Acidic pH increases cGMP accumulation through the OGR1/phospholipase C/Ca^{2+}/neuronal NOS pathway in N1E-115 neuronal cells

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Abstract

Neuronal NO synthase (nNOS)-mediated cGMP accumulation has been shown to affect a variety of neuronal cell activities, regardless of whether they are detrimental or beneficial, depending on the amount of their levels, under the physiological and pathological situations. In the present study, we examined the role of proton-sensing G protein-coupled receptors (GPCRs), which have been identified as new pH sensors, in the acidic pH-induced nNOS/cGMP activity in N1E-115 neuronal cells. In this cell line, ovarian cancer G protein-coupled receptor 1 (OGR1) and G protein-coupled receptor 4 (GPR4) mRNAs are expressed. An extracellular acidic pH increased cGMP accumulation, which was inhibited by nNOS-specific inhibitors. Acidic pH also activated phospholipase C/Ca^{2+} pathways and Akt-induced phosphorylation of nNOS at S1412, both of which have been shown to be critical regulatory mechanisms for nNOS activation. The acidic pH-induced activation of the phospholipase C/Ca^{2+} pathway, but not Akt/nNOS phosphorylation, was inhibited by small interfering RNA specific to OGR1 and YM-254890, an inhibitor of $G_{q/11}$ proteins, in association with the inhibition of cGMP accumulation. Moreover cGMP accumulation was inhibited by 2-aminoethoxydiphenyl borate, an inhibitor of inositol 1,4,5-trisphosphate channel; however, it was not by wortmannin, a phosphatidylinositol 3-kinase inhibitor, which inhibited Akt/nNOS phosphorylation. In conclusion, acidic pH stimulates cGMP accumulation preferentially through the OGR1/$G_{q/11}$ proteins/phospholipase C/Ca^{2+}/nNOS in N1E-115 neuronal cells. Akt-mediated phosphorylation of nNOS, however, does not appreciably contribute to the acidification-induced accumulation of cGMP.

Keywords: Acidification, nNOS, cGMP, OGR1, N1E-115 neuronal cell

1. Introduction

Extracellular acidic pH has been shown to take place with ischemia and neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, in which lactate and by-products of glycolysis are accumulated [1]. In the ischemic situation, for example, a lack of blood supply causes hypoxia and the inhibition of aerobic respiration and, thereby, increases lactic acid production through glycolysis, causing a decrease in pH to 6.1-6.8 [2, 3]. Acidic pH is thought to influence mitochondrial function and finally leads to neuronal cell death [4]. Recent studies have suggested that acid-sensing ion channels (ASICs) mediate neuronal cell death in the severe extracellular acidic pH of 6.0 to 5.0, which was associated with an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) [1]. Such a high proton concentration also stimulates sensory neurons, resulting in nociception through ASICs and transient receptor potential V1 (TRPV1) channels [5].

In addition to the ionotropic channels, which are stimulated by a severe acidic pH of 6.0 to 4.0, depending on their subtypes, recent studies have shown that OGR1 family G-protein coupled receptors (GPCRs), including ovarian cancer G protein-coupled receptor 1 (OGR1), G protein-coupled receptor 4 (GPR4), G2 accumulation (G2A), and T cell death-associated gene 8 (TDAG8), sense moderate extracellular pH of 8.0 to 6.0, resulting in the stimulation of intracellular signaling pathways [6-8]. For example, OGR1 is coupled to $G_{q/11}$ proteins and phospholipase C/Ca^{2+} signaling pathways, and TDAG8 and GPR4 are coupled to G_s proteins and adenylyl cyclase/cAMP pathways [6,8,9]. A moderate pH of higher than 6.0 has also shown to modulate a variety of neuronal cell activities [10], including neurotransmitter

release [11] and prevention of neuronal cell death [12,13]. However, the mechanisms underlying acidic pH-induced actions are poorly understood.

As for the beneficial neuronal cell activities, the roles of nNOS/NO/cGMP pathways have been extensively examined in neuronal cells: moderate NO production through nNOS mediates neuronal cell survival [14], neurite extension [15-17], neuronal glucose homeostasis [18], memory [19], and so on, although high NO production causes neuronal cell death and pathological situations [20-22]. Extracellular acidic pH effects on cGMP accumulation have not been previously reported, even for cell types other than neuronal cells. In the present study, therefore, we examined whether acidic pH regulates nNOS/cGMP activity and, if so, the roles of proton-sensing GPCRs and their signaling pathways, focusing especially on Akt and Ca^{2+} signaling pathways, both of which have been known to be critical for neuronal cell activities, including nNOS regulation [22].

2. Materials and Methods

2.1. Materials

Lipopolysaccharide (LPS), wortmannin, and anti-β-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO); N^{ω}-propyl-L-arginine (N-PLA) and (4S)-*N*-(4-Amino-5[aminoethyl]aminopentyl)-*N'*-nitroguanidine, TFA (nNOS inhibitor I) were from Calbiochem-Novabiochem Co. (San Diego, CA); [*myo*-2-^{3}H] inositol (23.0 Ci/mmol) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO);

2-aminoethoxydiphenyl borate (2-APB) was from Enzo Life Sciences, Inc. (Farmingdale, NY); anti-phosphorylated S1417 nNOS (PAI-032) antibody (for mouse S1412) and BCA Protein Assay were from Thermo Scientific (Rockford, IL); anti-nNOS (#4234), anti-eNOS (#9572), anti-phosphorylated Akt Ser473 (#9271) and anti-Akt (#9272) antibodies were from Cell Signaling Technology (Beverly, MA); anti-iNOS antibody (SA-200) was from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA); Cyclic GMP EIA Kit was from Cayman Chemical Co. (Ann Arbor, MI); Fura-2/acetoxymethylester (Fura-2/AM) was from Dojindo (Tokyo, Japan); SUMITOMO Nerve-Cell Culture System was from Sumitomo Bakelite Co., Ltd. (Tokyo, Japan); small interfering RNA (siRNA) specific for mouse OGR1 (si-OGR1, L-056512-00) and non-silencing RNAs (si-NS, D-001206-13) were from Dharmacon, Inc. (Lafayette, CO); Lipofectamine RNAiMAX Reagent was from Invitrogen (Carlsbad, CA) and RT-PCR probes specific for G2A (Mm00490809 and Hs00203431), GPR4 (Mm00558777 and Hs00947870), OGR1 (Mm01335272 and Hs00268858), TDAG8 (Mm00433695 and Hs01087326) and glyceraldehydes 3-phosphate dehydrogenase (GAPDH, 4352932E and 4352934E) were from Applied Biosystems (Foster City, CA). YM-254890 was a gift from Dr. M. Taniguchi of Astellas (Tsukuba, Japan). MG6 cells (RCB 2403) were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. The sources of all other reagents were the same as described previously [23-26].

2.2. Cell culture

N1E-115 cells, a mouse neuroblastoma cell line, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated 10% fetal bovine serum. For analyses of cell response, the cells were plated on culture dishes with poly-D-lysine, and then cultured in fresh medium without serum for another day. SH-SY5Y cells (from ECACC, EC94030304), a human neuroblastoma cell line, were grown in DMEM/Ham's F12 supplemented with 15% fetal bovine serum. Cortical neurons were isolated and cultured according to the instruction manual of SUMITOMO Nerve-Cell Culture System. In brief, the cerebral cortex of mouse at embryonic day 15-18 were cut into pieces and incubated for 20 min at 37°C in Enzyme Solution containing papain. Dissociated cells were cultured for 5 days in Neuron Culture Medium supplemented with glial conditioned medium but without serum. Human umbilical vein endothelial cells (HUVECs) (passage number between 5 and 12) were cultured, as previously described [27]. MG6 cells were a microglial cell line with human *c-myc* from C57BL/6 mouse, and were grown in DMEM supplemented with 10% FBS, 100 μM β-mercaptoethanol and 10 μg/ml insulin as described in [28]. The MG6 cells were treated with LPS (1 μg/ml) 24 h before preparation of protein extracts for NOS analysis. For preparation of cortical neurons, the study was carried out in strict accordance with the guidelines of the Animal Care and Experimentation Committee of Gunma University, and all animals were bred in the Institute of Animal Experience Research of Gunma University. The protocol was approved by the Animal Care and Experimentation Committee of Gunma University (Permit Number: 11-019).

2.3. cGMP measurement

N1E-115 cells on 12-well plates were washed and pre-incubated for 10 min at 37°C in a HEPES-buffered medium composed of 20 mM HEPES (pH 7.6), 134 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.0 mM $CaCl_2$, 2.5 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 5 mM glucose, and 0.1% BSA. The medium was then replaced with the same medium (0.2 ml) containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) under an appropriate pH. After an incubation at 37°C for the indicated time, the reaction was terminated by the addition of 0.5 N HCl (25 μl). The cells were immediately frozen and store at -20 until cGMP assay. Cyclic GMP in the acid fraction was measured according to the instruction manual of cGMP EIA Kit. The protein content of the adherent cells was determined with the BCA Protein Assay.

2.4. Measurement of $[Ca^{2+}]_i$.

The cells on 10-cm dish were gently harvested from dishes with PBS containing 0.05% trypsin-EDTA. The cells were incubated with 1 μM Fura-2/AM and $[Ca^{2+}]_i$ was estimated from the changes in the intensities of 540 nm fluorescence obtained by the two excitations (340 nm and 380 nm), which were monitored by CAF-110 fluorometer (JASCO, Tokyo, Japan), as described previously [29].

2.5. Measurement of [^{3}H]inositol monophosphate (IP) production

The cells were cultured on 12-well dishes in the growth medium, and then the medium was changed to TCM199 (Sigma-Aldrich) containing 2 μCi/ml [^{3}H]inositol and 0.1% BSA and incubated for 24 h. After being washed once with the HEPES-buffered medium containing 0.1% BSA, the cells were preincubated for 10 min with 10 mM LiCl and incubated for further 15 min with the same medium under an appropriate pH to measure [^{3}H]IP, as described previously [30]. Data were normalized to 10^5 dpm of the radioactivity of unstimulated cells, and expressed as percentage of the basal values at pH 7.6.

2.6. Estimation of nNOS and Akt activation

Anti-phosphorylated antibodies against nNOS (phospho S1412 nNOS and nNOS) and Akt (phospho S473 Akt and Akt) were used for estimation of their activation. The serum-starved cells were incubated at 37°C in the HEPES-buffered medium containing 0.1% BSA together with test substances under an appropriate pH for the indicated times. Reactions were terminated by washing twice with ice-cold phosphate-buffered saline (PBS) and adding a lysis buffer composed of 50 mM HEPES, pH 7.0, 150 mM NaCl, 0.1% Nonidet P-40, 1% phosphatase inhibitor cocktail, and 1% proteinase inhibitor cocktail (Sigma-Aldrich). The recovered lysate was centrifuged at 14,000 x *g* for 20 min. The supernatant was resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the protein bands were detected by alkaline phosphatase method, as described previously [23].

2.7. Transfection of siRNA

The siRNA targeted for OGR1 receptor (si-OGR1) or non-silencing RNA (si-NS) was transfected into N1E-115 cells using Lipofectamine RNAiMAX Reagent as described previously [25]. In brief, the cells were harvested with 0.05% trypsin-EDTA and washed with RPMI 1640 medium with 10% FBS. The cell suspension (approximately ~10^7 cells in 9 ml) was mixed with siRNA solution (60 pmol siRNA and 10 μl of RNAiMAX Reagent in 1 ml Opti-MEMI), plated on 10-cm dishes in the same medium. After incubation for 24 h, the cells were dissociated again and re-plated on dishes coated with poly-D-lysine for the experiments of cGMP change, IP production, nNOS activation, RNA analysis and $[Ca^{2+}]_i$ change. The experiments were carried out 72 h after transfection.

2.8. RNA analysis

For analysis of mRNA for OGR1-family receptors, total RNA was prepared from neuronal cells according to the manufacturer's instructions for RNAiso Plus (TAKARA BIO INC, Otsu, Japan). Quantitative real-time PCR (RT-qPCR) was performed by TaqMan technology, as described previously [26]. The expression level of the target mRNA was normalized to the relative ratio (x 10^3) of the expression of GAPDH mRNA. The RT-qPCR assay was performed with 3 different RNA concentrations in each sample.

2.9. Data presentation

All experiments were performed in duplicate or triplicate. The results of multiple observations are presented as the mean ± SEM or as representative results from more than three different batches of cells unless otherwise stated.

3. Results

3.1. Extracellular acidification stimulates nNOS-mediated cGMP accumulation in N1E-115 cells

Acidic pH transiently induced a roughly 3-fold increase in cGMP accumulation with a peak at 1 min, and it was followed by a decrease to 2-fold increase, which was sustained for at least 10 min (Fig. 1A). A significant cGMP accumulation was observed at pH 6.8 and maximal at pH 5.9 (Fig. 1B). The cGMP accumulation was inhibited by nNOS-specific inhibitors (Fig. 1C) and nNOS expression was confirmed in N1E-115 cells (Fig. 1D). These results suggest that extracellular acidic pH stimulates the nNOS-mediated cGMP accumulation.

3.2. Extracellular acidification also activates phospholipase C/Ca^{2+} and Akt/nNOS phosphorylation pathways

Neuronal NOS has been shown to be activated by Ca^{2+}/calmodulin and/or Akt-mediated

phosphorylation of S1412-nNOS [31,32]. We next examined whether acidic pH induces either an increase in $[Ca^{2+}]_i$ or activation of the Akt/nNOS phosphorylation. Acidic pH induced a transient increase in $[Ca^{2+}]_i$ (Fig. 2A), which was unchanged by the removal of extracellular Ca^{2+} (Fig. 2A). Moreover, the Ca^{2+} response was accompanied by IP production, reflecting an activation of phospholipase C (Fig. 2B). These results suggest that Ca^{2+} is mobilized from an intracellular pool through phospholipase C activation. Acidic pH also stimulated phosphorylation of Akt (S473) and nNOS (S1412), both of which have been shown to be active forms of the enzymes, in a manner dependent on time (Supplementary Fig. S1) and dose (Fig. 2C). These results suggest that extracellular acidification also stimulates either the phospholipase C/Ca^{2+} pathway or Akt/nNOS pathway.

3.3. Role of OGR1 and $G_{q/11}$ protein in acidic pH-induced actions

Since the antibodies specific to proton-sensing GPCRs are not available, we examined the mRNA expression of proton-sensing GPCRs; *i.e.,* OGR1, GPR4, TDAG8, and G2A. Among them, OGR1 and GPR4 are expressed in N1E-115 cells (Fig. 3A). Mouse cortical neurons also express both OGR1 and GPR4, and human SH-SY-5Y neuronal cells express OGR1 but not GPR4 (Supplementary Fig. S2). Thus, OGR1 seems to be universally expressed in neuronal cells. The role of OGR1 was examined by small interfering RNA (siRNA) specific to the receptor gene: OGR1-siRNA specifically inhibited the expression of OGR1 mRNA (Supplementary Fig. S3), which was associated with a remarkable inhibition of acidic pH-induced cGMP accumulation (Fig. 3B). Unfortunately, however, GPR4-siRNA

failed to inhibit the GPR4 mRNA expression (data not shown), and therefore, the role of GPR4 in the acidic pH was not further examined. OGR1-siRNA also inhibited acidification-induced $[Ca^{2+}]_i$ increase (Fig. 3C) and phospholipase C activation (Fig. 3D). On the other hand, neither Akt nor nNOS phosphorylation in response to acidic pH was appreciably affected by OGR1-siRNA (Fig. 3E). Similarly to OGR1-siRNA effects, YM-254890, a specific inhibitor for $G_{q/11}$ proteins, inhibited acidic pH-induced cGMP accumulation, $[Ca^{2+}]_i$ increase, and phospholipase C activation and barely affected Akt/nNOS phosphorylation (Fig. 5A-D). These results suggest that the OGR1/$G_{q/11}$ proteins mediate acidic pH-induced activation of phospholipase C/Ca^{2+}, which resulted in cGMP accumulation; however, the OGR1/$G_{q/11}$ protein signaling is not the main pathway for the acidic pH-induced Akt/nNOS phosphorylation.

3.4. Ca^{2+} signaling, but not Akt/nNOS phosphorylation, is preferentially coupled to nNOS-mediated cGMP accumulation

To further confirm the role of Ca^{2+} signaling and Akt/nNOS phosphorylation, we examined the effects of inhibition of these cellular activities on cGMP accumulation. 2-APB, an inhibitor of the inositol 1,4,5-trisphosphate ($InsP_3$) channel, inhibited the acidic pH-induced $[Ca^{2+}]_i$ increase (Fig. 5A), which was accompanied by an inhibition of cGMP accumulation (Fig. 5B). Akt phosphorylation is well known to be regulated by phosphatidylinositol 3-kinase (PI3K). In fact, as shown in Fig. 5C, wortmannin, a PI3K inhibitor, blocked acidic pH-induced phosphorylation of either Akt or nNOS. Under these

conditions, however, the inhibitor did not appreciably affect acidic pH-induced cGMP accumulation (Fig. 5D).

4. Discussion

As for the regulatory mechanisms of neuronal cell activities in acidic microenvironments, previous studies have focused on ionotropic receptors, such as TRPV1 and ASICs. Recent studies have shown that a novel type of proton sensors or metabotropic proton-sensing GPCRs are also working in a variety of biological systems; however, the role of proton-sensing GPCRs have not yet been characterized in neuronal cells. In the present study, we show for the first time the involvement of OGR1, a proton-sensing GPCR, in the acidic pH regulation of an nNOS/cGMP system, a critical intracellular signaling system of neuronal cell activities, in N1E-115 neuronal cells. A proposed signaling mechanism by which acidic pH regulates the nNOS/cGMP system through OGR1 is shown (Fig. 6).

Regulatory mechanisms of the nNOS/cGMP system have been extensively investigated for ionotropic N-methyl-D-aspartic acid (NMDA)-type glutamate receptors [19,22,33]. There are at least two regulatory mechanisms for nNOS activation, *i.e.*, $[Ca^{2+}]_i$ increase, that directly activates nNOS through calmodulin [19,21], and phosphorylation of nNOS at S1412 by Akt [27]. The recent studies have shown that S1412-nNOS can also be phosphorylated by protein kinase A [33-35]. In N1E-115 cells, either $[Ca^{2+}]_i$ increase or Akt-mediated phosphorylation of S1412-nNOS was stimulated by extracellular acidic pH.

The $[Ca^{2+}]_i$ increase was accompanied by phospholipase C activation and remarkably

inhibited by siRNA-OGR1, YM-254890 (a $G_{q/11}$ protein inhibitor), and 2-APB (an $InsP_3$ channel inhibitor). These results suggest that the $[Ca^{2+}]_i$ increase is mainly mediated by OGR1/$G_{q/11}$ proteins/phospholipase C/$InsP_3$-induced intracellular Ca^{2+} mobilization and, thus ruling out the possible involvement of proton-sensing Ca^{2+} channels, such as ASICs and TRPV1, which are shown to be expressed in neuronal cells [1,36,37]. The acidic pH-induced cGMP accumulation was also inhibited by agents that inhibited the $[Ca^{2+}]_i$ increase, *i.e.*, siRNA-OGR1, YM-254890, and 2-APB, suggesting that an OGR1-mediated $[Ca^{2+}]_i$ increase is involved in the acidic pH-induced cGMP accumulation, probably through direct activation of nNOS by Ca^{2+}/calmodulin [19,21].

On the other hand, Akt-mediated nNOS phosphorylation at S1412 might not appreciably contribute to the regulation of cGMP metabolism in the cells. This conclusion was based on the following observations. First, wortmannin, which inhibits acidic pH-stimulated phosphorylation of either Akt at S473 or nNOS at S1412, had no significant effect on the cGMP accumulation. Second, the acidic pH-induced Akt phosphorylation was modulated by neither OGR1-siRNA nor YM-254890, suggesting that proton-sensing mechanisms other than OGR1/$G_{q/11}$ proteins may be involved. In N1E-115 cells, GPR4 is expressed. Unfortunately, however, siRNA-GPR4 failed to downregulate GPR4 mRNA expression in our system, although siRNA-OGR1 effectively inhibited the target receptor mRNA expression. It is not concluded, therefore, whether acidic pH-induced Akt phosphorylation is mediated by GPR4 in the current study. GPR4 is usually coupled to the G_s/cAMP signaling pathway. However, we could not detect a significant cAMP accumulation by acidic pH (data not shown), which dose not support the possible role of GPR4/cAMP pathways and, hence, protein kinase

A-induced phosphorylation of S1412 of nNOS [33-35] in acidic pH-induced actions in N1E-115 cells. Whatever the role of GPR4, the Akt/nNOS phosphorylation system does not seem to contribute to acidic pH-induced cGMP accumulation in N1E-115 cells.

The role of nNOS/cGMP has been extensively examined for neuronal cell activities, including cell survival, neurogenesis, learning, and memory [14,16-19]. Moderate extracellular acidic pH has also been shown to modulate neuronal cell activities, including neurotransmitter release and neuroprotection [11-13]. These results suggest that acidic pH-induced cGMP accumulation may cause beneficial neuronal activities. Elucidation of this is an important future project.

In conclusion, acidic pH stimulates cGMP accumulation preferentially through the OGR1/$G_{q/11}$ proteins/phospholipase C/Ca^{2+}/nNOS in N1E-115 neuronal cells. Although acidic pH also stimulates Akt-mediated phosphorylation of nNOS, another nNOS activation mechanism, the contribution of this system plays a minor role if any. The role of GPR4, another proton-sensing GPCR expressed in the cells, is currently unknown.

Competing interest

The authors declare no competing interest.

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(Figure Legends)

Fig. 1. Involvement of nNOS in acidic pH-induced accumulation of cGMP in N1E-115 cells. The cells were incubated in the HEPES-buffered medium (pH 7.6 or 6.1) for the indicated time (A) or at the indicated pH for 1min (B) in the presence of 0.5 mM IBMX. The cGMP content is expressed as percentages of the basal value (4.07 ± 0.81 pmol/mg protein in A and 4.60 ± 0.65 pmol/mg protein in B) at pH 7.6. (C) Effect of nNOS inhibitors on acidic pH-induced cGMP production. The cells were pre-incubated with or without 1 μM N-PLA or nNOS inhibitor I and incubated at pH 7.6 or 6.1 for 1 min in the presence of 0.5 mM IBMX. The result is expressed as percentages of the basal value (5.63 ± 0.67 pmol/mg protein) at pH 7.6 in the absence of the inhibitor. (D) Expression of NOS proteins in N1E-115 cells, HUVECs, and MG6 cells. Expression of nNOS, eNOS, and iNOS were analyzed by Western blot.

Fig. 2. Extracellular acidification induces increase in $[Ca^{2+}]_i$, production of IP, and phosphorylation of Akt or nNOS in N1E-115 cells. (A) Effect of Ca^{2+} chelation with EGTA on acidic pH-induced increase in $[Ca^{2+}]_i$. Left panel shows a typical $[Ca^{2+}]_i$ trace. At arrows, 500 mM EGTA (final concentration of 2.5 mM) or 1 N HCl (final pH of 6.7) were added. In right panel, the difference between peak and basal value was shown as the means ± SEM of three separate experiments. (B) Extracellular pH-dependent IP production. The cells labeled with [^{3}H]inositol were incubated for 15 min in the presence of 10 mM LiCl at the indicated pH. The results are expressed as percentages of basal value at pH 7.6, which was 696 ± 55

dpm. (C) Acidic pH induces phosphorylation of Akt at S473 and nNOS at S1412. N1E-115 cells were stimulated with the indicated pH for 5 min (Akt) or for 30 min (nNOS), and cell extracts were subjected to Western blot analysis for detection of total and phosphorylated proteins. The result is shown in duplicate and a representative from more than three different batches of cells.

Fig. 3. OGR1 mediates acidic pH-induced cGMP production and activation of phospholipase C/Ca^{2+} signaling, but not phosphorylation of Akt/nNOS. (A) Expression of OGR1-family receptor mRNAs estimated by RT-qPCR. $^{\#}$TDAG8 mRNA was undetectable. (B) Effect of siRNA specific to OGR1 (si-OGR1) on the accumulation of cGMP. The cells treated with non-silencing siRNAs (si-NS) or si-OGR1 were stimulated at the indicated pH for 1 min in the presence of 0.5 mM IBMX to measure cGMP. The cGMP content is expressed as percentages of the basal value at pH 7.6 in si-NS cells (4.51 ± 1.25 pmol/mg protein) and si-OGR1 cells (5.07 ± 1.62 pmol/mg protein), respectively. (C) Effect of si-OGR1 on acidic pH-induced increase in $[Ca^{2+}]_i$. A typical trace (left panel) and differences between peak and basal value (right panel) are shown. (D) Effect of si-OGR1 on acidic pH-induced IP production. The cells were incubated for 15 min in the presence of LiCl at pH 7.6 or pH 6.4 to measure IP release. Normalized basal value was 885 ± 29 at pH 7.6 for si-NS cells and 934 ± 45 at pH 7.6 for si-OGR1 cells. Effect of si-OGR1 was significant from si-NS in B-D (*$p <$ 0.05 and **$p <$ 0.01). (E) Acidic pH induces phosphorylation of Akt at S473 and nNOS at S1412. N1E-115 cells were stimulated with the indicated pH for 5 min. The result is shown in duplicate and a representative from more than two different batches of the cells.

Fig. 4. Involvement of $G_{q/11}$ proteins in acidic pH-induced cGMP accumulation and activation of phospholipase C/Ca^{2+} system, but not phosphorylation of Akt/nNOS. The cells were preincubated with 100 nM YM-2514890 or DMSO as a vehicle. (A) For accumulation of cGMP, the cells were incubated at pH 7.6 or 6.1 for 1 min in the presence of 0.5 mM IBMX. (B) Effect of YM-2514890 on $[Ca^{2+}]_i$ increase stimulated by 1 N HCl (final pH of 6.7). (C) For IP production, the cells were stimulated for 15 min at pH 7.6 or 6.2. (D) For phosphorylation of Akt at S473 and nNOS at S1412 by acidic pH, the cells were incubated at pH 7.6 or 6.1 for 5 min (Akt) or for 30 min (nNOS). Cell extracts were subjected to Western blot analysis for detection of total and phosphorylated proteins. The result is shown in duplicate and a representative from two different batches of the cells. **Effect of acidic pH was significant in A and C and effect of YM-254890 was significant in B ($p < 0.01$).

Fig. 5. Acidic pH-induced Ca^{2+} signaling is preferentially coupled to nNOS-mediated cGMP accumulation in N1E-115 cells. (A) Effect of 2-APB (75 μM) on $[Ca^{2+}]_i$ increase. Data are expressed as the difference between peak and basal value. (B) Effect of 2-APB on acidic pH-induced cGMP production. The cells were pre-incubated for 10 min in the HEPES-buffered medium (pH 7.6) with 75 μM 2-APB and incubated with the same medium (pH 7.6 or 6.1) for 1 min. (C) and (D) Effect of wortmannin on acidic pH-induced phosphorylation of Akt (S473) and nNOS (S1412) and cGMP accumulation. The cells were pre-incubated for 10 min at pH 7.6 with 100 nM wortmannin (Wort) and stimulated with the

indicated pH for 5 min (Akt) or for 30 min (nNOS) to analyze the total and phosphorylated proteins (C) and for 1 min to measure cGMP accumulation (D). The results are representative from more than two different batches of the cells.

Fig. 6. A proposed signaling mechanism by which acidic pH regulates the nNOS/cGMP system in N1E-115 cells. Acidic pH stimulates the cGMP production preferentially through OGR1/$G_{q/11}$ proteins/phospholipase C/Ca^{2+}/nNOS. Extracellular acidification also activates PI3K/Akt pathway leading to the phosphorylation of nNOS at S1412; however, this mechanism plays a minor role in the acidic pH-induced cGMP production. PLC, phospholipase C; GC, guanylyl cyclase. See text for more detail.

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Fig. 1 (↑)

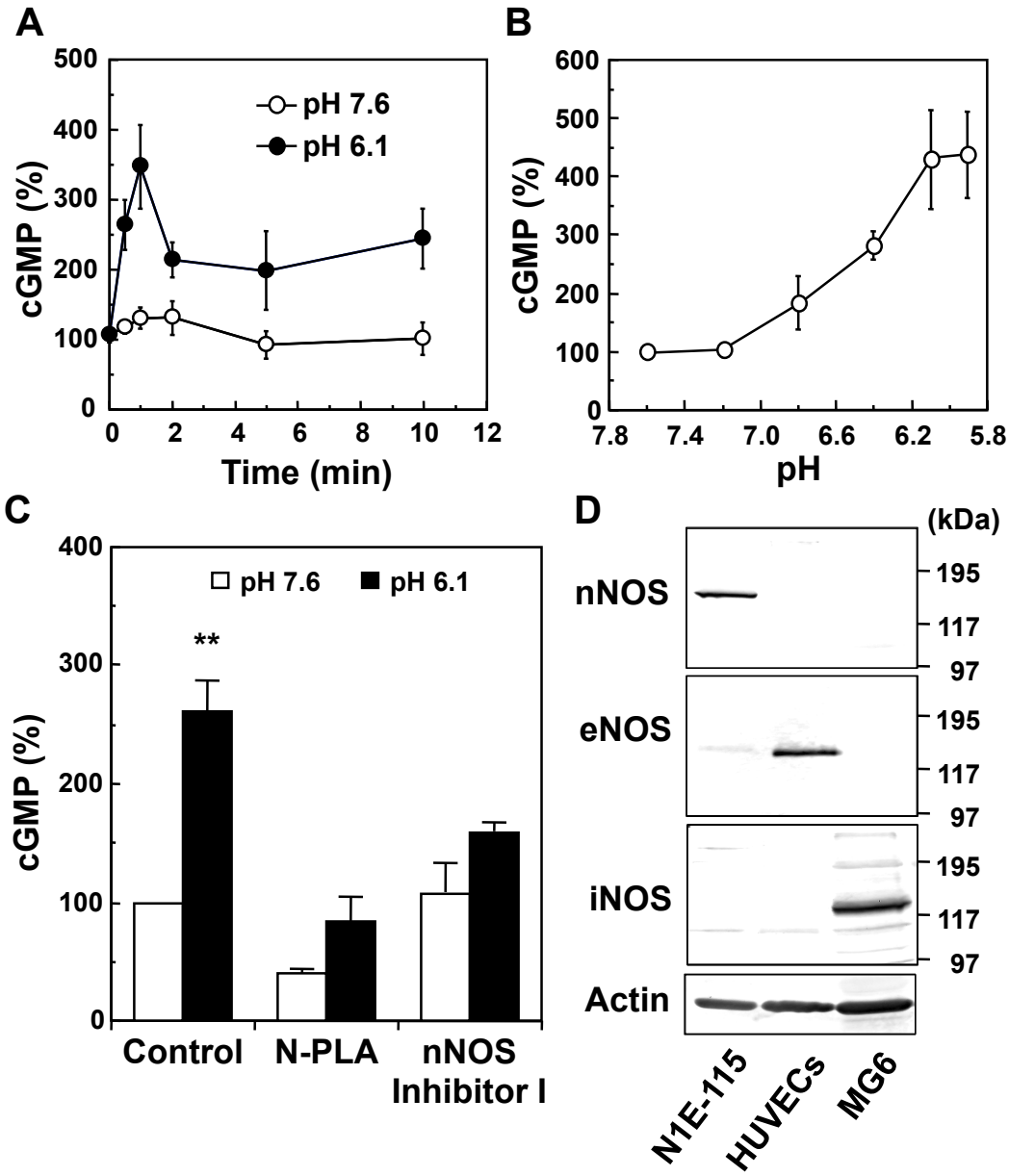

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Fig. 2 (↑)

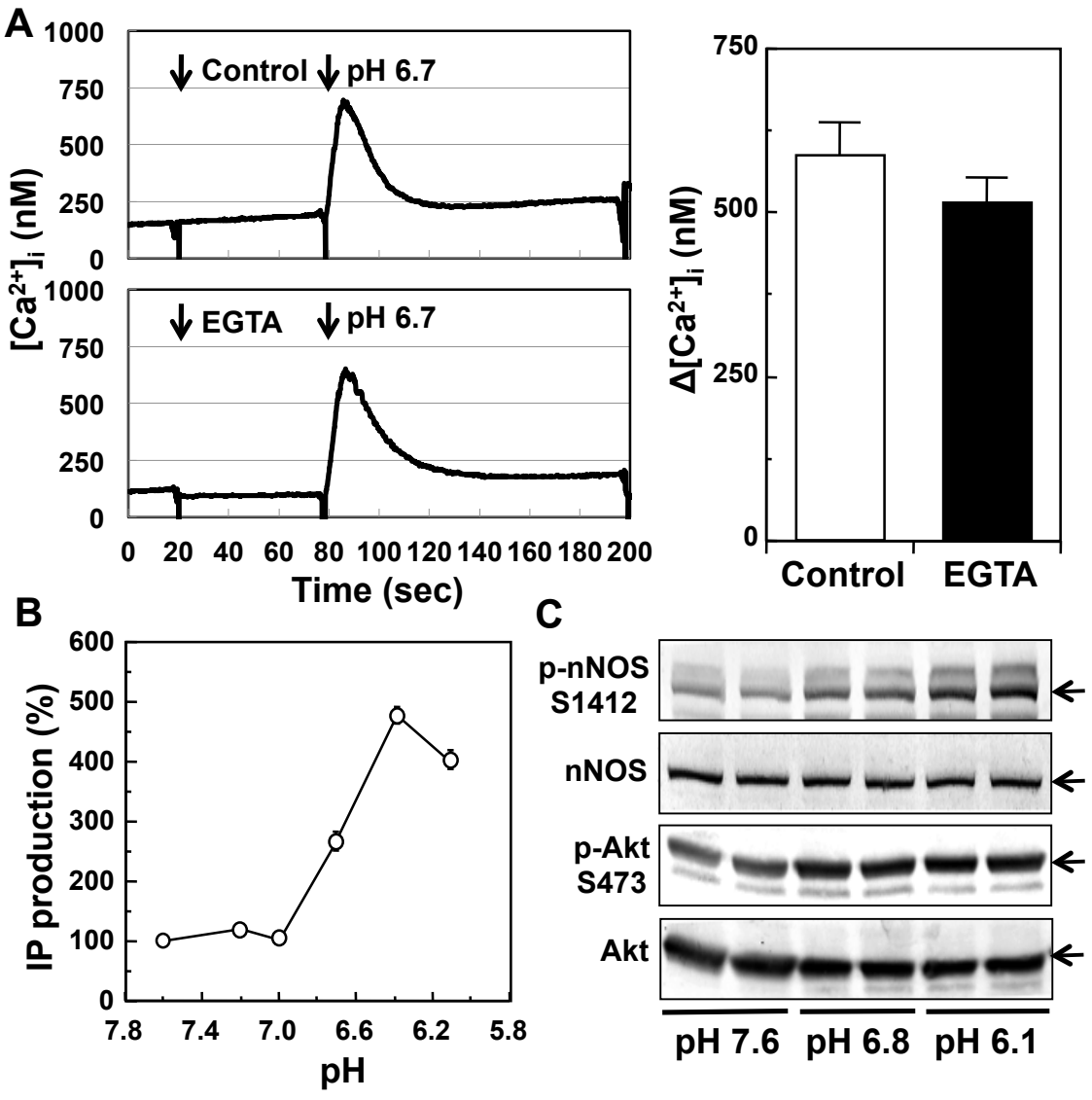

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Fig. 3 (↑)

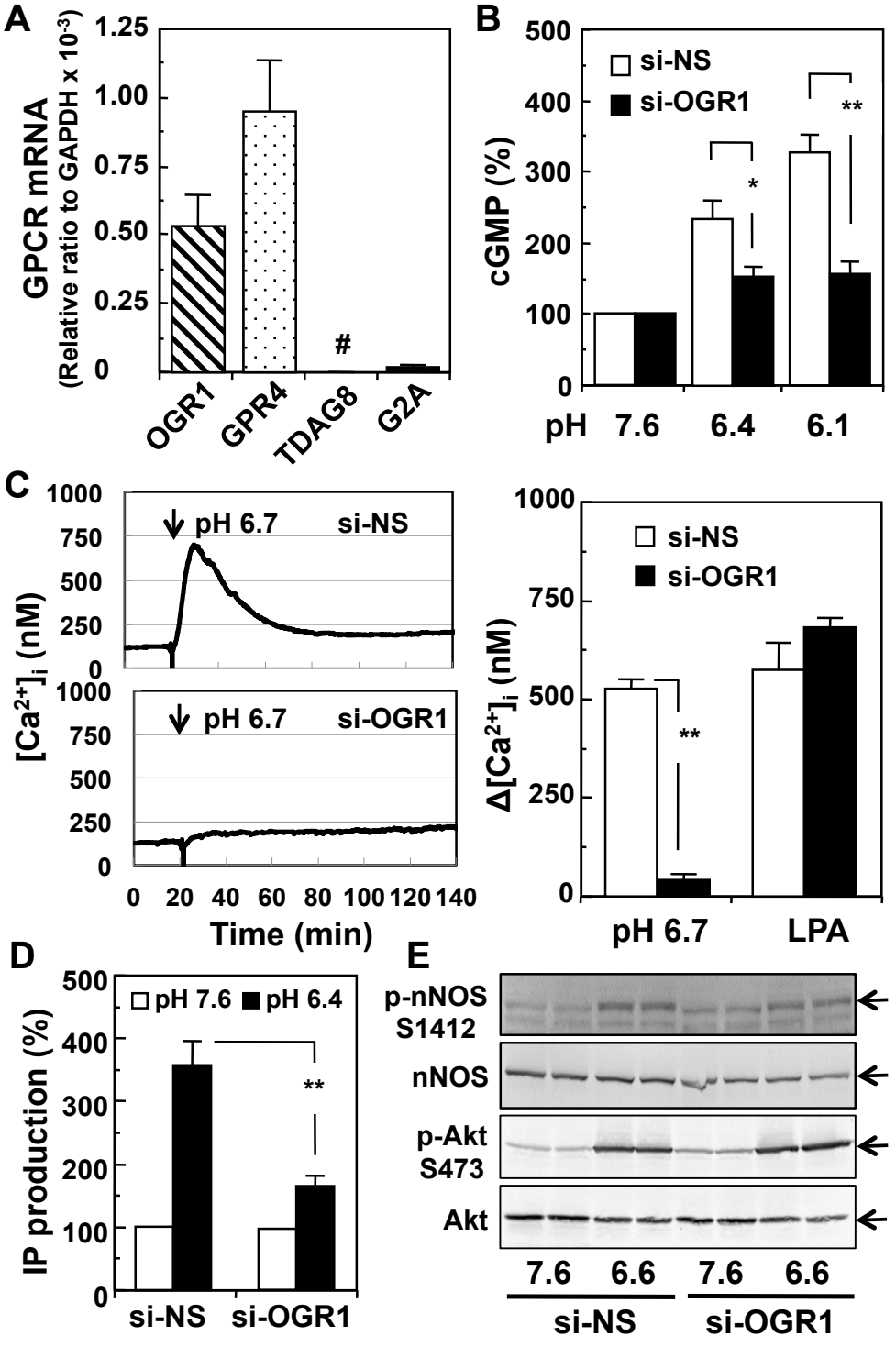

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Fig. 4 (↑)

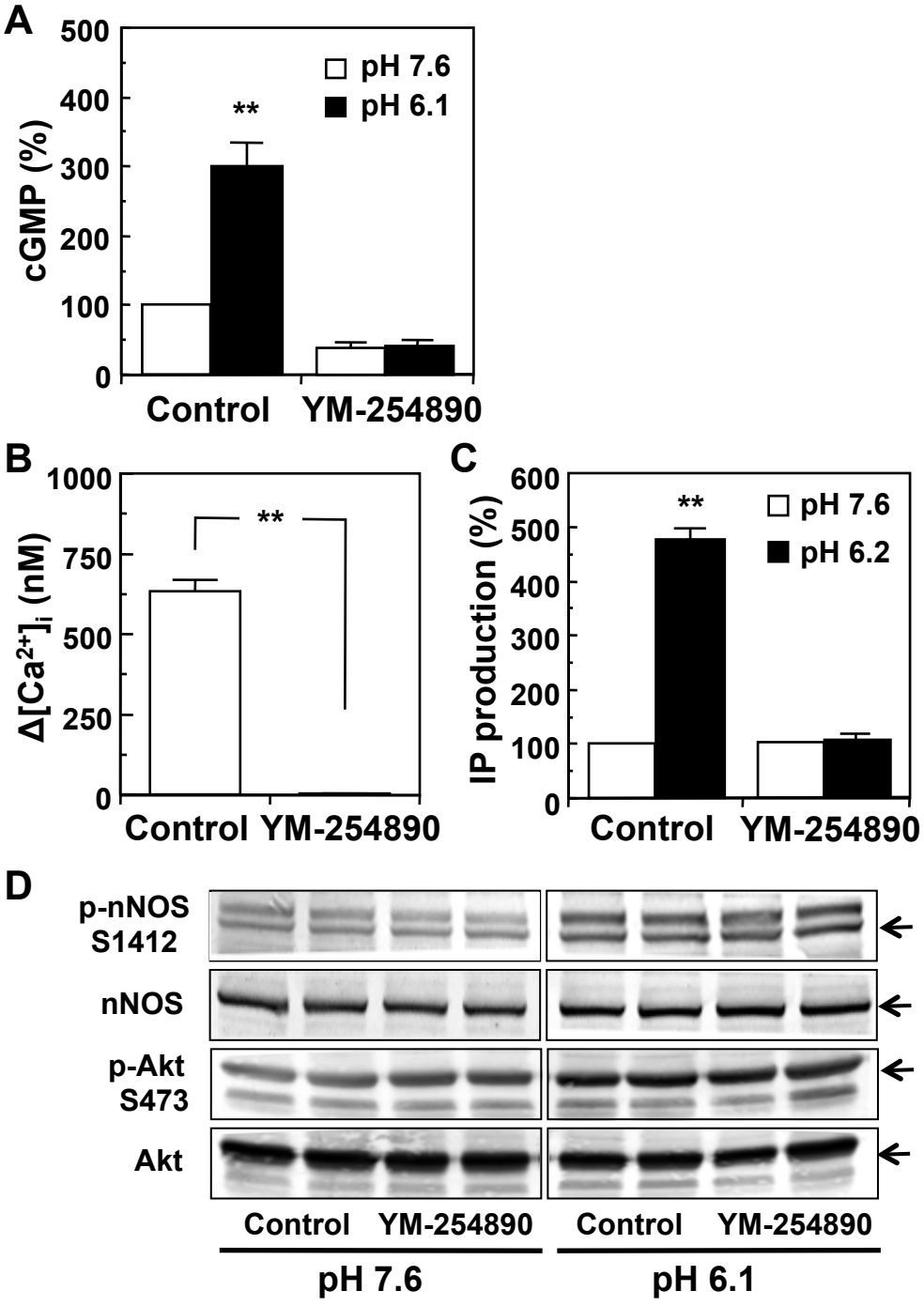

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Fig. 5 (↑)

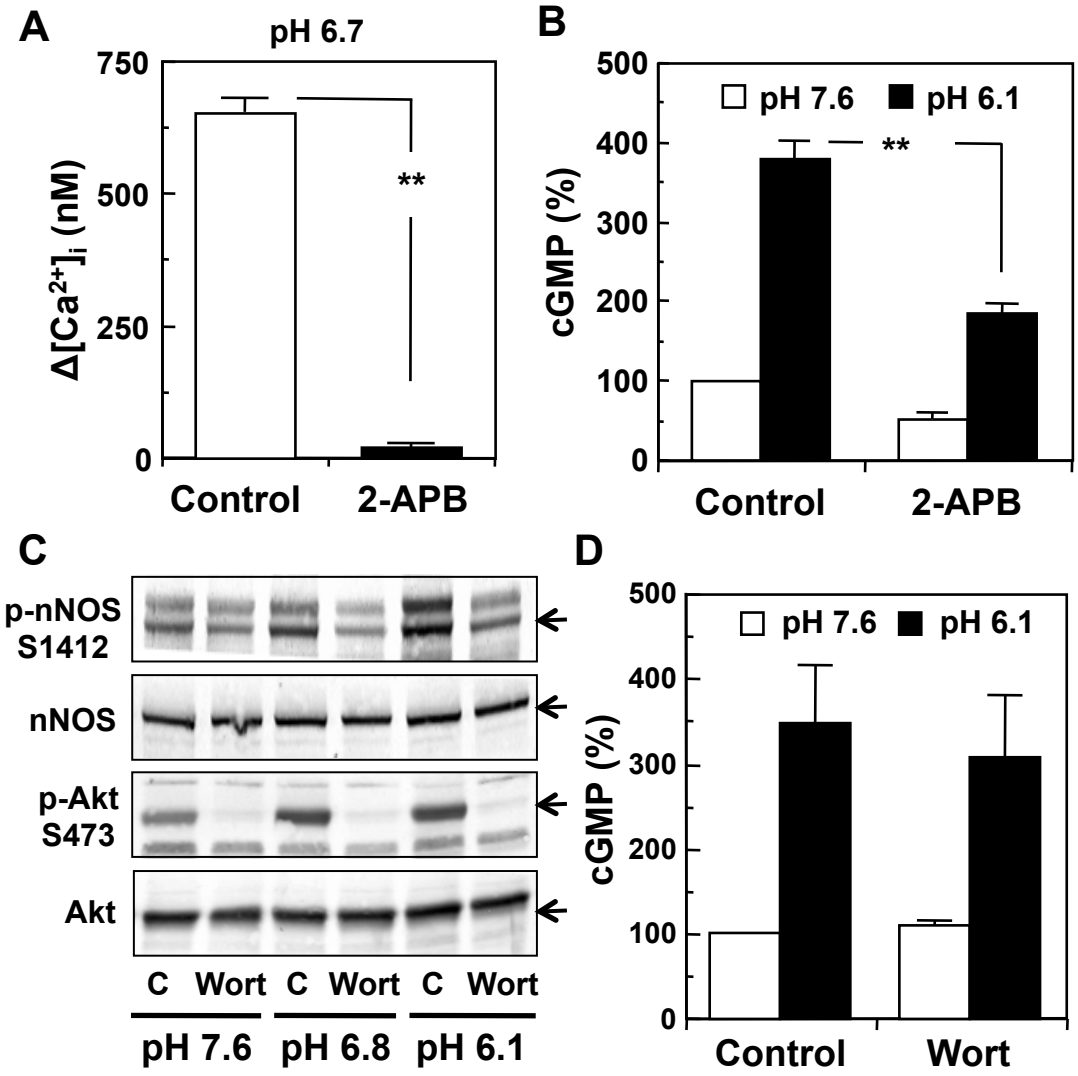

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Fig. 6 (↑)