Acid-sensing ion channels contribute to the increase in vesicular release from SH-SY5Y cells stimulated by extracellular protons

Qiu-Ju Xiong,1,6 Q. J. Xiong and Z.-L. Hu contributed equally to this work. Peng-Fei Wu,1 Lan Ni,1 Zhi-Fang Deng,1 Wen-Ning Wu,1 Jian-Guo Chen,1,2,3 and Fang Wang1,2,3

1Department of Pharmacology, School of Basic Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China; 2Key Laboratory of Neurological Diseases (HUST), Ministry of Education of China, Wuhan, Hubei, China; and 3The Key Laboratory for Drug Target Researches and Pharmacodynamic Evaluation of Hubei Province, Wuhan, China

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Address for reprint requests and other correspondence: F. Wang, Dept. of Pharmacology, School of Basic Medicine, Tongji Medical College, Huazhong Univ. of Science and Technology, Wuhan, 430030, China (e-mail: wangfang0322@yahoo.com.cn).

Acid-sensing ion channels (ASICs) contribute to the increase in vesicular release from SH-SY5Y cells stimulated by extracellular protons. Am J Physiol Cell Physiol 303: C376–C384, 2012. First published May 16, 2012; doi:10.1152/ajpcell.00067.2012.—Acid-sensing ion channels (ASICs) have been reported to play a role in the neuronal dopamine pathway, but the exact role in neurotransmitter release remains elusive. Human neuroblastoma SH-SY5Y is a dopaminergic neuronal cell line, which can release monoamine neurotransmitters. In this study, the expression of ASICs was identified in SH-SY5Y cells to further explore the role of ASICs in vesicular release stimulated by acid. We gathered evidence that ASICs could be detected in SH-SY5Y cells. In whole cell patch-clamp recording, a rapid decrease in extracellular pH evoked inward currents, which were reversibly inhibited by 100 μM amiloride. The currents were pH dependent, with a pH of half-maximal activation (pH0.5) of 6.01 ± 0.04. Furthermore, in calcium imaging and FM 1-43 dye labeling, it was shown that extracellular protons increased intracellular calcium levels and vesicular release in SH-SY5Y cells, which was attenuated by PCTx1 and amiloride. Interestingly, N-type calcium channel blockers inhibited the vesicular release induced by acidification. In conclusion, ASICs are functionally expressed in SH-SY5Y cells and involved in vesicular release stimulated by acidification. N-type calcium channels may be involved in the increase in vesicular release induced by acid. Our results provide a preliminary study on ASICs in SH-SY5Y cells and neurotransmitter release, which helps to further investigate the relationship between ASICs and dopaminergic neurons.

N-type calcium channels; acidification; neurotransmitter release

Acid-sensing ion channels (ASICs) are voltage-insensitive cation-permeable ion channels transiently activated by rapid extracellular acidification. They are members of the amiloride-sensitive degenerin/epithelial sodium channels (DEG/ENaC) family (15, 34, 38). Until now, four ASIC genes have been verified to encode at least six subunits (ASIC1a, 1b, 2a, 2b, 3, and 4) through alternative splicing. ASICs were initially found in nerve cell membrane (16) and now have been found to exist widely in both central and peripheral nervous systems, including cortical neurons (18), hippocampal neurons (39), sensory neurons (4), and glial cells (13). Different ASIC subunits are involved in a variety of neuronal activities such as ischemic neuronal injury (8, 41), sensory perception (19), and learning and memory (37). They are also found to be related to postoperative pain (7), temporal lobe epilepsy (20), multiple sclerosis (30), migraine headache (43), irritable bowel syndrome (21), and blood volume control (17).

In recent years, there has been increasing attention to the relationship between ASICs and dopaminergic neurons. It is known that ASICs are densely expressed in the striatum (1, 36), and the homomeric ASIC1a channel is the main subtype in medium spiny neurons (14). Moreover, ASIC-like currents are recorded in midbrain dopamine neurons (26). As for the role of ASICs in the dopamine pathway, amiloride, a nonspecific blocker of ASICs, has recently been proven to be protective in an MPTP model of Parkinson’s disease (3). Meanwhile, ASICs mediate learning in Caenorhabditis elegans by facilitating dopamine signaling (31). Therefore, the exact effect of ASICs on dopaminergic neurons needs further investigation.

Neurotransmitters are endogenous chemicals that transmit signals from a neuron to a target cell across a synapse; they are packaged into synaptic vesicles which are clustered beneath the membrane. ASICs are also reported to be involved in neurotransmitter release. For example, a heteromeric ASIC2a/ASIC2b channel has been implicated in sour taste sensing, and activation of the receptor by acid leads to depolarization of taste cells and transmitter release onto gustatory afferent neurons (28); ASIC1 knockout mice have an increased probability of neurotransmitter release through electrophysiological detection of microisland cultures of hippocampal neurons (6). In addition, increases in intracellular calcium concentration ([Ca2+]i) are required for the release of neurotransmitter in synaptic terminals (22), and ASIC1a can directly mediate Ca2+ influx and increase intracellular calcium levels in neurons (44). Human neuroblastoma SH-SY5Y is a dopaminergic neuronal cell line which has been used as an in vitro model for Parkinson’s disease. They exhibit calcium-dependent release of norepinephrine in response to membrane depolarization with 100 mM K+ (23). However, it is still unknown whether ASICs are expressed in SH-SY5Y cells, as well as the response of SH-SY5Y cells to extracellular acidification. In light of these issues, the effect of acid on neurotransmitter release in SH-SY5Y cells using the styryl dye FM 1-43 was observed to further understand the role of ASICs in vesicular release process.

In this study, we have confirmed the functional expression of ASICs in SH-SY5Y cells and that the activation of ASICs enhanced calcium-dependent vesicular release in response to acidification. Activation of N-type calcium channels evoked by
extracellular protons which induced membrane depolarization also contributed to the increase in vesicular release.

**MATERIALS AND METHODS**

**Materials.** Amiloride, Hoechst 33258, cyclopiazonic acid (CPA), and nifedipine were purchased from Sigma (St. Louis, MO). Fura-2/AM (Biotium, Hayward, CA) was dissolved in DMSO. PctX1, ω-conotoxin GVIA (ω-CTX), and ω-agatoxin TK (ω-AGX) were from Alomone (Alomone Labs, Jerusalem, Israel). Other general agents were purchased from commercial suppliers. All drugs were prepared as stock solutions and diluted to the final concentrations before application. The final concentration of DMSO was <0.05%.

**SH-SY5Y cell culture.** SH-SY5Y cells were maintained in DMEM/F-12 (GIBCO, Carlsbad, CA) supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin, and 2 mM l-glutamine at 37°C in a 5% CO2 humidified atmosphere. Medium was changed every 2 days and cells were passaged 3–5 days. The cells were grown to 70% confluence for the next experiment.

**RT-PCR and quantitative real-time PCR experiment.** Total RNA was extracted from SH-SY5Y cells using TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. The concentration and purity of RNA were determined spectrophotometrically at 260 nm. RNA (2 µg) was used for single-strand cDNA synthesis with the RevertAid First Strand cDNA Synthesis system for RT-PCR kits (Fermentas, Thermo Fisher Scientific). The primers used for PCR and quantitative real-time PCR (qPCR) analysis of ASIC1, ASIC2, ASIC3, and GAPDH were as follows: ASIC1 (140 bp) 5'-CAGATGGCTGAT-GAAAGCCA-3' (forward), 5'-AAGTGCGCAGGAGAGCAT-3' (reverse); ASIC2 (139 bp) 5'-TGACATTTGGTGCTAAATGG-3' (forward), 5'-ATCATGCTCCTCCCCTCT-3' (reverse); ASIC3 (107 bp) 5'-AGGGAGGAGTCCCAAGAGCT-3' (forward), 5'-GACACTCCA-TTCCCGAGGAGA-3' (reverse); and GAPDH (216 bp) 5'-ACATTTGGT-GCACATACCG-3' (forward), 5'-AGCCCATGACTCCACAC-GAC-3' (reverse). Two microliters of the first-strand product were used as a template in each 20 µl PCR reaction, under the following conditions: initial denaturation at 94°C for 3 min, PCR cycles: 94°C for 30 s, 55°C for 30 s, 72°C for 30 s repeated for 40 cycles; final elongation at 72°C for 10 min. Confirmation of the amplified product sizes was resolved by 2% agarose gel electrophoresis using PCR markers (Fermentas, Thermo Fisher Scientific). The protocol was 10 min at 95°C, 40 cycles of 10 s at 95°C, 30 s at 55°C, 10 min. The emitted light was imaged at 510 nm with a video camera

**Electrophysiological experiments.** Membrane currents were recorded in voltage-clamp mode with an EPC-10 amplifier (HEKA, Lambrecht, Germany) driven by Pulse/PulseFit software (HEKA). The procedure for whole cell patch-clamp recording was performed as described in our previous studies (12, 13), with minor modification. Borosilicate glass pipettes (1.5 mm diameter) were pulled with a two-stage microelectrode puller (Narishige, Japan), and the resistance of the pipettes ranged from 2 to 4 MΩ when filled with the following solution (in mM): 140 KCl, 10 NaCl, 1 MgCl2, 5 EGTA, 2 Mg-ATP, 0.25 CaCl2, 0.4 Na3VO4, 10 HEPES, and 10 glucose, adjusted to pH 7.4 with KOH. After a whole-cell configuration was established, the adjustment of capacitance compensation and series resistance compensation was done before recording. SH-SY5Y cells were voltage clamped at −80 mV. A multibarrel perfusion system was used to achieve a rapid exchange of extracellular solutions (in mM): 150 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 10 HEPES, and 10 glucose, adjusted to pH 7.4 or 6.0 with NaOH. For solutions with pH < 6.0, HEPES was replaced with MES for reliable pH buffering. Membrane potential was recorded in current-clamp mode. All experiments were performed at room temperature (20–25°C).

**Calcium imaging.** SH-SY5Y cells grown on glass coverslips were washed three times with extracellular solution and incubated with 1 µM fura-2/AM for 30 min at 37°C. Coverslips with fura-2-loaded cells were then mounted on a chamber positioned on the movable stage of an inverted microscope (Olympus IX-70, Tokyo, Japan), which is equipped with a calcium imaging system (TILL-Photonics). Fluorescence was excited at wavelengths of 340 nm for 150 ms and 380 nm for 50 ms at 1-s intervals by a monochromator (Polychrome IV), and the emitted light was imaged at 510 nm with a video camera (TILL-Photonics Image) through a X-70 fluor oil immersion lens (Olympus, Tokyo, Japan) and a 460 nm long-pass barrier filter. F340/F380 fluorescence ratio was recorded and analyzed with TILL software (version 4.0), which was used as an indicator of [Ca2+]i, independent of intracellular fura-2 concentration. All experiments were repeated at least three times using different batches of cells.

**FM 1-43 imaging.** Vesicular recycling in SH-SY5Y cells was visualized with an FM 1-43 FX dye (Invitrogen). Solution A was

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prepared as follows (in mM): 150 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, and glucose 10, adjusted to pH 7.4 or 6.0 with NaOH. CaCl₂ was replaced by 5 mM EGTA to get calcium-free solution B. First, the coverslips were removed from the culture medium and rinsed three times with solution A. Then, coverslips were immersed in alternative pH solution A with or without different stimulants (60 mM K⁺/H11001, amiloride, PcTx1, etc.) in the presence of 5 μg/ml FM 1-43 FX dye for 1 min at room temperature. After rinsing three times with solution B (adjust to pH 7.4) on ice, the stain was fixed with ice-cold 4% formaldehyde for 20 min. The coverslips were rinsed three times and then mounted with 30% glycerin. Finally, coverslips were visualized with an Olympus FV500 confocal microscope. It is essential to maintain the image acquisition parameters exactly the same when comparing vesicular release in control and test conditions. The fluorescence density in nerve terminals was quantified by Image-Pro Plus software (Olympus) to reflect neurotransmitter release. When being stimulated in the presence of dye, vesicular exocytosis was induced and came in contact with the dye, and then the dye was taken up by endocytosis of vesicle. Therefore, the increase in fluorescence density in the terminals reflects the vesicular release by the stimulation (35).

Statistical analysis. Data are presented as means ± SD. Comparison between two experiments groups was made using the two-tailed unpaired Student’s t-test. One-way ANOVA combined with post hoc least significant difference (LSD) analysis was used for the statistical analysis by employing SPSS 10.0 software (SPSS, Chicago, IL). Significance level was set at P < 0.05.

**RESULTS**

**Characteristics of ASIC expression in SH-SY5Y cells.** RT-PCR and Western blotting were used to identify the expression of ASICs in SH-SY5Y cells. qPCR was carried out to quantify the expression of mRNAs encoding the various ASICs in SH-SY5Y cells. PCR and qPCR were performed using the primer pairs listed in MATERIALS AND METHODS. The products of these PCR reactions are shown in Fig. 1A. The sizes of the expected PCR products for ASIC1, ASIC2, and ASIC3 were 140, 139, and 107 bp, respectively. The appearance of the expected fragments in PCR reactions indicated the presence of ASIC1, ASIC2, and ASIC3 in SH-SY5Y cells. qPCR analysis of SH-SY5Y cells revealed that using GAPDH as a reference, ASIC1 was the most abundant ASIC transcript (Fig. 1B). Compared with cortex neurons, ASIC1 in SH-SY5Y was 0.366 ± 0.154-fold, ASIC2 was 0.008 ± 0.001-fold, and ASIC3 was 0.033 ± 0.023-fold. Using cortex neurons as positive controls, preincubation with peptides as negative controls, and β-actin as reference controls, bands in Fig. 1C between 55 kDa and 72 kDa indicated the existence of ASIC1, ASIC2a, and ASIC3 in SH-SY5Y cells. Since TRPV1 channels are also activated by acid (25) and participate in vesicular release (11), we also tested the existence of TRPV1 using Western blot analysis. Figure 1C shows the absence of TRPV1 in SH-SY5Y cells. To determine cellular

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**Fig. 1.** Expression of acid-sensing ion channels (ASIC1, ASIC2, and ASIC3) and transient receptor potential vanilloid 1 (TRPV1) in SH-SY5Y cells. A: ASIC transcripts were detected in SH-SY5Y cells by RT-PCR. 1, 2, and 3 refer to ASIC1, ASIC2, and ASIC3, respectively. M, marker. Cortex neurons were used as positive controls. The control PCR of GAPDH (top) was used to validate the quality of each sample. B: quantitative real-time PCR analysis of SH-SY5Y cells revealed that, relative to GAPDH, ASIC1 had the highest ASIC transcript expression, followed by ASIC2 and ASIC3. C: representative Western blot indicated the existence of ASICs and absence of TRPV1 in SH-SY5Y cells. –, without peptides; +, preincubated with peptides as negative controls; a, cortical neurons; b, SH-SY5Y cells. D: representative colocalization of ASICs (green) and cell nuclei (dark blue). NC, preincubated with peptides as negative controls. Scale bars, 50 μm.
distribution of ASICs in SH-SY5Y cells, we used double-staining immunofluorescence. FITC was conjugated to anti-ASIC antibodies (green), and the cell nuclei (blue) were stained with Hoechst 33258. Cells that were preincubated with peptides were used as negative controls. As shown in Fig. 1D, ASIC1 and ASIC3 were mainly expressed in cellular membrane and cytoplasm; however, ASIC2a was located in the cell nucleus. The results indicated that ASICs, not TRPV1, were expressed in SH-SY5Y cells.

Electrophysiological properties and calcium permeability of ASICs in SH-SY5Y cells. As previously reported (34), a drop in extracellular pH to below 6.9 activates a fast-rising and rapid desensitizing inward ASIC current that was inhibited by amiloride. Here, SH-SY5Y cells were held at −80 mV and then a rapid decrease in extracellular pH from 7.4 was applied to the bath. The rapid inward currents were detected by whole cell patch-clamp recording in SH-SY5Y cells. After recording of two to three consecutive traces that had similar amplitude, amiloride or capsazepine (CPZ) was added to the extracellular solutions. The currents were reversibly inhibited by 100 μM amiloride, a nonspecific blocker of ASICs. The rate of inhibition was 62.5% ± 6.7% (n = 6, Fig. 2A); however, they did not respond to 10 μM CPZ, the TRPV1 antagonist. As shown in Fig. 2B, the currents induced by acid in SH-SY5Y cells were pH-dependent. The current amplitude was 61.48 ± 13.49 pA at pH 6.5; 137.71 ± 35.23 pA at pH 6.0; 234.4 ± 40.41 pA at pH 5.5; and 270.37 ± 14.82 pA at pH 5.0. The pH of half-maximal

Fig. 2. Electrophysiological properties and calcium permeability of ASICs in SH-SY5Y cells. A: representative currents evoked by extracellular pH dropping from 7.4 to 6.0 for 5 s in SH-SY5Y cells. Currents were reversibly inhibited by amiloride (100 μM). B: pH-dependence of ASIC-like currents recorded at −80 mV. Currents were induced by a drop of extracellular pH from 7.4 to 6.5 (n = 19), 6.0 (n = 25), 5.5 (n = 13), and 5.0 (n = 6), respectively. C: pH-response curve fitted by the Hill equation (three parameters): y = a+b/(c+b), where a is maximal current, b is the Hill coefficient, and c is the pH at which one-half of the channels are activated (pH50). pH50 was 6.01 ± 0.04 (mean ± SD). D: representative intracellular calcium concentration ([Ca2+]i) response of SH-SY5Y cells to acid solution (pH 6.0). Preincubation with PcTx1 (10 nM) for 5 min inhibited the elevation. E: the increase in [Ca2+]i was inhibited by amiloride (100 μM). F: amiloride itself did not affect the basic [Ca2+]i.
activation (pH0.5) was 6.01 ± 0.04 (Fig. 2C). To determine whether intracellular calcium ([Ca^{2+}]_i) levels rise detectably during ASIC activation by acidic extracellular medium application, we labeled cells with the calcium-sensing dye fura-2/AM. After a baseline period in standard extracellular solution of pH 7.4, pH 6.0 solution was locally applied with the rapid exchange system for 10 s before returning to pH 7.4. As shown in Fig. 2D, pretreatment with 10 nM PcTx1 (a specific blocker of ASIC1a) for 5 min inhibited the increase in [Ca^{2+}]_i, with an inhibition rate of 40.4% ± 2.9% (n = 15). Another unspecific antagonist, amiloride (100 μM), also attenuated the [Ca^{2+}]_i rise by acid, with an inhibition rate of 54.7% ± 6.8% (n = 20, Fig. 2E). Meanwhile, amiloride itself did not affect the basic [Ca^{2+}]_i (Fig. 2F). These results suggest that ASIC-like currents were recorded in SH-SY5Y cells and that they were calcium permeable.

ASICs are involved in vesicular release from SH-SY5Y cells in response to acidification. The styryl dye FM 1-43 is a powerful tool to track exocytosis, endocytosis, and recycling of secretory granules or vesicles (2). Here, SH-SY5Y cells were incubated with FM 1-43 FX, and vesicular release was quantified by comparing the mean fluorescence density in terminals. As shown in Fig. 3, the mean density of fluorescence was 20.25 ± 8.03 in control solution (pH 7.4, n = 17), and 60 mM K^+ largely augmented the fluorescence density to 71.89 ± 10.24 (n = 18, P < 0.05 vs. control). Meanwhile, pH 6.0 extracellular solution also increased the density to 55.69 ± 9.89 (n = 20, P < 0.05 vs. 60 mM K^+). However, amiloride and PcTx1 inhibited the acid-induced response. After cells were preincubated with 100 μM amiloride (pH 7.4) for 20 min, the density of fluorescence in terminals decreased to 24.03 ± 10.64 (n = 15, P < 0.05 vs. pH 6.0, Fig. 3). The increased density by acid stimulation also lowered to 35.62 ± 9.46 after preincubation with 10 nM PcTx1 (pH 7.4) for 20 min (n = 17, P < 0.05 vs. pH 6.0, Fig. 3C). These results suggest that ASICs, and especially ASIC1a, were involved in vesicular release in response to acidosis.

N-type calcium channels are involved in calcium-dependent vesicular release from SH-SY5Y cells by acid. It is well known that intracellular Ca^{2+} is a key messenger for neurotransmitter release. To test whether vesicular release from SH-SY5Y cells stimulated by acid was calcium dependent, extracellular calcium was chelated with EGTA (5 mM) in calcium-free solution. It was found that the increase in vesicular release by pH 6.0 extracellular solution was significantly decreased, with the mean density of fluorescence dropping from 55.87 ± 14.97 (pH 6.0, means ± SD) to 22.09 ± 10.81 (n = 20, P < 0.05 vs. pH 6.0, Fig. 4A). Next, CPA (1 mM) was preincubated for 20

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**Fig. 3. Vesicular release analysis in SH-SY5Y cells with FM 1-43 imaging.**

A: representative images from SH-SY5Y cells stimulated by 60 mM K^+ (n = 18) and pH 6.0 acid solution (n = 20). Scale bars, 50 μm. B: quantitative analysis of fluorescent signals. Mean density was normalized to control. *P < 0.05 vs. control, #P < 0.05 vs. 60 mM K^+ or pH 6.0 (ANOVA). C: typical FM 1-43 images from SH-SY5Y cells were visualized by detecting green fluorescent signals before (n = 15) and after (n = 15) pH 6.0 acid stimulation in the absence and presence of amiloride (100 μM, n = 15) or PcTx1 (10 nM, n = 17). Scale bars, 50 μm. Images in bottom row are a magnification of the rectangle areas in top row. Scale bars, 10 μm.
Fig. 4. N-type calcium channel involved in calcium-dependent vesicular release from SH-SY5Y cells by acid. Left: typical FM 1-43 imaging. Right: quantitative analysis normalized to control in SH-SY5Y cells. Green fluorescent signals were detected before and after acid (pH 6.0) stimulation. A: extCa\(^2+\)(-) refers to EGTA-treated calcium-free buffer solution (n = 20). B: cyclopiazonic acid (1 mM, CPA) was added to deplete intracellular calcium store (n = 9). C: nifedipine (10 \(\mu\)M), an L-type calcium channel blocker, had no effect on the increase of vesicular release (n = 15). D: \(\omega\)-conotoxin GVIA (20 nM, \(\omega\)-CTX), an N-type calcium channel blocker, decreased the fluorescence density rise (n = 16). E: \(\omega\)-agatoxin TK (20 nM, \(\omega\)-AGX), a P/Q-type calcium channel blocker, did not affect the density rise (n = 14). Scale bar, 50 \(\mu\)m. \(*P < 0.05\) vs. control, \#P < 0.05 vs. pH 6.0 (ANOVA).
min to deplete intracellular Ca\(^{2+}\) stores. The increased vesicular release stimulated by pH 6.0 solution was also inhibited (\(n = 9, P < 0.05\) vs. pH 6.0, Fig. 4B). Thus, vesicular release from SH-SY5Y cells stimulated by acidosis was calcium dependent, which was dependent on both extracellular and intracellular Ca\(^{2+}\) sources.

Considering that L-, N-, and P/Q-type calcium channels play roles in neurotransmitter release and that T-type calcium channel is responsible for neuronal oscillatory activity such as sleep/wakefulness regulation (42), L-, N-, and P/Q-type calcium channel blockers (nifedipine, \(\omega\)-CTX, and \(\omega\)-agatoxin TK) were preincubated for 20 min. As shown in Fig. 4D, \(\omega\)-conotoxin GVIA (\(\omega\)-CTX) significantly reduced the vesicular release, with the mean density of fluorescence dropping from 58.47 ± 7.55 (pH 6.0) to 42.69 ± 4.73 (\(n = 16, P < 0.05\) vs. pH 6.0), while nifedipine (Fig. 4C) and \(\omega\)-AGX (Fig. 4E) had no effect. To further confirm that the N-type calcium channel also mediated acid-evoked [Ca\(^{2+}\)]\(_i\) elevation, we performed calcium imaging experiments. As shown in Fig. 5, \(\omega\)-CTX inhibited the intracellular calcium increase by rapid pH changes from 7.4 to 6.0, and the inhibition rate was 21.5% ± 5.6% (\(n = 20, P < 0.05\) vs. pH 6.0). These results implicated that vesicular release from SH-SY5Y cells stimulated by acid was calcium dependent and that N-type calcium channels were involved in the process.

**Depolarization of SH-SY5Y cell membrane by ASIC activation.** Since voltage-gated calcium channels are activated when the cell membrane depolarizes, we then assessed whether ASIC activation caused cell membrane depolarization, thereby leading to activation of N-type calcium channels and consequent neurotransmitter release. The results showed that application of acidic extracellular solution induced a rapid depolarization of membrane potential in SH-SY5Y cells under the whole cell current-clamp recording (Fig. 6A). The amplitude of membrane potential was increased by 29.4 ± 6.9 mV (\(n = 11\)) and 54.5 ± 7.7 mV (\(n = 10,\) Fig. 6B) when extracellular solution dropped from pH 7.4 to pH 6.0 and pH 5.0, respectively. Thus, ASIC activation can induce membrane depolarization in SH-SY5Y cells.

**DISCUSSION**

In the present study, we found functional expression of ASICs in SH-SY5Y cells and its role in vesicular release stimulated by extracellular protons. N-type calcium channels participated in the process probably because the activation of ASICs depolarized cell membrane and then activated the calcium channels.

**Functional expression of ASICs in SH-SY5Y cells.** The variation of pH is a common and vital phenomenon in physiological conditions and pathological processes, especially in the brain. Electrical activity caused by neuronal transmission and some diseases such as seizures often induces rapid pH changes in the intracellular and extracellular fluid (5). ASICs, a kind of highly sensitive acid sensors that distribute in most nervous systems, will sense the pH variations and mediate various neural function and disorders, such as synaptic plasticity (37), pain (33), and seizure (45).

Since vesicular release has been reported to cause pH decrease in the synaptic cleft (24), and the SH-SY5Y cell line has similar characteristics to dopaminergic neurons (40), including neurotransmitter release, we therefore explored the role of ASICs in vesicular release here by using SH-SY5Y cell. In the present study, we first identified the gene and protein expressions of ASICs in SH-SY5Y cells. The expression of ASIC1 was much more than that of ASIC2 and ASIC3 in SH-SY5Y cells. Furthermore, ASIC1 and ASIC3 were mainly expressed in the cell nucleus. Then, the whole cell patch-clamp and calcium imaging experiments further demonstrated the properties of ASICs in SH-SY5Y cells. The ASIC currents were pH depen-
dent, with a pH_{0.5} of 6.01 \pm 0.04, and extracellular acidification elevated the intracellular calcium through ASIC channels. Therefore, functional ASICs were expressed in the SH-SY5Y cell line.

Although the SH-SY5Y cell line, derived from human neuroblastoma, is a kind of heterogeneous cell, it is different from other cell lines such as HEK293 and CHO cells. HEK293 cells, only used to express specific ASIC subunits to study the functional role of ASICs alone, are not neuronal in origin; thus, the cellular responses and signal transduction pathways in those heterologous cells are not representative of those in native neurons. Hence, our results that there are functional ASIC proteins in SH-SY5Y cells provide a new human source neuronal cell line to investigate the physiological and pathological roles of ASICs.

Role of ASICs in vesicular release stimulated by acid and possible mechanisms. ASICs have been reported to be involved in the neurotransmitter release; however, the results are controversial in different tissues (6, 28). The effect of second messengers on norepinephrine release in SH-SY5Y cells has been confirmed by high K^+ stimulation (23, 29); thus SH-SY5Y cells can be used as a tool to study vesicular release. The present study showed that acidosis stimulated vesicular release from SH-SY5Y cells and that ASIC blockers inhibited this response, suggesting that ASICs contribute to acid-induced vesicular release.

Considering that most cases of vesicular release are calcium dependent, it was also found that acidosis-induced vesicular release was due to [Ca^{2+}]_i elevation in SH-SY5Y cells. Since voltage-gated calcium channels are involved in neurotransmitter release, several calcium channel blockers were employed. In this study, N-type calcium channel blockers, not L- or P/Q-type calcium channel blockers, attenuated the increase in vesicular release by acid, indicating that the N-type calcium channel might be activated by extracellular acidification and permit Ca^{2+} influx to evoke vesicular release. It has been reported that proton inhibits NMDA receptors (10, 27, 32); thus, the NMDA receptor is surely excluded from the acidosis-induced [Ca^{2+}]_i rise. Then, how could acid activate the N-type calcium channel? Daniel et al. (9) reported that extracellular acidification can induce depolarization of oligodendrocyte progenitor (9). The threshold activation of Ca^{2+} currents through high-voltage-activated Ca^{2+} channels occurs at \(-40\) to \(-10\) mV (42). As expected, the resting membrane potential and magnitude of depolarization were dependent on the quality of the recording seal. In our present study, transient extracellular acidification depolarized the membrane potential from \(-60\) mV to around \(-30\) mV to 0 mV, which was enough to activate the N-type calcium channel. Thus, extracellular acidification activates ASICs which depolarizes the cell membrane and sequentially activates N-type calcium channels. However, there is a need for further investigation.

In summary, this study provides evidence that ASICs exist in SH-SY5Y cell lines and play an important role in the vesicular release stimulated by extracellular acidification. The present study lays a foundation for continued research on ASICs and vesicular release, which is needed for the development of new treatments for diseases in which there is a lack of transmitter release, especially Parkinson’s disease.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Q.-J.X., Z.-L.H., P.-F.W., J.-G.C., and F.W. conceived and designed the research; Q.-J.X., Z.-L.H., J.-G.C., and F.W. performed the experiments; Q.-J.X. analyzed the data; Q.-J.X. interpreted the results of the experiments; Q.-J.X. prepared the figures; Q.-J.X. drafted the manuscript; Z.-L.H., J.-G.C., and F.W. edited and revised the manuscript; F.W. approved the final version of the manuscript.

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