Pannexin 1, a large-pore membrane channel, contributes to hypotonicity-induced ATP release in Schwann cells

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Abstract

Pannexin 1 (Panx 1), as a large-pore membrane channel, is highly permeable to ATP and other signaling molecules. Previous studies have demonstrated the expression of Panx 1 in the nervous system, including astrocytes, microglia, and neurons. However, the distribution and function of Panx 1 in the peripheral nervous system are not clear. Blocking the function of Panx 1 pharmacologically (carbenoxolone and probenecid) or with small interfering RNA targeting pannexins can greatly reduce hypotonicity-induced ATP release. Treatment of Schwann cells with a Ras homolog family member (Rho) GTPase inhibitor and small interfering RNA targeting Rho or cytoskeleton disrupting agents, such as nocodazole or cytochalasin D, revealed that hypotonicity-induced ATP release depended on intracellular RhoA and the cytoskeleton. These findings suggest that Panx 1 participates in ATP release in Schwann cells by regulating RhoA and the cytoskeleton arrangement. This study was approved by the Animal Ethics Committee of Nantong University, China (No. S20180806-002) on August 5, 2018.

Key Words: ATP; cytoskeleton; injury; neuron; pannexin 1; peripheral nerve; Ras homolog family member A; Schwann cells

Chinese Library Classification No. R446; R741; Q493.8

Introduction

Schwann cells not only act as physical support cells for axons but also release multiple signaling molecules that interact with long axons. As the first response after sciatic nerve injury, these cells act by dedifferentiation, proliferation, and release of abundant signaling molecules to modulate nerve injury repair (Su et al., 2019; Wei et al., 2019). Among these signaling molecules, extracellular ATP can be released from Schwann cells as well as peripheral neurons (Fields and Stevens, 2000) and contributes to their communication (Lyons et al., 1995; Negro et al., 2016). For example, ATP release in Schwann cells is induced by glutamate (Liu and Bennett, 2003) or uridine triphosphate (Liu et al., 2005), and purinergic receptors are expressed in several peripheral nervous system cell types that mediate neuron-Schwann cell signaling transmission, which contributes to a variety of functions. ATP, as a key signaling molecule, mediates peripheral sensitization after sciatic nerve injury (Tsuda et al., 2010). Evidence for ATP release in Schwann cells is primarily focused on formation and delivery of intracellular vesicles (Shin et al., 2012). However, regulation of...
ATP release in Schwann cells is still incompletely understood. Pannexins (Panx) are large-pore membrane channels that are highly permeable to ATP and other signaling molecules. The Panx family mainly consists of three members: Panx 1, Panx 2, and Panx 3 (Baranova et al., 2004). Among these, Panx 1 is the best characterized and is nearly ubiquitously expressed in neurons and glial cells (Baranova et al., 2004). In microglia (Mousseau et al., 2018), astrocytes (Iglesias et al., 2009; Suadicani et al., 2012), and the dorsal root ganglion (Zhang et al., 2015), Panx 1 has been reported to mediate ATP release and other regulated functions. Nevertheless, the distribution of Panx 1 in Schwann cells and its role in regulating ATP release remain unclear.

Cell swelling is an early event that occurs under pathophysiological conditions in response to various types of injury, such as trauma, ischemia, and stroke (Vardjan et al., 2010). In this study, we explored the distribution and function of Panx 1 in Schwann cells and determined whether RhoA and the cytoskeleton are involved in its mechanism.

Materials and Methods

Ethics statement
Postnatal day 1–3 CD-1 mice (for primary Schwann cell cultures) and adult male mice (aged 6–8 weeks, specific-pathogen-free level) (for western blot assays) were provided by the Experimental Animal Center of Nantong University (SYXK (Su) 2015-0016, SYXK (Su) 2017-0046). Animal procedures were approved by the Animal Ethics Committee of Nantong University, China (No. S20180806-002) on August 5, 2018.

Cell culture, transfection, and incubation
Primary Schwann cell cultures were produced as previously described (Su et al., 2019). Briefly, cells were isolated from the sciatic nerves of newborn mice and plated on 100 μg/mL poly-D-lysine-coated 3 cm² dishes in Dulbecco’s modified Eagle medium (Sigma-Aldrich, St. Louis, MO, USA) containing (mM): 1 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose (Sigma-Aldrich) dissolved in dimethyl sulfoxide (DMSO) were incubated in buffer immediately before use. Vehicle solutions were mixed with DMSO at the same volume in hypotonic solutions. DMSO has no effect when incubated at a low concentration (<0.1%).

Quantitative real-time polymerase chain reaction
RNA obtained from Schwann cells was isolated using TRIzol/ chloroform (Invitrogen), and complementary DNA was synthesized with a Prime-Script RT reagent Kit (Takara, Dalian, Liaoning Province, China) according to the manufacturer’s instructions. Quantitative real-time polymerase chain reaction (RT-PCR) assays were conducted with SYBR Green and a Step-one RT-PCR system (Applied Biosystems, Foster City, CA, USA). The procedure was as follows: 95°C for 10 minutes and 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The levels of target mRNA were normalized to the endogenous control glyceraldehyde 3-phosphate dehydrogenase by the 2^(-ΔΔCT) method. Quantitative RT-PCR was used to identify the best siRNA sequence for knocking down the expression of target genes. All reactions were performed in triplicate. All primers and siRNAs used in this experiment are listed in Tables 1 and 2.

Immunofluorescence
The procedure was conducted as previously described (Su et al., 2019). Briefly, after fixing with 4% paraformaldehyde for 12 minutes, Schwann cells were permeabilized with 0.1% Triton X-100 for 10 minutes and blocked in 10% bovine serum albumin for 1 hour. Cells were processed for immunofluorescence with the following primary antibodies overnight at 4°C: S100β (mouse; 1:500; Sigma) and Panx 1 (rabbit; 1:200; Santa Cruz Biotechnology, Dallas, TX, USA). After washing, the cells were incubated with the following fluorescent secondary antibodies for 1 hour at room temperature: anti-mouse Alex-488 and anti-rabbit Cy3 (1:100; Jackson ImmunoResearch, West Grove, PA, USA). In the negative control, primary antibodies were replaced with phosphate buffered saline, and the other procedures were the same as above. Cells were imaged with a Nikon fluorescence microscope (Tokyo, Japan).

Western blot assay
Proteins were extracted from adult mouse brains and cultured Schwann cells with a radioimmunoprecipitation assay lysis buffer containing complete protease inhibitors (1:100; Roche, Basel, Switzerland) and 1 mM phenylmethylsulfonyl fluoride and measured with a bicinchoninic acid protein assay kit (Beyotime, Haimen, China). Isolated proteins (20 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis after boiling for 10 minutes at 95°C and then transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After blocking in 5% (w/v) bovine serum (Gibco, Carlsbad, CA, USA). Small interfering RNA (siRNA) sequences were transferred into cells using RNAiMAX (Invitrogen, Carlsbad, CA, USA) at 2 or 3 days post-plating, and scrambled siRNA was used as a control. The RhoA siRNA, Panx 1 siRNA, and a universal negative control siRNA sequences were provided by Biomics Biotechnologies Co., Ltd. (Nantong, Jiangsu Province, China).

The cells were rinsed twice with isotonic solution containing (mM): 140 NaCl, 1 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES (Sigma-Aldrich), and 10 glucose (Sigma-Aldrich) (pH 7.2–7.4, 290 mOsm) and pre-incubated as indicated in the following assay solutions (Liu et al., 2008). Hypotonic solutions (pH 7.2–7.4, 200 mOsm) were composed of a HEPES buffered solution containing (mM): 1 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose (pH 7.2–7.4, 20 mOsm) (Liu et al., 2008). Inhibitors including carboxenolone (CBX; Sigma-Aldrich), probenecid (Sigma-Aldrich), C3 transferase (Cytoskeleton, Denver, CO, USA), nucodazole (Sigma-Aldrich), and cytochalasin D (Sigma-Aldrich) dissolved in dimethyl sulfoxide (DMSO) were incubated in buffer immediately before use. Vehicle solutions were mixed with DMSO at the same volume in hypotonic solutions. DMSO has no effect when incubated at a low concentration (<0.1%).
Table 1 | Quantitative real-time polymerase chain reaction primers

| Gene   | Sense sequence (5’→3’) | Anti-sense sequence (5’→3’) |
|--------|------------------------|-----------------------------|
| Panx 1 | CCT CAT TAA CCT CAT TGT GTA T | CAT TGT AGC CTT CAG ACT TG |
| Panx 2 | TGT GGT CTA TAC TCG CTA TG | CTC CTG CTG GAT GTC TAG |
| Panx 3 | CTC AGA TTA TGG ACT ATG AAC | TCA GAA GGT AAC TTG GAG AAT AC |
| RhoA   | TGC TTG CTC ATA GTC TCT A | GGC GGT CAT AAT CTT CCT |

Panx: Pannexin; RhoA: Ras homolog family member A.

Results

Characterization of Panx 1 expression in mouse Schwann cells

To explore the expression of Panx in Schwann cells, we examined the mRNA levels in pure cultured Schwann cells by quantitative RT-PCR. Panx 1 is highly expressed compared with other subtypes (Figure 1A). Therefore, we focused on the role of Panx 1 in our study. Western blotting and double immunofluorescence labeling detected a single band and co-localization with S100β, a specific Schwann cell marker, respectively, in pure cultured Schwann cells (Chen et al., 2012) (Figure 1B and C). Immunohistochemistry analysis showed that Panx 1 co-localized with S100β in sciatic nerves (Figure 1D). Together, these results showed that Schwann cells express Panx 1.

Hypotonic solution-induced Panx 1 activation increases ATP release

We investigated the role of Panx 1 in regulating hypotonic solution-induced ATP release. Treatment with a hypotonic solution for 2, 4, 8, or 12 minutes induced significant increases in ATP release in cultured Schwann cells (Figure 2A). After examining the increases in ATP release at different time points, we chose hypotonic solution treatment for 12 minutes for use in subsequent studies. Notably, the effect of activated Panx 1 was abolished by pretreatment with two Panx blockers, CBX and probenecid (Karatas et al., 2013). Pretreatment with CBX (100 μM, 60 minutes) or probenecid (500 μM, 60 minutes) led to a reduction of ATP release induced by hypotonic solution in Schwann cells (Figure 2C). Because Panx 1 blockers may affect channels other than Panx 1 (Chekeni et al., 2010; Chen et al., 2014), we next used Panx 1 siRNA to knock down Panx 1 expression in Schwann cells. We initially screened and selected the most effective Panx 1 siRNAs in RSC96 cells or Schwann cells (Figure 3A–C). Surprisingly, we found that hypotonic solution could dramatically increase Panx 1 expression. However, the increase was markedly abolished after transfection with a Panx 1-specific siRNA (Figure 3D). Moreover, hypotonicity-induced ATP release was prevented with Panx 1 siRNA treatment but not with non-targeting siRNA treatment (Figure 3E).

During the same period, we detected LDH activity in cells under different conditions. Under all conditions, except for Triton-treated cells, only low levels of LDH were detected, suggesting that ATP release was not caused by cell lysis (Chen et al., 2012) (Figures 2B, D, and 3F). Thus, the findings demonstrate that Panx 1 activation is involved in ATP release induced by hypotonic challenge.

RhoA GTPase activity regulates Panx 1-mediated ATP release

We first examined the expression of RhoA under hypotonic conditions. The results showed that hypotonicity-induced RhoA upregulation was significantly attenuated by Panx 1 knockdown via siRNA (Figure 4A), suggesting that RhoA is important in regulating Panx 1-mediated ATP release. Next, we further investigated the role of RhoA with a Rho GTPase inhibitor and Rho-siRNA. Pretreatment with C3 transferase, a potent Rho GTPase inhibitor (2 μg/mL, 1 hour) reduced ATP release induced by hypotonic solution (Figure 4B). We next utilized Hepa1-6 cells to select the most effective siRNA for knockdown of RhoA to reduce its expression in Schwann cells. The results showed that the increase in ATP release induced by hypotonic solution was abolished but not in the scrambled siRNA group (Figure 4D–G). During the same period, we also detected LDH activity in the medium of treated and untreated cells to exclude the release of ATP as a side effect resulting from cell lysis (Figure 4C and H). Taken together, we concluded that RhoA GTPase is involved in Panx 1-mediated ATP release induced by a hypotonic solution in Schwann cells.
Cytoskeletal organization controls hypotonicity-induced ATP release

According to our immunostaining results in cultured Schwann cells, Panx 1 is expressed primarily on the surface of the cell membrane (Figure 1C). To evaluate the role of microtubules, cells were pretreated with the microtubule disrupting agent nocodazole (10 μM) for 2 hours before the addition of hypotonic solution for 12 minutes. The results revealed that hypotonic solution-induced ATP release was markedly attenuated in the nocodazole group (Figure 5A). Therefore, we then utilized cytochalasin D (1 μM) to disrupt actin filaments for 2 hours before hypotonic solution was added for 12 minutes. Hypotonic challenge-induced ATP release was markedly attenuated by treatment with cytochalasin D (Figure 5A). To exclude the possibility of cell lysis, an LDH assay was conducted at the same time (Figure 5B). Thus, we concluded that an intact cytoskeleton is essential for hypotonicity-induced ATP release.

Discussion

ATP acts as a key regulator in the peripheral nervous system in processes such as the formation and maintenance of neuropathic pain (Burnstock, 2017; Wei et al., 2019) and the mediation of interactions of neurons and Schwann cells (Fields and Stevens, 2000). Here, our work primarily revealed the contribution of Panx 1 to the regulation of ATP release and its regulatory mechanism. We suggest that emerging evidence of mechanisms of ATP release will help to develop potential treatments for diseases.
Panx 1 contributes to ATP release in hypotonic stress, and the effect was significantly attenuated under conditions of Panx 1 channel blockade with CBX or probenecid and siRNA knockdown of Panx 1. Connexins, such as CX43 and CX32 (De Vuyst et al., 2006), are expressed in Schwann cells and involved in ATP release (Zhao et al., 1999; Nualart-Martí et al., 2013). Moreover, we also found expression of CX43 in primary cultured Schwann cells (data not shown). Therefore, under different conditions, connexins and Panx 1 are both important for ATP release in Schwann cells.

The intercellular mechanisms that might regulate hypotonic solution-induced ATP release in Schwann cells are unknown. Hypotonic stress promotes Rho activation, as previously reported in some cells (Koyama et al., 2001; Kawa et al., 2003; Krepskyn et al., 2003). Our study suggests that RhoA signaling mediates hypotonicity-induced ATP release. We first demonstrated that hypotonic solution induced an increase in RhoA expression, and we showed that knocking down RhoA with siRNA-RhoA or adding the potential Rho GTPase inhibitor C3 transferase markedly impaired hypotonicity-induced ATP release. These results are consistent with those of a previous study (Seminario-Vidal et al., 2011), which showed that Rho-regulated Panx 1 channel opening is involved in ATP release in airway epithelial cells.

We have not further addressed the mechanism by which RhoA regulates Panx 1-induced ATP release. However, given that RhoA is a regulator of the cytoskeleton, the role of Panx 1 in regulating ATP release is of great interest. Membrane stretch induced by a hypotonic stimulus can result in Panx activation in several cell types and under certain conditions. In human bronchial epithelial cells, Panx 1 mediated ATP release under hypotonic conditions, which was markedly inhibited by Panx 1 RNA interference, CBX, and treatment with a Panx 1-selective blocking peptide, 2,3-pannexin 1 (Seminario-Vidal et al., 2011). Here, we showed that

Figure 4 | Inhibition of RhoA activation reduced hypotonic solution-induced ATP release. (A) Relative expression of RhoA upon treatment with isotonic (Ctrl) or hypotonic (Hypo) solution after transfection with scrambled and Panx 1 siRNA (3). (B) ATP release was assessed in Schwann cells treated with C3 transferase. Dimethyl sulfoxide at the same volume in isotonic solution was used as a control (Ctrl). (C) Lactate dehydrogenase (LDH) release was assessed under the same conditions as ATP release in B. (D) RhoA mRNA levels in Schwann cells treated with scrambled siRNA (NC) and RhoA-siRNA (1–3). (E, F) Western blot images and mean analysis showing the levels of RhoA protein in Hepa 1–6 cells from the scrambled (Ctrl) and Panx 1-siRNA (1–3) groups. (G) ATP was assessed in scrambled siRNA- and RhoA-siRNA (3)-treated Schwann cells. Cells were treated with isotonic solution after transfection with scrambled siRNA as a control. (H) LDH release was assessed under the same conditions as ATP release. Data are expressed as the mean ± SEM (n = 3). *P < 0.05, **P < 0.01 (one-way analysis of variance or Student’s t-test). GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; LDH: lactate dehydrogenase; Panx 1: Pannexin 1; RhoA: Ras homolog family member A; siRNA: small interfering RNA; Vehicle: dimethyl sulfoxide.

Figure 5 | An intact cytoskeleton is required for ATP release during hypotonic challenge. (A, B) Schwann cells were incubated with nocodazole (Noco) or cytochalasin D (CytoD) for 2 hours before treatment with hypotonic (Hypo) solutions. ATP (A) and lactate dehydrogenase (LDH) (B) release were assessed under different conditions. Dimethyl sulfoxide at the same volume in isotonic solution was used as a control (Ctrl). Data are expressed as the mean ± SEM (n = 3). *P < 0.05, **P < 0.01 (one-way analysis of variance or Student’s t-test). LDH: Lactate dehydrogenase; Vehicle: dimethyl sulfoxide.

Our study provides new information about the role of Panx 1 in regulating ATP release in Schwann cells. We first determined that Panx 1 is highly expressed in Schwann cells. Furthermore, Panx 1-mediated ATP release induced by a hypotonic stimulus is markedly reduced after treatment with Panx 1 and RhoA siRNA and cellular cytoskeleton blockers. Therefore, our findings demonstrate that Panx 1 contributes to ATP release in Schwann cells, and this process is accompanied by RhoA activation and cytoskeleton rearrangement.

It is well known that the Panx channels are highly permeable to ATP. The ATP that interacts with purinoreceptors is an important inflammatory mediator released by activated cells after injury or trauma (Burnstock, 2006, 2017). Our previous study revealed that Schwann cells express several subtypes of P2X receptors (Su et al., 2019). Schwann cells can release ATP in both physiological and pathological conditions, and this released ATP further activates Schwann cells and neurons to release signaling molecules. Therefore, the role of Panx 1 in regulating ATP release is of great interest.

Membrane stretch induced by a hypotonic stimulus can result in Panx activation in several cell types and under certain conditions. In human bronchial epithelial cells, Panx 1 mediated ATP release under hypotonic conditions, which was markedly inhibited by Panx 1 RNA interference, CBX, and treatment with a Panx 1-selective blocking peptide, 2,3-pannexin 1 (Seminario-Vidal et al., 2011). Here, we showed that

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Furthermore, ATP released by Panx 1 can increase intracellular calcium spread to the surrounding cells (Scemes and Giamei, 2006; Spray and Hanani, 2019). Overall, increased intracellular calcium may have a specific effect on hypertonicity-induced ATP release, but this still requires further investigation. In this Panx 1 in Schwann cells following hypertonic-induced ATP release. An increased Panx 1 level is an important regulator in hypertonicity-induced ATP release. Moreover, using blockers and RhoA knockdown via siRNA, we found that RhoA signaling and cytoskeletal organization regulate Panx 1-mediated ATP release. Our study provides new evidence that expression of Panx 1 in Schwann cells contributes to ATP release.

Author contributions: Study design: GC, ZYW; experimental implementation and data analysis: ZYW, HLQ; experimental assistance and data collection: YJD, DW, ZML, WFS, YYY, WXS; manuscript writing: ZYW, GC; experimental supervision: GC. All authors approved the final version of the manuscript.

Conflict of interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Financial support: This study was supported by the National Natural Science Foundation of China, Nos. 31900718 (to ZYW), 31872773 (to GC); the National Key Research and Development Program of China, No. 2017YFA0104704 (to GC). Basic Research Program of the Education Department of Jiangsu Province of China, Nos. 19KJB180024 (to ZYW), 18KB180020 (to WXS); Postdoctoral Science Foundation of China, No. 2019M651925 (to ZYW), Jiangsu Students’ Platform for Innovation and Entrepreneurship Training Program of China, No. 2018103040312 (to YJD); Six Talent Peaks Project in Jiangsu Province of China (ref. No.WSN-2018-62). The funders had no role in the study design, conduct of experiment, data collection and analysis, decision to publish, or preparation of the manuscript.

Institutional review board statement: The study was approved by the Animal Ethics Committee of Nantong University, China (No. 520180806-002) on August 5, 2018.

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Data sharing statement: Datasets analyzed during the current study are available from the corresponding author on reasonable request.

Plagiarism check: Checked twice by iThenticate.

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