because it has been demonstrated that both chemicals specifically block L-type Ca^{2+} channel (5, 6). Pretreatment of PC12 cells with nifedipine for 10 min completely inhibited APGW-amide-induced $[Ca^{2+}]_i$ elevation (Fig. 2A). Similarly, verapamil also exerted similar inhibition on $[Ca^{2+}]_i$ increase induced by APGW-amide (Fig. 2B). Bonferroni /Dunn's multiple comparisons revealed that the values of

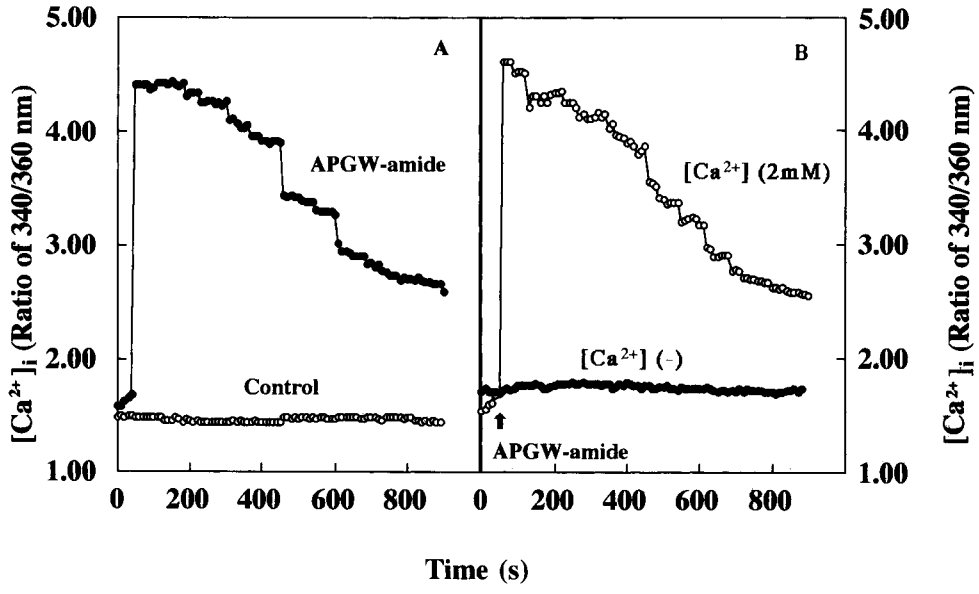

Fig. 1. $[Ca^{2+}]_i$ elevation induced by APGW-amide and the effect of $[Ca^{2+}]_o$. A: APGW-amide-induced $[Ca^{2+}]_i$ elevation in PC12 cells. PC12 cell monolayer was loaded with 4 μM Fura-2/AM for 1.5 h at 37°C. After washing and post-incubation, the cells were stimulated with 30 μM APGW-amide (●) and KRB (○). Data are representative of three experiments. B: Effect of extracellular Ca^{2+} on $[Ca^{2+}]_i$ elevation induced by APGW-amide. PC12 cells were loaded with 4 μM Fura-2/AM for 1.5 h at 37°C, followed by washing and incubation for 30 min with KRB containing 2 mM Ca^{2+} (○), for 30 min with Ca^{2+}-free KRB, and with Ca^{2+}-free KRB containing 1 mM EGTA for the final 5 min (●). The arrow indicates APGW-amide addition. Data are representative of three experiments.

the control, nifedipine and verapamil were significantly different from that of the APGW-amide group. These results indicate that APGW-amide opens L-type voltage-operated Ca^{2+} channels.

It has also been reported that PKC mediates regulation of Ca^{2+} channels in PC12 cells (3). The involvement of PKC in APGW-amide-elevated $[Ca^{2+}]_i$ was examined. For this purpose, a PKC inhibitor calphostin C was employed. As shown in Fig. 3, calphostin C (5 μM) completely prevented APGW-amide-induced $[Ca^{2+}]_i$ increase. The impli-

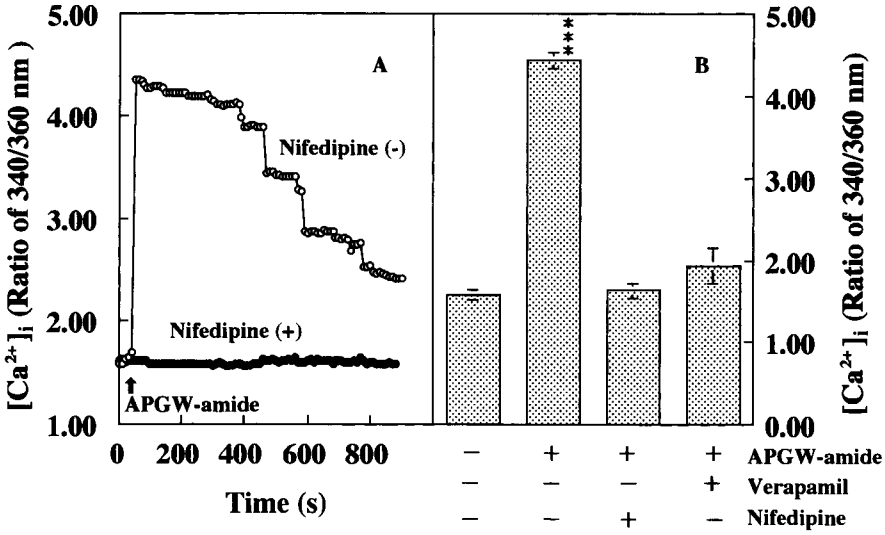

Fig. 2. Effect of L-type voltage-operated Ca^{2+} channel blocker nifedipine and verapamil on APGW-amide-induced $[Ca^{2+}]_i$ elevation. A: PC12 cells were loaded with 4 μM Fura-2/AM for 1.5 h at 37°C and washed followed by incubation for 10 min with 1 μM nifedipine (●) and KRB (○). The arrow indicates APGW-amide addition. Data are representative of three experiments. B: PC12 cells were loaded with 4 μM Fura-2/AM for 1.5 h at 37°C and were washed followed by incubation for 10 min with 1 μM verapamil. Data shown are means ± S.D. of 3 separate experiments, each with 5 – 7 PC12 cells. The statistical analysis was done with ANOVA (***P<0.001, Bonferroni/Dunn's multiple comparisons).

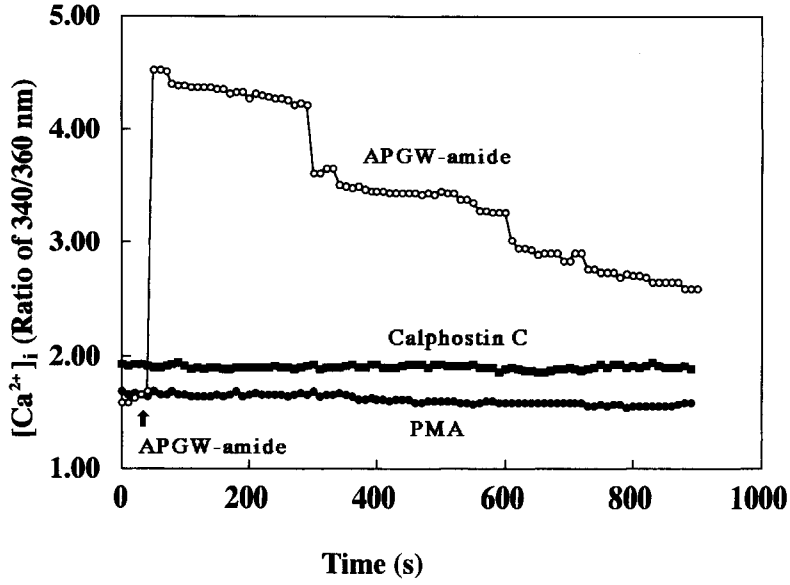

Fig. 3. Effects of PKC inhibitor calphostin C and PKC-down regulation on APWG-amide-induced $[Ca^{2+}]_i$ elevation. PC12 cells were loaded with 4 μM Fura-2/AM for 1.5 h at 37°C and were washed followed by incubation for 30 min with 10 μM calphostin C (■) and KRB (○). For PKC-down regulation, PC12 cells were incubated with 500 nM PMA for 24 h (●). Data are representative of three experiments. The arrow indicates APGW-amide addition.

cation of PKC on Ca^{2+} mobilization was also investigated in PKC down-regulated PC12 cells. We used a PKC activity blocker PMA to determine whether PKC was conclusively involved in the elevation of $[Ca^{2+}]_i$ in PC12 cells. On long-term exposure to 500 nM PMA for 24 h, PKC was abolished almost completely without any effects on the cell viability (Y. Ito et al., unpublished data). After incubation of PC12 cells with 500 nM PMA for 24 h, the elevation of $[Ca^{2+}]_i$ by APGW-amide was no longer observed (Fig. 3).

We have reported previously that APGW-amide exerts modulatory effects on ionic current *Achatina* neurons and suggested that APGW-amide affects the molluscan intracellular signal transduction systems (1, 2). In the present study, we wanted to determine 1) if the APGW-amide originally found in molluscan neurons is also effective in mammalian excitable cells, and 2) whether APGW-amide opens Ca^{2+} channels and increases $[Ca^{2+}]i$. For this purpose, PC12 cells are the most suitable preparation, because the PC12 cell line is a classic excitable cell line and has often been used for studies concerning signal transduction and its mechanisms (3, 4).

The results obtained here demonstrated that APGW-amide elevates $[Ca^{2+}]_i$ in PC12 cells. Although several mechanisms have been proposed for the $[Ca^{2+}]_i$ elevation, the exact mechanism remains to be elucidated. The finding that the depletion of extracellular Ca^{2+} by simple omission of Ca^{2+} and then by chelating residual Ca^{2+} with EGTA abolished APGW-amide-induced $[Ca^{2+}]_i$ elevation (Fig. 1B) indicates that extracellular Ca^{2+} is an important regulator of $[Ca^{2+}]_i$ elevation in PC12 cells. This fact leads to the possibility that voltage-operated Ca^{2+} channels play a role in the elevation of $[Ca^{2+}]_i$. At present, at least six types of Ca^{2+} channels (designated T-, N-, L-, P- Q- and R-type) have been distinguished on the basis of electrophysiological and pharmacological properties. Among them, L-type Ca^{2+} channel is mainly expressed in PC12 cells. Molecular biological studies have demonstrated that L-type Ca^{2+} channels show a greater diversity in their composition, which has been proposed to be a complex of proteins consisting of $\alpha 1$, $\alpha 2/\delta$, β and γ subunits. The $\alpha 1$ subunit is present in Ca^{2+} channels that are highly sensitive to dihydropyridines (10). Nifedipine and verapamil have been shown to bind to the $\alpha 1$ subunit of L-type Ca^{2+} channel (11) and inhibit $[Ca^{2+}]_i$ elevation in neuromuscular synapses (5) and PC12 cells (6). In this study, both of the L-type Ca^{2+} channel blockers almost completely inhibited APGW-amide-elevated $[Ca^{2+}]_i$, indicating APGW-amide opens L-type voltage-operated Ca^{2+} channels.

PKC is a family of serine/threonine specific protein kinases and highly expressed in the central nervous system. The enzyme has been implicated in regulation of neuronal excitability, neurotransmitter release, cellular proliferation and gene expression (12); and it has been considered as an important regulator in signal transduction pathways (3). It has been reported that PKC-dependent neurosecretion, $[Ca^{2+}]_i$ elevation (3, 13) and NA release (14) are mediated through the activation of L-type Ca^{2+} channels. A recent report showed that the L-type Ca^{2+} channel current in rat pinealocytes is regulated by PKC-mediated phosphorylation (15). In our study, the PKC-specific inhibitor calphostin C completely blocked APGW-amide-induced $[Ca^{2+}]_i$ increase and also the prolonged exposure of PC12 cells to PMA for 24 h prevented the $[Ca^{2+}]_i$ increase. In conclusion, the results obtained here indicate that APGW-amide elevates $[Ca^{2+}]_i$ in PC12 cells by eliciting Ca^{2+} influx via L-type voltage-operated Ca^{2+} channel activated by PKC. Another possibility that the activation of PKC is mediated by Ca^{2+} influx through L-type voltage-operated Ca^{2+} channel opened by APGW-amide cannot be excluded.

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REFERENCES

1 Han XY, Salunga TL, Zhang W, Takeuchi H and Matsunami K: Modulation by APGW-amide, an *Achatina* endogenous inhibitory tetrapeptide, of currents induced by neuroactive compounds on *Achatina* neurons: amines and amino acids. Gen Pharmacol **29**, 523 - 530 (1997)

2 Han XY, Salunga TL, Zhang W, Takeuchi H and Matsunami K: Modulation by APGW-amide, an *Achatina* endogenous inhibitory tetrapeptide, of currents induced by neuroactive compounds on *Achatina* neurons: peptides. Gen Pharmacol **29**, 531 - 538 (1997)

3 Harris KM, Kongsamut S and Miller RJ: Protein kinase C mediated regulation of calcium channels in PC-12 pheochromocytoma cells. Biochem Biophys Res Commun **134**, 1298 - 1305 (1986)

4 Ito Y, Nakashima S, Kanoh H and Nozawa Y: Implication of Ca^{2+}-dependent protein tyrosine phosphorylation in carbachol-induced phospholipase D activation in rat pheochromocytoma PC12 cells. J Neurochem **68**, 419 - 425 (1997)

5 Fu WM and Huang FL: L-Type Ca^{2+} channel is involved in the regulation of spontaneous transmitter release at developing neuromuscular synapses. Neuroscience **58**, 131 - 140 (1994)

6 Suh BC and Kim KT: Inhibition by ethaverine of catecholamine secretion through blocking L-type Ca^{2+} channels in PC12 cells. Biochem Pharmacol **47**, 1262 - 1266 (1994)

7 Liu GJ, Santos DE, Takeuchi H, Minakata H, Nomoto K, Kubota I, Ikeda T and Muneoka Y: APGW-amide as an inhibitory neurotransmitter of *Achatina fulica Férussac*. Biochem Biophys Res Commun **177**, 27 - 33 (1991)

8 Sakai T, Okano Y, Nozawa Y and Oka N: Different protein kinase C isozymes could modulate bradykinin-induced extracellular calcium-dependent and -independent increases in osteoblast-like MC3T3 cells. Cell Calcium **13**, 329 - 340 (1992)

9 Di Virgilio F, Pozzan T, Wollheim CB, Vicentini LM and

Meldolesi J: Tumour promoter phorbol myristate acetate inhibits Ca^{2+} influx through voltage-gated Ca^{2+} channels in two secretory cell lines, PC12 and RIN m5F. J Biol Chem **261**, 32 - 35 (1986)

10 Tang S, Yatani A, Bahinski A, Mori Y and Schwartz A: Molecular location of regions in the L-type calcium channel critical for dihydropyridine action. Neuron **11**, 1013 - 1021 (1993)

11 Antonios TF and MacGregor GA: Some similarities and differences between verapamil and the dihydropyridines. J Hypertension **16**, S31 - S34 (1998)

12 Kaczmarek LK: The role of protein kinase C in the regulation of ion channels and neurotransmitter release. Trends Neurosci **10**, 30 - 34 (1987)

13 Tanaka K, Shibuya I, Nagamoto T, Yamashita H and Kanno T: Pituitary adenylate cyclase-activating polypeptide causes rapid Ca^{2+} release from intracellular store and long lasting Ca^{2+} influx mediated by Na^{+} influx-dependent membrane depolarization in bovine adrenal chromaffin cells. Endocrinology **137**, 956 - 966 (1996)

14 Oda H, Murayama T and Nomura Y: Effects of protein kinase C and A activation on ATP-stimulated release of [^{3}H]noradrenaline from PC12 cells. J Biochem **118**, 325 - 331 (1995)

15 Chic CL, Li B, Karpinski E and Ho AK: Regulation of the L-type Ca^{2+} channel current in rat pinealocytes: role of basal phosphorylation. J Neurochem **72**, 73 - 80 (1999)