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Extracellular ATP and P2Y2 receptors mediate intercellular Ca²⁺ waves induced by mechanical stimulation in submandibular gland cells: role of mitochondrial regulation of store operated Ca²⁺ entry

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SUMMARY

Coordination of Ca²⁺ signaling among cells contributes to synchronization of salivary gland cell function. However, mechanisms that underlie this signaling remain elusive. Here, intercellular Ca²⁺ waves (ICW) in submandibular gland cells were investigated using fura-2 fluorescence imaging. Mechanical stimulation of single cells induced ICW propagation from the stimulated cells through ~7 layers of cells or ~120 μm. Our findings indicate that an extracellular ATP-dependent pathway is involved because the purinergic receptor antagonist suramin and the ATP hydrolyzing enzyme apyrase blocked ICW propagation. However, the gap junction uncoupler oleamide had no effect. ATP is released from mechanically stimulated cells possibly through opening of mechanosensitive maxi-anion channels, and does not appear to be directly linked to cytosolic Ca²⁺. The ICW is propagated by diffusing ATP, which activates purinergic receptors in neighboring cells. This purinergic signaling induces a Ca²⁺ transient that is dependent on Ca²⁺ release via IP₃ receptors in the ER and store operated Ca²⁺ entry (SOCE). Finally, inhibition of mitochondrial Ca²⁺ uptake modified ICW indicating an important role of these organelles in this phenomenon. These studies increase our understanding of purinergic receptor signaling in salivary gland cells, and its role as a coordination mechanism of Ca²⁺ signals induced by mechanical stimulation.

Keywords

Mechanical stimulation; purinergic receptor; Ca²⁺ wave; IP₃ receptor; store operated Ca²⁺ entry; maxi-anion channel; mitochondria

1. INTRODUCTION

Intracellular Ca²⁺ signaling has a central role in the regulation of salivary gland cell function [1]. Once increased in the cytosol, Ca²⁺ activates various ion channels and transporters such

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as the Ca^{2+} -activated K^+ or Cl^- channels, and Na^+ - K^+ - 2Cl^- transporters that are involved in saliva production and modification [1–3]. In a multi-cellular system, coordination of this Ca^{2+} signal between cells is important for synchronized and effective tissue function.

Intercellular Ca^{2+} wave (ICW) propagation has been reported in many different cell types such as respiratory tract epithelial [4], glial [5], aortic epithelial [6], liver epithelial [7], osteoblastic [8], and renin secreting juxtaglomerular [9] cells. Two main pathways are currently proposed as underlying mechanisms of ICW propagation. One pathway is extracellular while the other is mediated through gap junction intercellular communication (GJIC). Two apposing connexin hemichannels form gap junctions between cells, which allow direct intercellular communication through passage of small signaling molecules such as Ca^{2+} and inositol 1,4,5-triphosphate (IP_3) in the GJIC pathway. Previous studies in salivary gland cells indicate that GJIC is involved in the synchronization or propagation of Ca^{2+} signals. Muscarinic stimulation induced a synchronized Ca^{2+} signal among individual acinar cells that was disrupted by pretreatment with the gap junction uncoupler octanol in rat submandibular glands [10]. In blowfly salivary glands, IP_3 microinjection induced propagation of a Ca^{2+} wave through gap junctions [11].

ATP and purinergic (P_2) receptor dependent signaling form a common extracellular pathway that, like GJIC, also contributes to ICW propagation. Various stimuli like muscarinic receptor activation, mechanical stress, and hypoxic conditions cause a release of ATP via exocytosis or ion channels [12]. Two different subtypes of P_2 receptors are involved in extracellular ATP-dependent signaling. P_2X subtype receptors are Ca^{2+} permeable ion channels and the cytosolic Ca^{2+} increase upon P_2X receptors activation is dependent on extracellular Ca^{2+} . P_2Y subtype receptors are G-protein coupled receptors that activate phospholipase C (PLC) which generates IP_3 . This IP_3 induces a Ca^{2+} release via IP_3 receptors in the endoplasmic reticulum (ER). Depletion of the ER Ca^{2+} store by IP_3 receptor activation can further induce Ca^{2+} influx from the extracellular fluid through store operated Ca^{2+} channels (SOCC). Subtypes of both the P_2X (P_2X_4 , P_2X_7) and P_2Y (P_2Y_1 , P_2Y_2) receptors have been identified in different salivary gland cells [13].

Salivary glands experience repetitive mechanical stress during mastication. Myoepithelial cells, which contain myosin, contract to generate direct mechanical stimulation of salivary gland cells. Furthermore, mechanical stimulation, e.g. in chewing gum, has been suggested as an alternative treatment for xerostomia patients to increase saliva production [14,15]. However, the detailed mechanisms underlying this cell signaling induced by mechanical stimulation are not clearly understood in salivary gland cells.

Here, we investigated the mechanism of ICW induced by mechanical stimulation in a monolayer of human submandibular gland (HSG) cells and in freshly isolated submandibular gland tissues using fluorescence Ca^{2+} imaging. The results below demonstrate that the propagation of Ca^{2+} waves from the mechanically stimulated cells to the neighboring cells relies on extracellular ATP-dependent signaling. Pharmacological characterization revealed that P_2Y_2 subtype receptors are involved in ICW. Our data also indicate that mechanosensitive maxi-anion channels are likely candidates for the ATP-release pathway in mechanically stimulated cells. Both intracellular Ca^{2+} release from the ER and Ca^{2+} influx from the extracellular medium contribute to ICW. Finally, mitochondria were found to play an important role by actively regulating the Ca^{2+} mobilization pathway.

2. METHODS

2.1. Cell culture

HSG cells were grown in MEM (minimum essential medium Eagle, Mediatec Inc.) containing 2 mM glutamine and supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin. Cells were maintained at 37°C in a humidified 5% CO₂ incubator and passed twice a week.

2.2. Preparation of native submandibular gland cells

Native submandibular gland cells were freshly prepared as previously described [16,17] with some modifications. In brief, Sprague-Dawley rats were sedated by 100% CO₂ and decapitated using a guillotine. Submandibular glands were surgically removed from the ventral cervical area and finely minced in MEM supplemented with 20 mM HEPES. The minced tissues were digested for 20 min with a collagenase (100 Unit/mL CLS type II, Wathington Biochemical Co.). The remaining collagenase was washed out twice and followed by gentle agitations for further dissociation of the tissues. Animals were carefully handled and sacrificed in accordance with the guide lines of National Institutes of Health (NIH) and University Committee on Animal Resource in New York University.

2.3. Cytosolic Ca²⁺ measurements

HSG cells were seeded on 25 mm No.1 glass coverslips (Fisher Scientific) at least 1 day before the experiment. Coverslips were transferred to Rose chambers and washed with imaging buffer containing (in mM): 145 NaCl, 4.5 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 5 HEPES, 5.5 glucose, and pH 7.4 was adjusted with NaOH. 5 μM Fura-2 acetoxymethyl ester (Fura-2 AM, Invitrogen), a cell permeable fluorescence Ca²⁺ indicator, was loaded for 20 min at 37°C. 0.02% Pluronic F-127 (Invitrogen) was used to help dispersion of Fura-2 AM. After dye loading, the remaining Fura-2 AM in the extracellular medium was removed by washing twice with the same imaging buffer and incubated another 20 min. The cell chamber with its custom-built regulated heating block was mounted on an inverted microscope (Eclipse TE2000-E, Nikon). The freshly isolated submandibular gland cells were preloaded with 5 μM Fura-2 AM and kept on ice. After transferring the native tissues to the same cell chamber, cells were equilibrated in the imaging buffer for 30 min before starting experiments. Cells were observed with a 20x objective (S fluor, numerical aperture, 0.75; Nikon) and fluorescence micrographic images were captured at 510 nm using a digital CCD camera (CoolSNAP HQ2, Photometrics) and an imaging software (NIS-Element AR 3.0, Nikon) after alternating excitations at 340 and 380 nm. Fura-2 ratio ($F_{\text{ratio}} = 340/380$) images were displayed and the F_{ratio} values from the regions of interest (ROIs) drawn on individual cells were monitored during the experiments and analyzed later offline with NIS-Element AR 3.0 software. The ICW propagation was measured by counting cell layers that showed peak amplitudes at least 0.1 F_{ratio} values increase (ΔF_{ratio}). To minimize UV light exposure, 4×4 binning function was used. All experiments were performed at 37±1°C.

2.5. Mitochondrial Ca²⁺ measurements

Cells were loaded with 5 μM Rhod-2AM (Invitrogen) for 20 min at room temperature, washed with imaging buffer, and incubated another 20 min at 37°C. For the co-localization study, mitochondria were visualized by transiently expressing the mitochondria matrix targeted pAc-GFP1 (mtGFP, a generous gift of E. Pavlov of University of Calgary) using a modified polyethylenimine (PEI, Sigma) transfection method originally described by Boussif et al [18]. Briefly, 2 μg of plasmid were mixed with 10 μL of PEI stock (1 g/L in distilled water, pH 7.4) in 500 μL culture medium without FBS and incubated for 15 min at room temperature to polymerize PEI/DNA. Cells were incubated with this mixture for 2 hours at 37°C and washed

with normal culture medium containing FBS. After 24 hours, mtGFP expression was checked on a fluorescence microscope. Both Rhod-2 and mtGFP fluorescence images were deconvoluted using a built-in deconvolution module in NIS-Element AR 3.0 software.

2.6. Mechanical stimulation

Micropipettes of ~1 μm diameter were fabricated using a preconfigured program in a horizontal pipette puller (Model P-87, Sutter Instrument Co.). The tips of micropipettes were sealed and polished with a microforge (MF-83, Narishige). These micropipettes were used to briefly touch the top of target cells in a monolayer of HSG cells using a micromanipulator (MO-303, Narishige) to provide the initial mechanical stimulation and initiate the ICW. The freshly isolated submandibular gland cells were mechanically stimulated with a side wall of the micropipettes to avoid rotation and movement of tissues.

2.7. Microinjection

HSG cells were microinjected with 10 mM Lucifer Yellow CH lithium salt (Invitrogen) using a micropipette connected to a pressure microinjector (IM-30, Narishige). Dye coupling was confirmed up to 30 min after microinjection.

2.8. Pharmacological agents

ATP, UTP, suramin, apyrase, SK&F96365, thapsigargin, 2-aminoethoxydiphenyl borate (2-APB), *p*-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP), oligomycin, GdCl_3 , LaCl_3 , 5-nitro-2-(phenylpropylamino)-benzoate (NPPB), carbenoxolone, glibenclamide, verapamil, and carbamylcholine chloride (carbachol) were purchased from Sigma. U73122 and its inactive analog U73343 were purchased from Biomol International. Brilliant Blue G was purchased from Alfa Aesar. 2-[(4-Fluorobenzoyl)amino]-benzoic acid methyl ester (Exo1) was purchased from Tocris Bioscience. Concentrated stock solutions were prepared in either distilled water or dimethyl sulfoxide (DMSO) and kept at -20°C . The concentration of DMSO in the final solution was less than 0.1%. DMSO alone tested up to 0.5% had no effect on ICW or ATP-induced Ca^{2+} responses.

2.9. Statistical analysis

Data were presented as mean $\pm$ S.D. and number of cells tested (n). For statistical analysis, independent two-sample Student's *t*-test were applied with an equal variance assumption to calculate p-values using an Origin 8.0 software (OriginLab Corp.). Two independent groups were considered statistically different if p-values <0.05 .

3. RESULTS

3.1. Mechanical stimulation induces intercellular Ca^{2+} wave propagation in HSG cells

A monolayer of HSG cells was used to mimic the microenvironment of a salivary gland during mastication and investigate the intercellular Ca^{2+} wave (ICW) induced by mechanical stimulation. The cell clusters were 300–400 μm in diameter and contained 150–300 cells. Focal mechanical stimulation of single cells in the center of cell clusters was achieved by gently touching the target cell for less than 1 sec with a sealed micropipette. The induced ICW was monitored by Fura-2 fluorescence ratio imaging. At the time of focal mechanical stimulation, there was an initial small increase in cytosolic Ca^{2+} concentration in the target cell. The amplitude of this Ca^{2+} signal reached a peak value 2–3 sec after mechanical stimulation. Without perfusion of the extracellular medium, this Ca^{2+} signal propagated from the target cell to the surrounding 7.2 ± 2.2 layers of cells ($\sim 120 \mu\text{m}$, $n=21$) with a speed of $\sim 15 \mu\text{m}/\text{sec}$ in all directions (Fig. 1A and a movie in Suppl. Fig. 1). Therefore, about 200 cells showed

synchronous Ca^{2+} signals in less than 10 seconds by focal mechanical stimulation of a single cell. The Ca^{2+} waves lasted up to ~2 minutes before returning to resting cytosolic Ca^{2+} level.

The ICW were propagated through an extracellular pathway. This Ca^{2+} wave was propagated to neighboring cells that did not have direct physical contact (Fig. 1B). Interestingly, the direction of Ca^{2+} wave propagation was affected by the flow of extracellular medium. With a flow rate of ~6mL/min, the Ca^{2+} wave was propagated more than 200 μm in the direction of perfusion whereas propagation against the flow was limited to less than 20 μm (Fig. 1C). In order to evaluate any contribution of GJIC pathway to propagation, cells were pretreated with a gap junction uncoupler oleamide (100 μM); this treatment had no effect on ICW (Fig. 1D). Furthermore, HSG cells showed poor dye coupling between cells when microinjected with Lucifer yellow (Suppl. Fig. 2). These results indicate that an extracellular pathway, but not GJIC, is used to propagate ICW induced by focal mechanical stimulation in HSG cells.

The possible involvement of signaling through P2 receptors was then examined to further characterize this extracellular pathway. Addition of the P2 receptor inhibitor suramin (Fig. 1E) or the ATP hydrolyzing enzyme apyrase (Fig. 1F) significantly decreased the ICW propagation induced by mechanical stimulation to 2.5 ± 1.1 ($n=10$, $p<0.001$) and 1.2 ± 0.9 ($n=10$, $p<0.001$) layers of cells, respectively. In summary, these findings indicate that ATP and P2 receptors are involved in the propagation of the ICW through an extracellular pathway.

3.2. P2Y2 receptor subtype is responsible for ICW propagation

Once the involvement of an extracellular ATP-dependent pathway was revealed, further pharmacological characterization was performed to identify the subtype of the P2 receptors involved in ICW induced by mechanical stimulation. Phospholipase C (PLC) and IP_3 dependent signaling are activated by metabotropic P2Y receptors, but not by ionotropic P2X receptors. Pretreatment with 10 μM U73122, a PLC inhibitor, completely abolished ICW induced by mechanical stimulation (Fig. 2B). However, its inactive analog U73343 had no effect on ICW (Fig. 2B). This finding suggests that the effect of U73122 is not due to nonspecific effects but is related to PLC inhibition. These findings indicate that P2Y receptors are involved.

The affinity for purines and pyrimidines was used to identify the P2 receptor subtype responsible for these ICW. Addition of ATP to the extracellular solution induced dose-dependent Ca^{2+} increases with a half effective concentration (EC_{50}) of 1.3 ± 0.05 μM and maximal responses at ~10 μM ATP (Fig. 2A). The Ca^{2+} responses were not sustained at >100 μM ATP, but decreased with time. Once the cytosolic Ca^{2+} concentration returned to basal level still in the presence of 100 μM ATP, further addition of up to 500 μM ATP failed to induce subsequent Ca^{2+} responses. UTP activates only P2Y2 and P2Y4 receptors [13]. P2Y2 has equal potency and efficacy with UTP and ATP, whereas P2Y4 is preferentially activated by UTP [13]. Addition of UTP induced dose-dependent Ca^{2+} responses similar to ATP (Fig. 2A). The EC_{50} for UTP was 0.91 ± 0.06 μM and maximal responses were at ~10 μM UTP. Higher concentrations of UTP also showed the same desensitization of the Ca^{2+} response as ATP. After desensitization by 200 μM UTP, mechanical stimulation did not induce further ICW (Fig. 2B). Previously, benzoyl (Bz)ATP sensitive P2z, or P2X7, receptors as well as UTP sensitive P2u, or P2Y2, receptors were identified in HSG cells [19]. However, a specific inhibitor P2X7 receptor, brilliant blue G, had no effect on ICW induced by mechanical stimulation (Fig. 3B), suggesting that P2X7 receptors are not involved. Taken together, these results indicate that UTP-sensitive and PLC-linked metabotropic P2 receptors, likely P2Y2, are involved in propagation of ICW induced by mechanical stimulation.

3.3. Mechanism of ATP release from the target cells

What is the signal that triggers ATP release from the mechanically stimulated cells? Previous reports showed that mechanical stimulation or cell swelling induces ATP release from different cell types [9,20,21]. Mechanosensitive channels such as maxi-anion channels, connexin hemichannels, pannexin1 channels, P2X7 receptors, and anion transporters were proposed as ATP-release mechanisms. Thus, we tested whether the inhibitors of these pathways affect the mechanical stimulation induced ICW in our model. Among various inhibitors, we found that Gd^{3+} and a broad spectrum anion channel inhibitor NPPB effectively inhibited ICW (Fig. 3A and 3B). Since Gd^{3+} and NPPB are also known to block connexin hemichannels and pannexin1 channels as well as maxi-anion channels, the effect of carbenoxolone, which blocks both connexin hemichannels and pannexin1 channels, was tested. However, carbenoxolone did not inhibit ICW (Fig. 3B), suggesting that the effect of Gd^{3+} is not due to the inhibition of connexin hemichannels or pannexin1 channels. Furthermore, inhibitor of P2X7 receptors brilliant blue G, cystic fibrosis transmembrane regulator (CFTR) regulated Cl^- channel inhibitor glibenclamide, multidrug resistant (MDR1) P-glycoprotein regulated Cl^- channel inhibitor verapamil, and inhibitor of exocytosis exo1 had no effect on ICW (Fig. 3B). These data suggest that mechanical stimulation itself may be the signal for ATP release from the target cells, and mechanosensitive maxi-anion channels are good candidates to provide a route for ATP release in our model.

We further examined whether the Ca^{2+} increase causes a secondary ATP release in the neighboring cells during ICW propagation. Carbachol is known to activate M3 muscarinic receptors and increase IP_3 and cytosolic Ca^{2+} in salivary gland cells [1]. Thus, we examined whether suramin, a P2 receptor inhibitor, affected the dose response of carbachol-induced Ca^{2+} responses. If carbachol-induced Ca^{2+} increase causes ATP release, then, it was expected that the P2 receptor would have some contribution to the calcium increase. However, the carbachol induced Ca^{2+} responses were not affected by 200 μM suramin (Suppl. Fig. 3). These data suggest that Ca^{2+} may not be the direct triggering signal for ATP release.

3.4. Ca^{2+} mobilization mechanism during the ICW

Activation of P2Y2 receptors induces Ca^{2+} release via IP_3 receptors in the ER. Thus, it was expected that this ICW still could be induced by mechanical stimulation without Ca^{2+} influx from the extracellular medium. As expected, mechanical stimulation still induced ICW in the absence of extracellular Ca^{2+} (no added Ca^{2+} plus 1 mM EGTA), although the peak amplitudes of these waves were decreased (Fig. 4A). The effect of the store operated Ca^{2+} channel (SOCC) inhibitor, SK&F96365, on ICW was determined to further evaluate the Ca^{2+} influx pathway. In the presence of normal extracellular Ca^{2+} , SK&F96365 (30 μM) also decreased the peak amplitude of the Ca^{2+} wave without inhibiting ICW (Fig. 4B). These findings indicate that intracellular Ca^{2+} release alone is enough to propagate ICW. However, Ca^{2+} influx through SOCC may also be involved in the regulation of the amplitude of the ICW.

In order to determine the contribution of the ER calcium reservoir to ICW, the effect of pretreatment with thapsigargin was determined. Thapsigargin is an ER Ca^{2+} pump inhibitor that effectively empties the ER store of Ca^{2+} . The intracellular Ca^{2+} release after removal of extracellular Ca^{2+} is dependent on the ER Ca^{2+} store because thapsigargin (2 μM) completely abolished the ICW induced by mechanical stimulation (Fig. 4C). Consistent with the effect of the PLC inhibitor U73122 (Fig. 2B), pretreatment with an IP_3 receptor inhibitor 2-aminoethoxydiphenyl borate (2-APB, 50 μM) in the absence of extracellular Ca^{2+} also completely abolished ICW (Fig. 4D). The involvement of IP_3 receptors and SOCC in this Ca^{2+} mobilization further supports a mechanism reliant on P2Y2 receptor signaling during ICW.

Unlike the Ca^{2+} signals in the neighboring cells, the Ca^{2+} signal in the target cells was not completely inhibited by suramin and apyrase (Fig. 1B and 1C). These findings suggest that there might be some additional Ca^{2+} mobilization mechanisms in the target cells other than autocrine activation of its own P2 receptors. Removal of extracellular Ca^{2+} markedly decreased the Ca^{2+} signal in the target cells (Fig. 4), indicating that a Ca^{2+} influx pathway is activated by mechanical stimulation. Thus, we examined the effect of 500 μM Gd^{3+} , an inhibitor of mechanosensitive cation channels, on the Ca^{2+} signal in the target cells. Above, we showed that 30 μM Gd^{3+} effectively inhibited the ICW propagation, but did not inhibit the Ca^{2+} increase in the target cells (Fig. 3A). However, 500 μM Gd^{3+} significantly decreased the Ca^{2+} signal in the target cells (Fig. 4E). These results indicate that both autocrine activation of P2Y2 and mechanosensitive cation channels contributed to the Ca^{2+} signal in the target cells.

3.5. Role of mitochondria in the regulation of ICW

Mitochondria buffer intracellular Ca^{2+} [22], which precipitated our investigation of the role of mitochondrial Ca^{2+} uptake in ICW. HSG cells were loaded with a mitochondrial Ca^{2+} indicator Rhod-2AM in order to determine if mechanical stimulation induces these organelles to take up Ca^{2+} . Mechanical stimulation induced an increase of Rhod-2 fluorescence signal in a punctuate pattern around nucleus (Fig. 5A). This pattern perfectly co-localized with mitochondria targeted GFP (mtGFP) fluorescence (Fig. 5A). This mitochondrial Ca^{2+} uptake during ICW propagation was completely inhibited by treatment with the uncoupler FCCP (2.5 μM) and ATP synthase inhibitor oligomycin (2.5 $\mu\text{g}/\text{mL}$, not shown). Next, the impact of eliminating mitochondrial Ca^{2+} buffering with FCCP and oligomycin on the ICW was determined. The peak amplitude of the ICW induced by mechanical stimulation and the Ca^{2+} response to extracellular ATP additions were both significantly reduced when mitochondrial Ca^{2+} uptake was inhibited (Fig. 5B and C). However, oligomycin alone did not affect these Ca^{2+} waves or responses (not shown). These results indicate mitochondria play an important role in regulation of these Ca^{2+} transients.

Inhibition of mitochondrial Ca^{2+} uptake modified store operated calcium entry (SOCE) but not IP_3 -dependent release of intracellular Ca^{2+} from the ER. In the absence of extracellular Ca^{2+} (no added Ca^{2+} plus 1 mM EGTA), IP_3 -dependent Ca^{2+} release was induced by the addition of 10 μM ATP to the medium. The peak amplitude of this intracellular Ca^{2+} release was not affected by the inhibition of mitochondrial Ca^{2+} uptake (Fig. 6). However, both peak amplitude and rate of SOCE after re-introduction of normal extracellular Ca^{2+} were significantly decreased (Fig. 6C). These results indicate that mitochondria actively regulate SOCE, which affects repetitive Ca^{2+} responses elicited by mechanical stimulation and ATP additions.

3.6. ICW in freshly isolated submandibular gland cells

Finally, we examined the possibility that the P2Y2 receptor dependent ICW can be induced by mechanical stimulation in freshly isolated rat submandibular gland ductal cells. Previously, the existence of P2Y2, or P2u, receptors was reported in native submandibular gland cells [16,17,23]. Thus, we first checked whether P2Y2 receptor agonist UTP can induce any Ca^{2+} signaling in freshly isolated rat submandibular gland cells. Similar to previous reports, a small increase of cytosolic Ca^{2+} was induced by 100 μM UTP (Fig. 7A), which was ~ 30% of the amplitude observed in HSG cells. Furthermore, mechanical stimulation also induced ICW in native rat submandibular gland cells (Fig. 7B). These results suggest that UTP sensitive P2u, or P2Y2, receptors can generate ICW induced by mechanical stimulation in native submandibular gland tissues as well as the HSG cell line.

4. DISCUSSION

In the present study, the mechanism of intercellular Ca^{2+} wave (ICW) propagation induced by mechanical stimulation in HSG cells and freshly isolated submandibular gland cells was investigated (proposed model shown in Fig. 8). Extracellular ATP and P2Y2 receptor dependent signaling is central to the resulting Ca^{2+} wave propagation (Fig. 1 and Fig. 2). Our data suggest that a mechanosensitive maxi anion channel is a good candidate for the ATP release mechanism (Fig. 3). We found that both IP_3 -dependent Ca^{2+} release from the ER and SOCE contribute to the cytosolic Ca^{2+} signal during ICW propagation (Fig. 4). Interestingly, mitochondria are actively involved in the regulation of SOCE, which contributes to fast store refilling during repetitive mechanical or P2 receptor stimulation (Fig. 5 and Fig. 6).

4.1. P2 receptors in salivary gland cells

The role of extracellular ATP and P2 receptor signaling in salivary glands is not completely understood. It seems that this signaling is involved in production and modification of saliva by modulating various ion transporters through cytosolic Ca^{2+} or protein kinase C (PKC) dependent signaling [12,13,17]. In our experimental model, we found that ATP and P2Y2 receptors can mediate the propagation of ICW induced by mechanical stimulation. However, extracellular ATP-dependent signaling will be more complex in vivo because multiple subtypes of P2 receptors such as P2Y1, P2Y2, P2X4, P2X7 have been reported in different salivary gland cells [13]. P2Y2 and P2X7 receptors were previously described by others in HSG cells [18]. P2Y2 receptors are known to be activated by low micromolar range of ATP, whereas P2X7 receptors are activated by a higher (>1 mM) concentration of ATP in the presence of normal extracellular Mg^{2+} [18]. High affinity receptors such as P2Y2 and P2X4 are probably required for this intercellular communication because of the high activity of ectonucleotidases at the cell surface and in saliva under in vivo conditions [12]. However, depending on the amount of ATP released, other lower affinity P2 receptors may also be involved in vivo. Recently, it was reported that P2X7 receptors are involved in nucleotide induced fluid secretion in mouse submandibular gland [24]. However, excessive amounts of ATP in the extracellular medium are also known to cause apoptosis or necrosis by forming a large pore constituted by this low affinity P2 receptor [13].

In native rat submandibular gland cells, specific localizations of P2 receptor subtypes were identified. UTP sensitive P2u or P2Y2, receptors exist on the basolateral side whereas BzATP sensitive P2z or P2X7, receptors exist on the luminal side of acinar and ductal cells [16,17]. The amplitude of Ca^{2+} responses and Cl^- currents after P2u receptor stimulation was $\sim 1/3$ of P2z receptor stimulation, suggesting that a significant level of P2u receptors are expressed in native tissues [16]. Consistent with these observations, $100\text{ }\mu\text{M}$ UTP induced a small increase in cytosolic Ca^{2+} (Fig. 7A). Mechanical stimulation also induced ICW in freshly isolated rat submandibular gland cells (Fig. 7B). However, the Ca^{2+} response and the propagation of ICW were much smaller than that observed in HSG cells, which is possibly due to a lower expression level of P2Y2. In contrast, mechanical stimulation in submandibular gland cells from mouse did not elicit ICW. The differences in Ca^{2+} responses between rat and mouse may be due to different P2Y2 expression level, isolation procedures, or their sensitivity to mechanical stimulation. Interestingly, the expression level of P2Y2 receptors in acinar and intercalated ductal cells of salivary glands are up-regulated during tissue injury [25] and in a mouse model of Sjögren's syndrome [26]. Thus, P2Y2 based ICW may play a larger role under these pathophysiological conditions.

P2Y2 receptor up-regulation is apparently part of a cell survival mechanism. P2Y2 receptor activation induces ERK, and $\text{PI}_3\text{K}/\text{Akt}$ dependent cell survival signaling [27]. Ca^{2+} signaling induced by muscarinic receptor activation is the major stimulus for saliva secretion and is significantly decreased in certain diseases like Sjögren's syndrome [24,25]. Hence, P2Y2

receptors may provide an alternative target to alleviate complications due to dry mouth or xerostomia while helping cells to survive under these pathological conditions. However, the experiments in these pathological conditions are beyond the scope of present study.

4.2. Gap junction intercellular communication

In our experimental model, unlike previous reports [10,11], gap junction intercellular communication (GJIC) does not contribute to the propagation of ICW. However, there are still possibilities that gap junctions as well as P2 receptors are involved in synchronization or coupling of local Ca^{2+} signaling under in vivo conditions. Connexin 32 and 43 were previously identified in acinar and myoepithelial cells [28]. However, connexin hemi-channels, which did not form gap junctions, can also contribute to extracellular ATP and P2 receptor signaling by providing an ATP-release pathway [29]. Additionally, the gap junction inhibitors such as octanol, heptanol, and glycyrrhetinic acid, which are often used to prove the involvement of GJIC, are not specific and also may directly interfere with intracellular Ca^{2+} signaling [30]. In the present study, oleamide, a more specific inhibitor of gap junctions than other inhibitors, did not affect ICW (Fig. 1D) and untreated cells did not spread Lucifer yellow staining to neighboring cells (Suppl. Fig. 2).

4.3. ATP release mechanism

Mechanosensitive maxi-anion channels are possible candidates for the ATP release mechanism in our model (Fig. 8). The activity of maxi-anion channels was previously recorded in wide variety of cell types including cardiomyocytes [31], macular densa cells [21], astrocytes [20], and mammary fibroblastic C127 cells. However, the molecular identity of maxi anion channel is not yet clearly known [32]. A voltage dependent anion-selective channel (VDAC) resembling mitochondrial VDAC was considered as a candidate for maxi-anion channel because maxi-anion channels have biophysical properties similar to mitochondrial VDAC. However, a recent knockout study indicates that none of the mitochondrial VDAC subtypes are responsible for the maxi-anion channel activity in the plasma membrane [33]. The pore-sizing study using a non-electrolyte exclusion method indicates that the pore diameter of the maxi-anion channel (~2.6 nm) is large enough for the passage of ATP, whose diameter is ~1.2 nm [34].

Several other pathways were also proposed for ATP release mechanisms in other cell types which include connexin hemichannel, pannexin1 channel, P2X7 receptor, CFTR regulated Cl^- channel, p-glycoprotein regulated Cl^- channel, and exocytosis. Among these pathways, connexin hemichannel, pannexin1 channel, P2X7 receptor as well as the maxi-anion channel are known to be sensitive to mechanical stimulation or swelling. Gd^{3+} and some anion channel inhibitors such as NPPB, DIDS, and SITS, which inhibit maxi-anion channel, effectively block ATP release induced by cell swelling [20,35]. Even if these anion channel inhibitors also inhibit connexin hemichannel, pannexin1 channel, and P2X7 receptors, the absence of an effect of carbenoxolone, an inhibitor of both connexin and pannexin1 hemichannels, or brilliant blue G, a P2X7 receptor inhibitor on ICW, suggests that the maxi-anion channel, rather than connexin hemichannels, pannexin1 or P2X7 receptors, is a candidate for ATP release mechanism in our model (Fig. 3). Vesicular pathway was also examined (Fig. 3B), but its' role seems to be minor at least in ductal cells. We hope more specific inhibitors or molecular approaches become available for maxi-anion channels in the near future.

4.4. Ca^{2+} mobilization mechanism

The cytosolic Ca^{2+} increase upon mechanical stimulation in the target cells is contributed by at least two different mechanisms. One is possibly through direct activation of Ca^{2+} permeable mechanosensitive nonselective cation channels [36] and the other is an autocrine activation of its own P2Y2 receptor secondary to ATP release. We showed that Gd^{3+} decreased the Ca^{2+}

signal induced by mechanical stimulation in the target cells at 500 μM (Fig. 4E), whereas 30 μM was enough to effectively inhibit ICW (Fig. 3A). We speculate that the binding affinity of Gd^{3+} to mechanosensitive cation channels and maxi-anion channels are different; the maxi-anion channels may have high affinity Gd^{3+} binding sites.

For subsequent ICW in the surrounding cells, both IP_3 -dependent Ca^{2+} release from the ER and SOCE contribute to cytosolic Ca^{2+} increase after P2Y_2 receptor activation (Fig. 4). Since the ER Ca^{2+} store is limited, further Ca^{2+} influx from the extracellular medium is necessary for a more sustained or repetitive cytosolic Ca^{2+} signals. ER-store depletion is known to activate Ca^{2+} release activated Ca^{2+} channel (CRAC)/or SOCC in the plasma membrane. Recently, it was found that the specific interaction between the ER Ca^{2+} sensor stromal interaction molecule 1 (STIM1) and SOCC (ORAI1/TRPC1 channels) underlies the activation of SOCE [37,38]. The contribution of extracellular Ca^{2+} influx and intracellular Ca^{2+} release to cytosolic Ca^{2+} signaling will vary depending on which P2 receptor signaling pathway underlies the ICW in different cell types.

4.5. Role of mitochondria

Mitochondria, located next to ER and plasma membrane, regulate local Ca^{2+} concentrations as well as metabolic state. IP_3 receptors and SOCC are known to be regulated by local Ca^{2+} and ATP concentration [39]. Mitochondrial regulation of these Ca^{2+} mobilization pathways seems to be cell type specific. In rat basophilic leukemia cells, inhibition of mitochondrial Ca^{2+} uptake specifically caused a decrease in SOCE without affecting IP_3 -dependent Ca^{2+} release [40]. However, in smooth muscle cells, Ca^{2+} release from IP_3 receptor is decreased by mitochondrial depolarization [41]. Here, we found that inhibition of mitochondrial Ca^{2+} uptake caused a decrease in the amplitude of ICW induced by repetitive mechanical stimulations (Fig. 5B). This decrease of Ca^{2+} responses in the FCCP/Oligomycin treated group is not simply due to a decrease in ATP release but may be due to the regulation of Ca^{2+} mobilization pathway by mitochondria. Importantly, additions of the same amount of extracellular ATP still showed decreased Ca^{2+} responses in FCCP/Oligomycin treated group. Furthermore, inhibition of mitochondrial Ca^{2+} uptake specifically decreased and slowed SOCE without affecting Ca^{2+} release from IP_3 receptor in HSG cells (Fig. 6). Since oligomycin alone did not affect the repetitive Ca^{2+} signaling, mitochondrial regulation of SOCC is probably due to changes in the local concentration of Ca^{2+} rather than ATP. We speculate that the local Ca^{2+} concentration just next to SOCC is dynamically regulated by mitochondrial Ca^{2+} uptake, which in turn regulates Ca^{2+} dependent inactivation of SOCC (Fig. 8) [39]. It is noteworthy that the basal cytosolic Ca^{2+} levels after mechanical stimulation or ATP addition remained slightly elevated in a FCCP/oligomycin treated group compared to that of control (Fig. 5B). This residual cytosolic Ca^{2+} might also help to keep SOCC inactivated and hence inhibit ER store refilling in between mechanical or P2 receptor stimulations.

5. CONCLUSIONS

The present study enhances our understanding about how the extracellular ATP and P2Y_2 receptor dependent signaling pathway mediates intercellular Ca^{2+} signaling, which synchronizes salivary gland cell function. Mitochondria were found to have a specific role in regulating the Ca^{2+} mobilization mechanism in salivary gland cells. Finally, this information identifies P2Y_2 receptors as an alternative therapeutic target to alleviate dry mouth symptoms in many different oral pathologies related to salivary hypo-function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The intercellular Ca^{2+} waves (ICW) induced by mechanical stimulation are dependent on extracellular ATP. Fura-2 fluorescence ratio ($F_{\text{ratio}} 340/380$) images are shown before (-1 sec), at the time of (0 sec), and 5 and 10 seconds after the focal mechanical stimulation of cells indicated by asterisks (*). Scale bar in A is $100 \mu\text{m}$ for all images. Cells were pretreated with pharmacological agents for 30 min before mechanical stimulation. The ICW induced by mechanical stimulation was propagated in **A.** within a cluster of cells and **B.** between cell clusters with no direct physical contact as indicated by arrows at 10 seconds. **C.** Perfusion of the extracellular medium, (flow rate at $\sim 6 \text{ ml/min}$), modifies the ICW. The direction of perfusion is indicated by an arrow at 10 second. The ICW were monitored after pretreatment with **D.** $100 \mu\text{M}$ oleamide, a gap junction blocker, **E.** $200 \mu\text{M}$ suramin, a P2 receptor inhibitor, or **F.** 100 unit apyrase, an ATP hydrolyzing enzyme, as indicated.

Figure 2.

The purinergic receptor subtype designated P2Y2 is involved in propagation of this ICW. The P2Y2 receptor subtype is activated by ATP and UTP and linked to phospholipase C (PLC) activity. **A.** Typical Fura-2 fluorescence ratio (ΔF_{ratio}) traces (left) show the dose dependence of the Ca^{2+} transients induced by 1 μM to 100 μM ATP (top) and UTP (bottom). Right side shows the corresponding dependence of the peak amplitude of the Fura-2 fluorescence ratio (ΔF_{ratio}) as a function of ATP and UTP concentration as indicated. The EC_{50} values were $1.30 \pm 0.05 \mu\text{M}$ ATP and $0.91 \pm 0.06 \mu\text{M}$ UTP. EC_{50} values were obtained from the best fit using Hill's equation, $y = V_{\text{max}} * x^n / (k^n + x^n)$, where V_{max} is maximal response, x is the concentration of agonist, k is EC_{50} , and n is Hill's coefficient. **B.** Fura-2 fluorescence ratio ($F_{\text{ratio}} 340/380$) images illustrate the effects on ICW induced by mechanical stimulation of P2Y2 desensitization with 500 μM UTP, inhibition of PLC by U73122, and negative control with its inactive analog U73343. Other conditions as in Figure 1.

Figure 3.

Effects of various inhibitors for ATP release mechanisms. **A.** Fura-2 ratio images show the effect of Gd^{3+} (30 μM), a maxi-anion channel inhibitor, and NPPB (100 μM), a universal anion channel inhibitor on ICW propagation. Addition of 10 μM ATP at the end of Gd^{3+} experiment shows Gd^{3+} did not inhibit the ATP-induced Ca^{2+} response. **B.** Bar graph shows the number of cell layers to which the ICW propagated in each condition. Gd^{3+} (30 μM) and NPPB (100 μM) decreased ICW propagation to 0.4 ± 0.52 ($n=10$) and 2.8 ± 1.48 ($n=10$) cell layers, respectively. The following agents did not affect ICW propagation: gap junction hemichannel and Pannexin1 channel inhibitor carbenoxolone (100 μM), P2X7 receptor inhibitor brilliant blue G (1 μM), cystic fibrosis transmembrane regulator (CFTR) regulated Cl^- channel inhibitor

glibenclamide (200 μ M), multidrug resistant (MDR1) p-glycoprotein inhibitor verapamil (20 μ M), and exocytosis inhibitor exo1 (100 μ M). *** indicates $p < 0.001$. Other conditions as in Figure 1.

Figure 4. The Ca^{2+} mobilization mechanisms during ICW include IP_3 -dependent Ca^{2+} release from the ER and SOCE. Fura-2 ratio images show the effect of **A** removing extracellular Ca^{2+} , **B** pretreatment with 30 μM SK&F96365 (store operated Ca^{2+} entry inhibitor), **C** removal of extracellular Ca^{2+} and 2 μM thapsigargin (SERCA pump inhibitor), and **D** addition of 50 μM 2-APB (IP_3 receptor inhibitor) on ICW propagation induced by mechanical stimulation. **E**. Effect of 500 μM Gd^{3+} , a mechanosensitive channel inhibitor, on target cells. Other conditions as in Figure 1.

Figure 5.

Mitochondria modulate Ca^{2+} transients induced by mechanical stimulation or ATP additions. **A.** Left two images show mitochondrial Ca^{2+} levels of HSG cells loaded with Rhod-2AM. These cells were transiently transfected with pAc-GFP1 (mtGFP) which encodes a GFP protein targeted to mitochondria. Mechanical stimulation (MS) increased rhod-2 fluorescence (red), which co-localized with mtGFP (green) and appears yellow in the merged image on the right. Scale bar is 20 μm for all images. **B.** Fura-2 ratio traces show Ca^{2+} waves induced by MS in cells that were not (control) and were treated with 2.5 μM of FCCP and 2.5 $\mu\text{g/ml}$ oligomycin. **C.** Fura-2 ratio traces show Ca^{2+} responses induced by repetitive additions of 10 μM ATP without (control) and with FCCP/Oligomycin treatment. Other conditions as in Figure 1.

Figure 6.

Mitochondria regulate SOCE without affecting ER Ca^{2+} release. **A.** Fura-2 ratio traces show both ER Ca^{2+} release and SOCE without (control) or with 2.5 μ M FCCP and 2.5 μ g/mL oligomycin treatment as indicated. ER Ca^{2+} release was induced by 10 μ M ATP in the absence of extracellular Ca^{2+} (1st transient). SOCE was induced by reintroduction of 1.8 mM extracellular Ca^{2+} in the presence of ATP (2nd transient). **B.** Bar graph shows the amplitude of ER Ca^{2+} release without (control, $\Delta F_{ratio}=1.06\pm0.32$, n=121 cells from 3 experiments) and with FCCP/Oligomycin ($\Delta F_{ratio}=1.00\pm0.30$, n=160 cells from 3 experiments) treatment. **C.** Bar graphs show the peak amplitude of SOCE (left) and the rate of SOCE (right, rate was calculated from (peak amplitude/time to peak)) without (control, peak amplitude and rate of SOCE were $\Delta F_{ratio}=1.51\pm0.33$ and $\Delta F_{ratio}/sec=0.036\pm0.008$) and with FCCP/Oligomycin (peak amplitude and rate of SOCE were $\Delta F_{ratio}=0.26\pm0.27$ and $\Delta F_{ratio}/sec=0.003\pm0.003$) treatment. *** indicates $p<0.001$. Other conditions as in Figure 1.

Figure 7.

Ca^{2+} responses in rat submandibular gland ductal cells. **A.** Fura-2 ratio images show that 100 μM UTP, a P2u or P2Y2 receptor agonist, induces a Ca^{2+} increase in freshly isolated rat submandibular gland ductal cells. Scale bar indicates 40 μm for all images. **B.** Mechanical stimulation induced ICW in native rat submandibular gland ductal cells. The location of mechanically stimulated cells was indicated by a dashed line at time -1 second. Arrows in the 10 second image indicates ICW propagation. Graphs below show line scans of fura-2 ratio (F_{ratio}) before (black) and after (red) mechanical stimulation. Location of line scans is indicated at -1 second image. X-scale indicates the distance from the stimulated cells. Scale bar indicates 30 μm for all images. Other conditions as in Figure 1.

Figure 8.

Mechanical stimulation induced intercellular Ca²⁺ wave (ICW) propagation model in submandibular gland cells. **A.** Mechanical stimulation (MS) directly activates mechanosensitive maxi-anion channels (mAC) to induce ATP release into extracellular medium. **B.** Ca²⁺ mobilization mechanisms in a single cell during ICW propagation. **(a)** ATP binding to P2Y2 receptors in the cell surface activates PLC, which generates IP₃. This IP₃ activates IP₃ receptors in the ER and causes a Ca²⁺ release. Depletion of Ca²⁺ stores in the ER further activates SOCC to induce Ca²⁺ influx from the extracellular medium. Under normal conditions, mitochondria efficiently take up local Ca²⁺ and this prevents SOCC inactivation. **(b)** Inhibition of mitochondrial Ca²⁺ uptake increases the local Ca²⁺ concentrations, which causes Ca²⁺-dependent inactivation of SOCC.