TMEM16A Ca$^{2+}$-activated Cl$^{-}$ channel inhibition ameliorates acute pancreatitis via the IP$_3$/Ca$^{2+}$/NFkB/IL-6 signaling pathway

Qinghua Wang$^{a,b,1}$, Lichuan Bai$^{a}$, Shuya Luo$^{a}$, Tianyu Wang$^{a}$, Fan Yang$^{a}$, Jialin Xia$^{a}$, Hui Wang$^{a}$, Ke Ma$^{a}$, Mei Liu$^{a}$, Shuwei Wu$^{a}$, Huijie Wang$^{a}$, Shibin Guo$^{c}$, Xiaohong Sun$^{d}$, Qinghuan Xiao$^{a,a}$

$^{a}$Department of Ion Channel Pharmacology, School of Pharmacy, China Medical University, Shenyang 110122, China
$^{b}$Department of Experimental Center, The Affiliated Hospital of Liaoning University of Traditional Chinese Medicine, Shenyang 110032, China
$^{c}$Department of Gastroenterological Endoscopy, the First Affiliated Hospital of Dalian Medical University, Dalian 116011, China
$^{d}$Department of Neurology, The Fourth Affiliated Hospital of China Medical University, Shenyang 110032, China

**GRAPHICAL ABSTRACT**

A positive activation loop between TMEM16A and the IP$_3$/Ca$^{2+}$/NFkB/IL-6 pathway is important for Ca$^{2+}$ elevation, NFkB activation and IL-6 release, and thus cooperatively promotes the pathogenesis of AP.

**Abbreviations:** AP, acute pancreatitis; T16Ainh-A01, TMEM16A inhibitor-A01; CaCCinh-A01, Ca$^{2+}$-activated Cl$^{-}$ channel inhibitor-A01; PACs, pancreatic acinar cells; CCK, cholecystokinin; IP$_3$R, inositol 1,4,5-trisphosphate receptor; ER, endoplasmic reticulum; IL-6, interleukin 6; IL-6R, interleukin 6 receptor; STAT3, signal transducers and activators of transcription 3; NFkB, nuclear factor-kB; shRNAs, short hairpin RNAs; FBS, fetal bovine serum; EGFP, green fluorescent protein; RIPA, radio immunoprecipitation assay; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; Tris, tris(hydroxymethyl)aminomethane; NP-40, Nonidet P-40; HEPES, N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid; NMDG, N-methyl-D-glucamine; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetyloxymethyl ester; WT, wild type; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; CFBE, cystic fibrosis bronchial epithelial.

Peer review under responsibility of Cairo University.

* Corresponding author at: Department of Ion Channel Pharmacology, School of Pharmacy, China Medical University, No. 77 Puhe Road, Shenyang North New Area, Shenyang 110122, China.

E-mail address: qinghuaxiao12345@163.com (Q. Xiao).

Q.W. and L.B. contributed equally to this work.

https://doi.org/10.1016/j.jare.2020.01.006
2090-1232/© 2020 Production and hosting by Elsevier B.V. on behalf of Cairo University.
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
Acute pancreatitis (AP) is an inflammatory disease with a severity ranging from mild edema in the pancreas to severe inflammation with systemic involvement [1]. The incidence of AP is approximately 13–45 cases/100,000 people and is increasing worldwide [2]. No therapeutic agents that inhibit the progression of AP are currently available [3]. Therefore, it is urgent to develop a novel agent that can ameliorate AP.

TMEM16A (anoctamin 1) is a Ca2+-activated Cl channel that participates in various physiological functions ranging from the secretion of epithelial fluid and the contraction of skeletal and smooth muscles to blood pressure control [4–6]. Recent studies have revealed that TMEM16A expression is upregulated in many diseases including cancer, hypertension, and cystic fibrosis [6–8]. In addition, TMEM16A overexpression is found in many inflammation-associated diseases such as asthma, cystic fibrosis, and chronic rhinosinusitis [8–11]. TMEM16A inhibition by its inhibitors such as T16Ainh-A01 (TMEM16A inhibitor-A01) and CaCCinh-A01 (Ca2+-activated Cl channel inhibitor-A01) (the structure of the inhibitors is shown in Fig. S1) blocks the development of cancer and inflammation [8,9]. Therefore, TMEM16A inhibitors may be promising for treating TMEM16A overexpression-associated inflammatory diseases.

TMEM16A has been immunohistochemically detected in the pancreatic tissues [12], including in acinar cells [13,14], islets [15], and duct cells [16], as well as in pancreatic cancer cells [17]. TMEM16A mediates Ca2+-activated Cl− and HCO3− transport in pancreatic acinar cells (PACs) [18,19]. TMEM16A-mediated Cl− secretion may function as a main driving force for secreting fluids in PACs [14]. TMEM16A-mediated HCO3− transport in PACs is important for luminal pH regulation, and TMEM16A inhibition increases luminal acidosis in AP induced by supramaximal cholestokinin (CCK) treatment [19]. However, although TMEM16A has been implicated in luminal pH regulation in AP, no studies have investigated whether TMEM16A contributes to the pathogenesis of AP.

Sustained intracellular Ca2+ elevation in PACs activates trypsinogen, causes mitochondrial dysfunction, induces NFkB activation, and has been recognized as a critical mechanism underlying the pathogenesis of AP [3,20]. Many known stimuli that induce AP, such as alcohol, CCK hyperstimulation, and bile acids, elicit sustained Ca2+ elevation in PACs by activating the inositol 1,4,5-triphosphate receptor (IP3R) Ca2+ channel in the endoplasmic reticulum (ER) [20]. TMEM16A binds directly to IP3R and is activated by IP3R-mediated Ca2+ release in dorsal root ganglia cells and HeLa cells (the human epithelial cancer cells of the cervix that have been maintained in culture since 1951 and are often used in research) [21,22], though this direct interaction between TMEM16A and IP3R is not found in cerebral artery smooth muscles. In addition, several lines of evidence have shown that TMEM16A overexpression increases intracellular Ca2+ concentrations [17,22]. However, it is unknown whether TMEM16A is involved in the pathogenesis of AP by increasing IP3R-mediated Ca2+ elevation.

Here, we studied the mechanism of TMEM16A channels in AP induced by cerulein, a CCK analog that is experimentally used for creating AP models. Both animal and cellular studies showed that TMEM16A expression was upregulated in PACs. TMEM16A upregulation was caused by interleukin 6 (IL-6)/IL-6 receptor (IL-6R)/signal transducers and activators of transcription 3 (STAT3) signaling activation, and TMEM16A overexpression increases IL-6 secretion via IP3R/Ca2+/NFkB signaling activation in pancreatic acinar cells. TMEM16A inhibition may be a new potential strategy for treating AP.

Materials and methods

Animals

The Animal Ethics Committee of Liaoning University of Traditional Chinese Medicine approved the experimental protocol for animal use (No. 2019YS (DW)-024-01). All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. C57BL/6N mice (male, 8–10 weeks old, weighing 20–24 g) were housed at 25 °C with 3.3. water and food ad libitum.
Mouse model of cerulein-induced AP

The mouse model of AP was created by a series of 7 intraperitoneal injections of supramaximal cerulein (50 μg/kg; Sigma-Aldrich, USA) spaced one hour apart. To investigate the time course of TMEM16A expression in AP mice, 24 mice were randomly assigned to 4 groups (n = 6 per group) based on sacrifice time at 0, 6, 12, and 24 h after the last cerulein injection. To study the in vivo effect of T16Ainh-A01, 18 mice were randomly assigned to 3 groups (n = 6 per group): the control group, the AP group, and the AP + T16Ainh-A01 group. For the AP + T16Ainh-A01 group, AP mice received intraperitoneal injection of T16Ainh-A01 (1 mg/kg) 30 min prior to the first cerulein injection. Equal volumes of normal saline were injected in control mice. The mice were sacrificed 12 h after the last cerulein injection. Blood and pancreatic tissues were collected for further analysis.

Histological examination of the pancreas

Pancreatic tissue sections (5 μm thick) were stained with hematoxylin and eosin. The severity of pancreatitis was assessed using a scoring system that evaluated four pathological parameters: edema (0–4), acinar cell necrosis (0–4), inflammation (0–4), and fibrosis (0–4). The scoring system that evaluated four pathological parameters:

Cell culture and transfection

Pancreatic acinar AR42J cells (ATCC, Manassas, USA) were cultured in RPMI 1640 medium (HyClone) supplemented with 20% fetal bovine serum (FBS) and 1% penicillin and streptomycin to prevent bacterial contamination in a humid incubator (37°C, 5% CO₂). The cell model of AP was created by treating cells with cerulein (10 nM) for 24 h.

AR42J cells were transfected with TMEM16A-expressing plasmids in the pEGFP-N1 vector and the control empty vector or with TMEM16A-shRNAs and scrambled control shRNAs in the pGPU6-EGFP vector (constructed by Shanghai GenePharma, China) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocols. The pEGFP-N1 vector and the pGPU6-EGFP vector encode enhanced green fluorescent protein (EGFP), which exhibits green fluorescence under a fluorescence microscope and can be used as a reporter to detect the transfected cells.

Western blot

For TMEM16A expression, pancreatic tissues or AR42J cells were homogenized in radio immunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology, China). For NFκB/p65 (65 kD) nuclear translocation, nuclear and cytoplasmic pools were generated using the nuclear and cytoplasmatic protein extraction kit (KeyGEN, China). After protein separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblot transfer, the membranes were incubated with primary antibodies against TMEM16A (1:2,000), STAT3 (1:1,000), phosphorylated STAT3 (p-STAT3; 1:1,000), NFκB/p65 (1:1,000) or IP3R (1:1,000) overnight at 4°C, followed by secondary antibodies (1:10,000) at room temperature for 1 h. Bands were visualized using chemiluminescence detection agents. All primary and secondary antibodies were from Abcam Biotechnology, UK.

Enzyme-linked immunosorbent assay (ELISA)

The IL-6 levels in the AR42J cell culture medium and in the mouse serum and pancreatic tissues were determined using an IL-6 ELISA kit (AMEKO, Shanghai, China) according to the manufacturer’s protocols and were detected using a microplate reader (Bio-Rad, USA).

Co-immunoprecipitation

AR42J cells were homogenized for 30 min in ice-cold RIPA lysis buffer containing 50 mM Tris(hydroxymethyl)aminomethane-thane-HCl (Tris-HCl: pH 7.4), 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.25% sodium deoxycholate, sodium orthovanadate, ethylenediaminetetraacetic acid (EDTA) and aprotinin, a protease inhibitor that inhibits proteolysis (Absin Biotechnology, China). After centrifugation, the supernatant was incubated with anti-TMEM16A antibodies or anti-IP3R antibodies overnight at 4°C, followed by incubation with pre-cleaned protein A/G agarose beads (20 μl) for 2 h at 4°C. The beads were then centrifuged at 3,000 rpm for 3 min at 4°C, washed with lysis buffer, and resuspended in the sample buffer. The samples were then analyzed by Western blot.

Measurement of intracellular Ca²⁺

AR42J cells were loaded with the cell-permeable fluorescent Ca²⁺ dye fluo-4- acetoxyethylmethyl ester (Fluo-4-AM) (2 μM, Invitrogen, USA) and 0.1% F127 (Invitrogen, USA) for 50 min at 37°C in Hank’s solution, which provided physiological pH, osmotic balance and essential inorganic ions. The cells were plated on a coverslip in Hank’s solution without Ca²⁺ (containing 5 mM Ca²⁺ chelator ethylene glycol-bis(2-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA)). The intracellular Ca²⁺ concentration in response to cerulein (10 nM) was measured using a confocal microscope (Nikon C2 plus, Japan) (excitation wavelength, 485 nm; emission wavelength, 515 nm). Fluo-4 fluorescence signal normalized to the resting level (F/F0) was used for analysis.

Patch clamp recordings

The patch clamp technique was used to record Cl⁻ currents in a whole-cell configuration. A P97 puller (Sutter Instruments, CA) was used to make electrodes with resistances of ~2-4 MΩ when filled with pipette solution. The data were recorded using Clampex 10 software on a computer connected to an Axopatch 200B amplifier via a Digidata system (Molecular Devices, CA, USA). AR42J cells were voltage clamped at a holding potential of 0 mV. Voltage ramps from −100 to +100 mV were applied at an interval of 10 s. Pipette solutions contained (in mM): 146 CsCl, 2 MgCl₂, 0.5 EGTA and 8 HEPEES (N-2-hydroxyethyl-piperazine-N’-2-ethanesulfonic acid), pH 7.3, adjusted with NMDG (N-methyl-D-glucamine). External solutions contained (in mM): 144 NaCl, 4 KCl, 5 EGTA, 1 MgCl₂, 10 glucose, and 10 HEPEES (pH 7.3). Cerulein was applied to the external solution to record Ca²⁺-activated Cl⁻ currents activated by IP3R.

Statistical analysis

Origin9 software was used for graphical presentations. Clampfit10 software was used for analyzing current traces. SPSS 13.0 software was used for statistical analyses. Student’s t-test or one-way analysis of variance (ANOVA) was used to compare the differences between groups. Spearman correlation analysis was used to evaluate the association of TMEM16A expression with the IL-6 levels in AP mice. P < 0.05 was considered to be statistically significant.
Results

**TMEM16A expression is upregulated in cerulein-induced AP**

We examined TMEM16A expression in cerulein-induced AP mice. Histological examination showed that cerulein treatment induced pancreatic damage in the mice (Fig. 1A, B). TMEM16A expression was upregulated in the pancreatic tissue of AP mice (Fig. 1C). Consistent with the animal model of cerulein-induced AP, TMEM16A expression was upregulated in AR42J cells following cerulein treatment for 6–24 h (Fig. 1D).

**TMEM16A expression correlates with IL-6 levels in cerulein-induced AP mice**

It is known that IL-6 is increased in AP and contributes to the pathogenesis of AP [25,26]. We examined the IL-6 levels in the pancreatic tissue and the serum of AP mice using ELISA. The IL-6...
levels in the pancreatic tissues and in the serum were increased in AP mice 6–24 h after cerulein injection (Fig. 1E, F). The amount of TMEM16A protein was significantly correlated with IL-6 levels in the pancreatic tissues (r = 0.99, p = 0.011; Fig. 1G) and in the serum (r = 0.99, p = 0.0057; Fig. 1F), suggesting that IL-6 may promote TMEM16A expression in AP.

IL-6 promotes TMEM16A expression via the IL-6R/STAT3 pathway in cerulein-induced AP

The ELISA results confirmed that the IL-6 concentration in the culture medium was significantly increased after cerulein treatment for 24 h (Fig. 2A), suggesting that IL-6 secretion was
increased in the AR42J cell model of AP. IL-6 (0.25 μg/ml) treatment increased TMEM16A expression in AR42J cells (Fig. 2B). Antibodies against IL-6 receptor (anti-IL-6R) reduced IL-6-induced STAT3 activation and TMEM16A upregulation in AR42J cells (Fig. 2C-E). The STAT3 inhibitor JSI-124 inhibited IL-6-induced TMEM16A upregulation in AR42J cells (Fig. 2F). IL-6R antibodies and JSI-124 significantly inhibited cerulein-induced TMEM16A upregulation in AR42J cells (Fig. 2G, H). These findings suggested that increased IL-6 secretion from acinar cells after cerulein treatment promoted TMEM16A expression via IL-6R/STAT3 signaling activation.

TMEM16A channels and IP3R activate each other in AR42J cells

We performed co-immunoprecipitation experiments to investigate whether TMEM16A and IP3R directly bind to each other in AR42J cells. The co-immunoprecipitation results showed that TMEM16A directly interacted with IP3R in AR42J cells (Fig. 3A). We then investigated whether IP3R-mediated Ca2+ release activated TMEM16A Cl–/C0 currents in AR42J cells by acute application of cerulein. Under the condition of Ca2+ extracellular solution to exclude the possible effect of Ca2+ influx, whole-cell Cl–/C0 currents in AR42J cells were gradually activated after the application of cerulein. The currents activated by cerulein exhibited outward rectification at the beginning of cerulein application and showed linear voltage-current relationships when currents were maximally activated, exhibiting the characteristic feature of TMEM16A currents (Fig. 3B-D). Cerulein-induced Cl– currents were significantly reduced by TMEM16A-shRNA treatment (Fig. 3B-D). The TMEM16A inhibitor T16Ainh-A01 inhibited cerulein-induced Cl– currents in AR42J cells (Fig. 3F). Furthermore, T16Ainh-A01 inhibited the...
IP$_3$-mediated Ca$^{2+}$ release induced by cerulein application (Fig. 3G, H). Taken together, these results suggested that TMEM16A and IP$_3$R directly interacted with and activated each other in AR42J cells.

**TMEM16A overexpression activates NF$\kappa$B signaling via Ca$^{2+}$ in AR42J cells**

Since NF$\kappa$B activation by Ca$^{2+}$ in PACs contributes to the pathogenesis of AP [25,27], we further investigated whether TMEM16A activates NF$\kappa$B signaling in AR42J cells. TMEM16A overexpression increased the nuclear expression of NFkB/p65 and decreased the cytosolic expression of NFkB/p65 (Fig. 4A). TMEM16A overexpression also increased the IL-6 secretion from AR42J cells (Fig. 4B). These results suggested that TMEM16A promoted NFkB activation in AR42J cells. Furthermore, the Ca$^{2+}$ chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N$^0$N$^0$-tetraacetic acid-acetyloxymethyl ester (BAPTA-AM) (Fig. S1C) inhibited TMEM16A overexpression-induced NFkB activation (Fig. 4C, D). These findings suggested that TMEM16A activated NFkB signaling by increasing intracellular Ca$^{2+}$.

**TMEM16A knockdown blocks cerulein-induced NFkB activation**

We next investigated the effect of TMEM16A inhibition on NFkB activation in the AR42J cell model of cerulein-induced AP. BAPTA-AM treatment inhibited cerulein-induced (24 h) NFkB activation (Fig. 5A), suggesting that Ca$^{2+}$ signaling was critical for NFkB activation in cerulein-induced AP. Cerulein-induced NFkB activation and IL-6 secretion were inhibited by TMEM16A-shRNA treatment (Fig. 5B, C), suggesting that TMEM16A mediated NFkB activation and IL-6 secretion in cerulein-induced AP.

**TMEM16A inhibitors ameliorate cerulein-induced AP via inhibition of channel activities and protein expression**

We investigated the effect of TMEM16A inhibitors on NFkB activation in cerulein-induced AP. T16Ainh-A01 treatment inhibited cerulein-induced NFkB activation (Fig. 6A, B), suggesting that inhibition of TMEM16A channel function reduced the cerulein-induced NFkB activation in AR42J cells. To further confirm whether TMEM16A channel activities were essential for NFkB activation, we transfected AR42J cells with D444EEEEEAVKD452 TMEM16A mutants with reduced channel activities [28]. Compared with wild-type (WT) TMEM16A, overexpression of D444EEEEEAVKD452 mutants resulted in less NFkB activation (Fig. 6C), suggesting that TMEM16A channel activity was essential for NFkB activation.

We then examined whether T16Ainh-A01 inhibited TMEM16A expression in cerulein-treated AR42J cells. The cerulein-induced increase in TMEM16A expression was inhibited by T16Ainh-A01 treatment (Fig. 6D). This effect was also found in the in vivo mouse model of cerulein-induced AP (Fig. 6E). Furthermore, histological examination showed that T16Ainh-A01 reduced the pancreatic damage in AP mice (Fig. 6F, G). T16Ainh-A01 treatment inhibited the IL-6 level in the pancreatic tissues and the serum of AP mice (Fig. 6H, I). These findings indicated that TMEM16A inhibition ameliorated AP.

**Discussion**

Our study demonstrated that TMEM16A expression was upregulated in PACs via the IL-6/IL-6R/STAT3 signaling pathway in cerulein-induced AP, and increased TMEM16A expression activated NFkB signaling and promoted IL-6 secretion by increasing IP$_3$R-mediated Ca$^{2+}$ release in acinar cells (Fig. 7). Thus, these
results reveal a positive activation loop between TMEM16A and IL-6 in PACs. Since Ca^{2+}, NF_{κ}B, and IL-6 are known contributors to the pathogenesis of AP [20,27], our findings suggest that TMEM16A may aggravate AP by promoting Ca^{2+} elevation, NF_{κ}B activation and IL-6 release. Furthermore, TMEM16A inhibition by TMEM16A-shRNA or T16Ainh-A01 was able to block NF_{κ}B activation and IL-6 secretion in the cellular model of AP, and T16inh-A01 treatment ameliorated pancreatic damage and reduced the IL-6 levels in the AP animal model. Our findings suggest that TMEM16A inhibition may be a new strategy for treating AP.

TMEM16A expression is upregulated in inflammation [10], and TMEM16A overexpression contributes to inflammation-associated respiratory diseases such as cystic fibrosis, chronic rhinosinusitis, and asthma [8,9,11]. Inflammatory cytokines such as IL-4 and IL-13 increase TMEM16A expression by activating STAT6 in airway epithelial cells and biliary epithelial cells [10,29,30]. We previously found that TMEM16A expression is upregulated by epidermal growth factor (EGF)/EGF receptor (EGFR)/STAT3 signaling activation in breast cancer cells [31]. The current study showed that TMEM16A expression was correlated with the IL-6 levels in the pancreatic tissues and in the serum of AP mice, and IL-6 promoted TMEM16A expression in PACs by activating the IL-6R/STAT3 signaling pathway. Furthermore, TMEM16A overexpression resulted in an increase in IL-6 secretion in PACs, and TMEM16A knockdown reduced IL-6 secretion in cerulein-treated cells. These findings suggest that TMEM16A expression and IL-6 secretion mutually activate each other in PACs, and the positive activation loop between TMEM16A and IL-6 may be important for the maintenance of persistent high TMEM16A expression and the constitutive secretion of IL-6 in PACs during AP. Since IL-6 is a pro-inflammatory cytokine that contributes to the development of AP [25], our findings also suggest that TMEM16A upregulation in PACs may promote AP by increasing IL-6 secretion.

Sustained Ca^{2+} elevation is an early cellular event in PACs during AP [3,20,27]. NF_{κ}B activation and the subsequent release of many pro-inflammatory cytokines including IL-6 play key roles in the pathogenesis of AP [25,27]. Several studies have demonstrated that Ca^{2+} is required for NF_{κ}B activation in PACs treated with cerulein or bile acids [27,32]. Consistent with these studies, we found that the Ca^{2+} chelator BAPTA-AM reduced cerulein-induced NF_{κ}B activation in PACs. In addition, TMEM16A inhibition by T16Ainh-A01 reduced the IP_{3}R-mediated Ca^{2+} release induced by cerulein, and BAPTA-AM treatment inhibited the TMEM16A overexpression-induced NF_{κ}B activation in PACs. Thus, TMEM16A overexpression activates NF_{κ}B by increasing Ca^{2+} levels. Furthermore, TMEM16A inhibition by shRNAs or T16Ainh-A01 reduced the cerulein-induced NF_{κ}B activation. Therefore, our findings suggest that TMEM16A may promote AP by increasing intracellular Ca^{2+} concentrations and subsequently activating NF_{κ}B in PACs.

TMEM16A is expressed in the ER–plasma membrane contact sites [21,22], where ion channels such as IP_{3}R are expressed and participate in the regulation of Ca^{2+} signaling [33]. TMEM16A directly binds to IP_{3}R and is activated by IP_{3}R-mediated Ca^{2+} release in the dorsal root ganglia and HeLa cells [21,22]. Furthermore, TMEM16A knockdown inhibits ATP-induced Ca^{2+} release in HeLa cells and human cystic fibrosis bronchial epithelial (CFBE)
However, the binding of TMEM16A to IP$_3$R is cell type-dependent, since TMEM16A does not co-immunoprecipitate with IP$_3$R in cerebral artery smooth muscles [23]. In addition, TMEM16A has been found to control EGF-induced Ca$^{2+}$ signaling in pancreatic cancer cells [17]. Here, we found that TMEM16A and IP$_3$R were co-immunoprecipitated in PACs. TMEM16A channels were activated by Ca$^{2+}$ release from IP$_3$R activation by cerulein. In turn, TMEM16A inhibition by T16Ainh-A01 reduced IP$_3$R-mediated Ca$^{2+}$ release. These results demonstrate that IP$_3$R-mediated Ca$^{2+}$ release can activate TMEM16A channels, and TMEM16A channels can promote IP$_3$R-mediated Ca$^{2+}$ release from the ER. Thus, our findings suggest that TMEM16A and IP$_3$R directly interact with and activate each other, thus forming a positive activation loop between TMEM16A and IP$_3$R.
Intracellular Ca\(^{2+}\) elevation subsequently activates NFXb signaling, resulting in an increase in IL-6 secretion from acinar cells. IL-6 further promotes TMEM16A expression via the IL-6/STAT3 signaling pathway. Therefore, a positive activation loop between TMEM16A and the IP3R/Ca\(^{2+}\)/NFXb/IL-6 pathway is important for Ca\(^{2+}\) elevation, NFXb activation and IL-6 release, and thus cooperatively promotes the pathogenesis of AP.

**Fig. 7.** The mechanisms by which TMEM16A promotes the pathogenesis of AP by activating the IP3R/Ca\(^{2+}\)/NFκB signaling pathways. TMEM16A is upregulated by IL-6 via the IL-6/STAT3 signaling pathway. Increased TMEM16A expression promotes intracellular Ca\(^{2+}\) release from the ER via direct interaction with IP3R. Intracellular Ca\(^{2+}\) elevation subsequently activates NFXb signaling, resulting in an increase in IL-6 secretion from acinar cells. IL-6 further promotes TMEM16A expression via the IL-6/STAT3 signaling pathway. Therefore, a positive activation loop between TMEM16A and the IP3R/Ca\(^{2+}\)/NFXb/IL-6 pathway is important for Ca\(^{2+}\) elevation, NFXb activation and IL-6 release, and thus cooperatively promotes the pathogenesis of AP.

Conclusions

Our study identified a novel mechanism by which TMEM16A channels are upregulated in PACs via IL-6/IL-6R/STAT3 signaling activation, and TMEM16A overexpression activates IP3R/Ca\(^{2+}\)/NFXb/IL-6 signaling in PACs (Fig. 7). The mutual activation between TMEM16A and IL-6 may be essential for persistent high TMEM16A expression, sustained Ca\(^{2+}\) elevation, and constitutive NFXb activation and IL-6 secretion in PACs, all of which are known to contribute to the pathogenesis of AP. Furthermore, TMEM16A inhibition by T16Ainh-A01 reduced the serum IL-6 levels and ameliorated pancreatic damage in AP mice. Therefore, our findings suggest that targeting TMEM16A may represent a novel therapy for AP. The identification of novel TMEM16A inhibitors may be promising for the treatment of AP. Consistent with this idea, some agents such as sikonin that inhibit TMEM16A [38] can ameliorate AP in mice [39].

**Compliance with ethics requirements**

All Institutional and National Guidelines for the care and use of animals (fisheries) were followed.

**Declaration of Competing Interest**

All authors declare no conflicts of interest.

**Acknowledgements**

This work was supported by grants from the National Natural Science Foundation of China (No. 81572613 and No. 31371145 to Qinghuan Xiao; No. 81702611 to Hui Wang), the Liaoning Pandeng Scholar (to Qinghuan Xiao), the Natural Science Foundation of Liaoning Province (No. 2019-MS-222 to Qinghua Wang), and the Natural Science Foundation of Liaoning Province for Guidance Program (No. 20180551125 to Lichuan Bai).

**Appendix A. Supplementary material**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2020.01.006.

**References**

[1] Goodchild G, Chouhan M, Johnson GJ. Practical guide to the management of acute pancreatitis. Frontline Gastroenterol 2019;10:292–9. doi: https://doi.org/10.1136/flgastro-2018-101102.

[2] Roberts SE, Akbari A, Thorne K, Akkinson M, Evans PA. The incidence of acute pancreatitis: impact of social deprivation, alcohol consumption, seasonal and demographic factors. Aliment Pharmacol Ther 2013;38:539–48. doi: https://doi.org/10.1111/apt.12408.

[3] Habezon A, Gukovskaya AS, Pandol SJ. Acute pancreatitis: a multifaceted set of organelle and cellular interactions. Gastroenterology 2019;156:1941–50. doi: https://doi.org/10.1053.j.gastro.2018.11.082.

[4] Oh U, Jung J. Cellular functions of TMEM16a/anoctamin. Pflugers Arch 2016;468:443–53. doi: https://doi.org/10.1007/s00424-016-1790-0.

[5] Dayal A, Ng SF, Grabner M. Ca(2+)-activated Cl(-) channel TMEM16A/ANO1 identified in zebrafish skeletal muscle is crucial for action potential acceleration. Nat Commun 2019;10:115. doi: https://doi.org/10.1038/s41467-018-07018-2.

[6] Ma MM, Gao M, Guo KM, Wang M, Li XY, Zeng XL, et al. TMEM16A contributes to endothelial dysfunction by facilitating Nox2 NADPH oxidase-derived reactive oxygen species generation in hypertension. Hypertension 2017;69:892–901. doi: https://doi.org/10.1161/HYPTENSIONAHA.116.08874.

[7] Wang H, Zou L, Ma K, Yu J, Wu H, Wei M, et al. Cell-specific mechanisms of TMEM16A Ca(2+)-activated chloride channel in cancer. Mol Cancer 2017;16:152. doi: https://doi.org/10.1186/s12953-017-07720-x.

[8] Kunzelmann K, Oussingsawat J, Cabrula D, Dousova T, Bahr A, Janda M, et al. TMEM16A in cystic fibrosis: activating or inhibiting? Front Pharmacol 2019;10:3. doi: https://doi.org/10.3389/fphar.2019.00003.
Suzuki S, Miyasaka K, Jimi A, Funakoshi A. Induction of acute pancreatitis by Ousingsawat J, Martins JR, Schreiber R, Rock JR, Harfe BD, Kunzelmann K. Loss Caputo A, Caci E, Ferrera L, Pedemonte N, Barsanti C, Sondo E, et al. TMEM16A, of TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity. Science 2008;322:590–4. doi: https://doi.org/10.1126/science.1163318.

Zhang Y, Wang X, Wang H, Jiao J, Li Y, Fan E, et al. TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity. J Allergy Clin Immunol 2015;7:367–75. doi: https://doi.org/10.1016/j.jaci.2015.7.3.307.

Yang YD, Cho H, Koo JY, Tak MH, Cho Y, Shim WS, et al. TMEM16A confers receptor-activated calcium-dependent chloride conductance. Nature 2008;455:1210–5. doi: https://doi.org/10.1038/nature07313.

Han Y, Shewan AM, Thorn P. HCO3-transport through Cl- channel. Am J Physiol Cell Physiol 2013;304:C673–84. doi: https://doi.org/10.1152/ajpcell.00196.2012.

Wang J, Haanes KA, Novak I. Purinergic regulation of CFTR and Ca(2+)-trisphosphate receptor-mediated Ca2+ release. Gut 2017;66:301–13. doi: https://doi.org/10.1136/gutjnl-2016-310500.

Wang Y, Kayoumu A, Lu G, Xu P, Qu X, Chen L, et al. Experimental models in syrian golden hamster replicate human acute pancreatitis. Sci Rep 2016;6:28014. doi: https://doi.org/10.1038/srep28014.

Manohar M, Verma AK, Venkateshaiah SU, Sanders NL, Mishra A. Pathogenic mechanisms of pancreatitis. World J Gastrointest Pharmacol Ther 2017;8:10–25. doi: https://doi.org/10.1016/j.wjgpt.2017.11.10.

Suzuki S, Miyasaka K, Jimi A, Funakoshi A. Induction of acute pancreatitis by cerulian in human IL-6 gene transgenic mice. Procands 2000:21:86–92.

Jakkampudi A, Jangala R, Reddy BR, Mittal S, Nageshwar Reddy D, Talukdar R. NF-kappaB in acute pancreatitis: Mechanisms and therapeutic potential. Pancreatology 2016;16:477–88. doi: https://doi.org/10.1016/j.pan.2016.05.001.

Xiao Q, Cui Y. Acidic amino acids in the first intracellular loop contribute to voltage- and calcium-dependent gating of anoctamin1/TMEM16A. PLoS ONE 2014;9:e99376. doi: https://doi.org/10.1371/journal.pone.0099376.

Qin Y, Yang J, Sheikh AS, Shen S, Liu J, Jiang D. Interleukin-13 stimulates MUC5AC expression via a STAT6-TMEM16A-ERK1/2 pathway in human airway epithelial cells. Int Immunopharmacol 2016;40:106–14. doi: https://doi.org/10.1016/j.intimp.2016.08.013.

Dutta AK, Khmiji AK, Kreishe B, Bugde A, Dougherty M, Eisser V, et al. Identification and functional characterization of TMEM16A, a Ca2+-activated Cl- channel activated by extracellular nucleotides, in biliary epithelium. J Biol Chem 2011;286:756–76. doi: https://doi.org/10.1074/jbc.M110.164970.

Wang H, Yao F, Luo S, Ma K, Liu M, Bai L, et al. A mutual activation loop between the Ca2+-activated chloride channel TMEM16A and EGFR/STAT3 signaling promotes breast cancer tumorigenesis. Cancer Lett 2019;455:48–59. doi: https://doi.org/10.1016/j.canlet.2019.04.027.

Mušić K, Jin S, Orabi AE, Eisses JF, Javed TA, Le T, et al. Pancreatic acinar cell nuclear factor kappaB activation because of bile acid exposure is dependent on calcineurin. J Biol Chem 2013;288:21065–73. doi: https://doi.org/10.1074/jbc.M113.471442.

Chung WY, Jha A, Ahuja M, Mualem S. Ca(2+)- influx at the ER/PM junctions. Cell Calcium 2017;63:29–32. doi: https://doi.org/10.1016/j.ceca.2017.02.009.

Benedetto R, Ousingsawat J, Wanitchakool P, Zhang Y, Holtzman MJ, Amaral M, et al. Epithelial chloride transport by CFTR requires TMEM16A. Sci Rep 2017;7:12397. doi: https://doi.org/10.1038/s41598-017-01210-z.

Wang H, Cane MC, Mukherjee R, Stainpery P, Zhang X, Elliott V, et al. Caffeine protects against experimental acute pancreatitis by inhibition of inositol 1,4,5-trisphosphate receptor-mediated Ca2+ release. Gastroenterology 2017;153:301–13. doi: https://doi.org/10.1016/j.gastro.2015.10.026.

Bill A, Hall ML, Borawski J, Hodgson C, Jenkins J, Pichon P, et al. Small molecule-facilitated degradation of ANO1 protein: a new targeting approach for anticancer therapeutics. J Biol Chem 2014;289:11029–41. doi: https://doi.org/10.1074/jbc.M114.549188.

Raskin L, Pezzutto JM, von Sch Cleary R, et al. TMEM16A/ANO1 molecule-facilitated degradation of ANO1 protein: a new targeting approach for anticancer therapeutics. J Biol Chem 2014;289:11029–41. doi: https://doi.org/10.1074/jbc.M114.549188.

Jin X, Shah S, Liu Y, Zhang H, Lees M, Fu Z, et al. Activation of the CI-channel ANO1 by localized calcium signals in nociceptive sensory neurons requires coupling with the IP3 receptor. Sci Signal 2013;6:ra73. doi: https://doi.org/10.1126/scisignal.2004184.

Cabrera I, Benedetto R, Fonseca A, Wanitchakool P, Sianian L, Skryabin BV, et al. Differential effects of ancatamins on intracellular calcium signals. FASEB J 2017;31:2123–34. doi: https://doi.org/10.1096/fj.201607978R.

Wang Q, Leo MD, Narayanad D, Kuruvella KP, Jaggar JH. Local coupling of TRPC6 to ANO1/TMEM16A channels in smooth muscle cells amplifies vasoconstriction in cerebral arteries. Am J Physiol Cell Physiol 2016;310:C1001–9. doi: https://doi.org/10.1152/ajpcell.00097.2016.

Wang Y, Kayoumu A, Lu G, Xu P, Qu X, Chen L, et al. Experimental models in syrian golden hamster replicate human acute pancreatitis. Sci Rep 2016;6:28014. doi: https://doi.org/10.1038/srep28014.

Manohar M, Verma AK, Venkateshaiah SU, Sanders NL, Mishra A. Pathogenic mechanisms of pancreatitis. World J Gastrointest Pharmacol Ther 2017;8:10–25. doi: https://doi.org/10.1016/j.wjgpt.2017.11.10.

Suzuki S, Miyasaka K, Jimi A, Funakoshi A. Induction of acute pancreatitis by cerulian in human IL-6 gene transgenic mice. Procands 2000:21:86–92.

Xiao Q, Cui Y. Acidic amino acids in the first intracellular loop contribute to voltage- and calcium-dependent gating of anoctamin1/TMEM16A. PLoS ONE 2014;9:e99376. doi: https://doi.org/10.1371/journal.pone.0099376.

Qin Y, Yang J, Sheikh AS, Shen S, Liu J, Jiang D. Interleukin-13 stimulates MUC5AC expression via a STAT6-TMEM16A-ERK1/2 pathway in human airway epithelial cells. Int Immunopharmacol 2016;40:106–14. doi: https://doi.org/10.1016/j.intimp.2016.08.013.

Dutta AK, Khmiji AK, Kreishe B, Bugde A, Dougherty M, Eisser V, et al. Identification and functional characterization of TMEM16A, a Ca2+-activated Cl- channel activated by extracellular nucleotides, in biliary epithelium. J Biol Chem 2011;286:756–76. doi: https://doi.org/10.1074/jbc.M110.164970.

Wang H, Yao F, Luo S, Ma K, Liu M, Bai L, et al. A mutual activation loop between the Ca2+-activated chloride channel TMEM16A and EGFR/STAT3 signaling promotes breast cancer tumorigenesis. Cancer Lett 2019;455:48–59. doi: https://doi.org/10.1016/j.canlet.2019.04.027.

Mušić K, Jin S, Orabi AE, Eisses JF, Javed TA, Le T, et al. Pancreatic acinar cell nuclear factor kappaB activation because of bile acid exposure is dependent on calcineurin. J Biol Chem 2013;288:21065–73. doi: https://doi.org/10.1074/jbc.M113.471442.