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Moderate extracellular acidification inhibits capsaicin-induced cell death through regulating calcium mobilization, NF-κB translocation and ROS production in synoviocytes

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ABSTRACT

We previously show the expression of transient receptor potential vanilloid 1 (TRPV1) in primary synoviocytes from collagen-induced arthritis (CIA) rats. Capsaicin and lowered extracellular pH from 7.4 to 5.5 induce cell death through TRPV1-mediated Ca^{2+} entry and reactive oxygen species (ROS) production. However, under the pathological condition in rheumatoid arthritis, the synovial fluid is acidified to a moderate level (about pH 6.8). In the present study, we examined the effects of pH 6.8 on the TRPV1-mediated cell death. Our finding is different or even opposite from what was observed at pH 5.5. We found that the moderate extracellular acidification (from pH 7.4 to 6.8) inhibited the capsaicin-induced Ca^{2+} entry through attenuating the activity of TRPV1. In the mean time, it triggered a phospholipse C (PLC)-related Ca^{2+} release from intracellular stores. The nuclear translocation of NF-κB was found at pH 6.8, and this also depends on PLC activation. Moreover, the capsaicin-evoked massive ROS production and cell death were depressed at pH 6.8, both of which are dependent on the activation of PLC and NF-κB. Taken together, these results suggested that the moderate extracellular acidification inhibited the capsaicin-induced synoviocyte death through regulating Ca^{2+} mobilization, activating NF-κB nuclear translocation and depressing ROS production.

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disorder with complex etiology, which eventually leads to cartilage and bone destruction [1,2]. The acidosis of synovial fluid is an important feature of RA [3]. At the cellular level, the decrease in extracellular pH has been suggested to regulate the inflammatory process by stimulating synoviocyte proliferation, metabolic activation, and lactic acid production. However, the underlying intracellular signaling is largely unknown [4–6].

Extracellular protons can modulate the activities of a number of receptors or channels, including transient receptor potential vanilloid 1 (TRPV1) channels [7,8]. Our previous work demonstrates the functional expression of TRPV1 in primary synoviocytes from a collagen-induced arthritis (CIA) rat model [9]. Both the TRPV1 agonist capsaicin, and the decrease of the extracellular pH from 7.4 to 5.5 cause synoviocyte death through TRPV1-mediated Ca^{2+} entry and reactive oxygen species (ROS) production [9]. However, under the pathological condition in RA, the extracellular acidification is much more moderate and it rarely achieves such a low pH level [3,4]. Although the modest acidification (pH > 6) cannot activate TRPV1 channels directly [10], it can regulate the channel gating, sensitivity or capsaicin affinity [11–16]. The activity of TRPV1 has been reported to be either potentiated [11–14] or attenuated [15,16] by protons. In the present study, we addressed whether the moderate acidification in pathological processes can regulate TRPV1 and whether it enhanced the activation of TRPV1 like pH 5.5. We examined the cell responses to a modestly acidic pH 6.8, and investigated the influence of pH 6.8 on capsaicin-induced effects.

2. Materials and methods

2.1. Animals and reagents

Healthy male Wistar rats weighing 150–250 g were obtained from Institute of Health and Environmental Medicine, Academy of Military Medical Sciences (Tianjin, China). DMEM and fetal calf serum (FCS) were from Gibco (USA) and HyClone (USA), respectively. Fura-2/AM was purchased from Biotium (USA). NF-κB Activation-Nuclear Translocation Assay kits were purchased from Beyotime Institute of Biotechnology (Haimen, China). The rest of the reagents, including capsaicin, capsazepine, HEPES, EGTA, trypsin, collagenase, dimethylsulfoxide (DMSO), U73122, 8-(diethyl-lamino) octyl-3,4,

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5-trimethoxybenzoate hydrochloride (TMB-8), BAPTA-AM, dihydroethidium (DHE), 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide (MTT), 15-deoxy-delta12,14-prostaglandin J_2 (15dPGJ2) and diphenylene iodonium (DPI) were purchased from Sigma (USA).

2.2. Preparation of synovial fibroblasts

The collagen-induced arthritis (CIA) rat models were established as reported [17], and the synovial fibroblasts were isolated and cultured as described [18]. Briefly, rats with arthritis were sacrificed and hind limbs were excised. Synovial membranes of the knee joints were carefully separated, minced in D-Hanks, and then digested in 0.2% collagenase for 3 h at 37 °C in serum-free DMEM. After that, the cell suspension was centrifuged at 300*g* for 10 min and the isolated synovial cells were cultured in DMEM supplemented with 10% FCS in a humidified CO_2 incubator with 5% CO_2 at 37 °C. Cultured cells were subjected to a minimum of 6 passages to obtain a pure culture.

2.3. Measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$)

Synoviocytes were incubated in Hanks' balanced salt solution (HBSS) (NaCl 150 mM, KCl 5.4 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM and HEPES 10 mM, pH 7.4) with 5 μM fura-2/AM for 1 h at room temperature. After washing gently and extensively with HBSS, cells were bathed in fresh HBSS. $[Ca^{2+}]_c$ was measured by calcium imaging system built on an inverted fluorescence microscope (Olympus IX51). The ratiometric fluorescent Ca^{2+} indicator dye Fura-2 was excited alternately at 340/380 nm with a Lambda 10–2 optical filter changer (Sutter Instrument Company). Fluorescence images (filtered at 515 nm) were captured by a CCD camera (CoolSNAP fx-M, Roper Scientific Inc.) and quantitated with MetaFluor (Universal Imaging Corporation). $[Ca^{2+}]_c$ was represented by fluorescence intensity ratio of F340/F380. At least 3 experiments were done for each condition and about 5 individual cells were selected randomly for each experiment. One representative calcium trace was plotted to represent >10 similar traces. In addition, calcium-free HBSS was prepared by substituting $MgCl_2$ for $CaCl_2$ at the same concentration with 2 mM EGTA. For acidic condition, the pH of HBSS was adjusted to 6.8 with HCl.

2.4. Detection of intracellular reactive oxygen species (ROS)

Dihydroethidium (DHE), a reduced form of ethidium bromide, was used to detect and measure intracellular ROS. After stimulation with indicated reagents for 1 h, synoviocytes were incubated with 5 μM DHE in HBSS for 30 min at 37 °C, then rinsed twice and observed with a fluorescence microscope at 488 nm excitation and 610 nm emission. The fluorescent intensity represents the intracellular ROS level.

2.5. NF-κB localization by immunofluorescence

The detection of NF-κB nuclear translocation was carried out following the instruction of the kit. Briefly, synoviocytes on glass coverslips were incubated with or without various test treatments in HBSS for 1 h at 37 °C, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 for 15 min, and blocked with 5% BSA for 2 h at room temperature. The cells were then incubated overnight with the primary antibody against the p65 subunit of NF-κB at 4 °C, washed in PBS, and incubated with Cy3-labeled secondary antibody for 2 h at room temperature. Finally, the cells were stained with 2 μM DAPI for 5 min, and then observed using a fluorescence microscope.

2.6. Cell viability assay

Cell viability was tested with a MTT assay. Briefly, synoviocytes were seeded into 96-well plate at $\sim 1 \times 10^4$ cells/well in 100 μL DMEM with 10% FCS and incubated overnight before treating with or without test agents for the indicated time. Ten microliters MTT solution (10 mg/mL) was added into each well 4 h before the end of such treatments. The culture medium was aspirated and replaced with 100 μL lysis solution (50% DMSO and 50% ethanol). The absorbance at 570 nm (A_{570}) for each well was determined by an ELISA reader (Labsystems Multiskan Ascent). The percentage of living cells was calculated as $A_{570,test}/A_{570,control}$.

2.7. Statistical analysis

Data are presented as mean ± SEM, and the statistical significance was calculated according to Student's *t* test with $P < 0.05$ as the significant level.

3. Results

3.1. Moderate extracellular acidification reduced capsaicin-induced Ca^{2+} entry and triggered Ca^{2+} release

The effects of extracellular acidification (from pH 7.4 to 6.8) on the concentration of cytosolic Ca^{2+} were measured by calcium imaging system. A rise in the F340/F380 ratio indicated an increase in $[Ca^{2+}]_c$. Representative $[Ca^{2+}]_c$ profiles were shown in Fig. 1. The TRPV1 agonist capsaicin (100 μM) evoked a rapid $[Ca^{2+}]_c$ increase followed by a sustained plateau, and the $[Ca^{2+}]_c$ elevation was blocked in a calcium-free buffer or by the TRPV1 antagonist, capsazepine (2 μM). This finding indicated that the $[Ca^{2+}]_c$ increase, caused by capsaicin, was due to TRPV1-induced calcium entry (Fig. 1A). At pH 6.8, the sustained $[Ca^{2+}]_c$ plateau induced by capsaicin was reduced significantly, implicating the attenuation of TRPV1 (Fig. 1B). Stimulation of cells with capsaicin (100 μM) and a pH 6.8 solution together triggered a $[Ca^{2+}]_c$ increase with two peaks. The first sharp peak was eliminated by the pre-treatment with phospholipse C (PLC) inhibitor U73122 (20 μM), and the second peak was eliminated in the Ca^{2+}-free buffer (Fig. 1C). Moreover, the application of pH 6.8 solution alone evoked a single peak in $[Ca^{2+}]_c$, and this $[Ca^{2+}]_c$ increase was blocked by U73122 (20 μM) or Ca^{2+} release inhibitor TMB-8 (10 μM), but remained unaffected in the calcium-free buffer (Fig. 1D). These data suggested that the acidification to pH 6.8 may inhibit the capsaicin-induced Ca^{2+} entry by attenuating the activity of TRPV1, and, in the mean time, trigger a PLC-related Ca^{2+} release from intracellular endoplasmic reticulum stores.

3.2. Moderate acidification activated the nuclear translocation of NF-κB

In rheumatoid arthritis, NF-κB is a key regulator of the inflammatory gene transcription and proliferation. In unstimulated cells, NF-κB is retained as an inactive cytoplasmic complex by its inhibitor IκB. Upon stimulation, the phosphorylation and degradation of IκB release NF-κB for nuclear translocation. We studied the NF-κB translocation using a primary antibody against the p65 subunit of NF-κB and the nuclei dye DAPI (Fig. 2). Stimulation with capsaicin (100 μM) for 1 h had little influence on the localization of NF-κB (Fig. 2B) in comparison with control (Fig. 2A), whereas an acidic solution (pH 6.8) induced marked nuclear translocation of NF-κB (Fig. 2C). The pH 6.8-induced translocation was suppressed by a pretreatment with U73122 (20 μM) (Fig. 2D), suggesting the PLC activation is essential for NF-κB nuclear translocation.

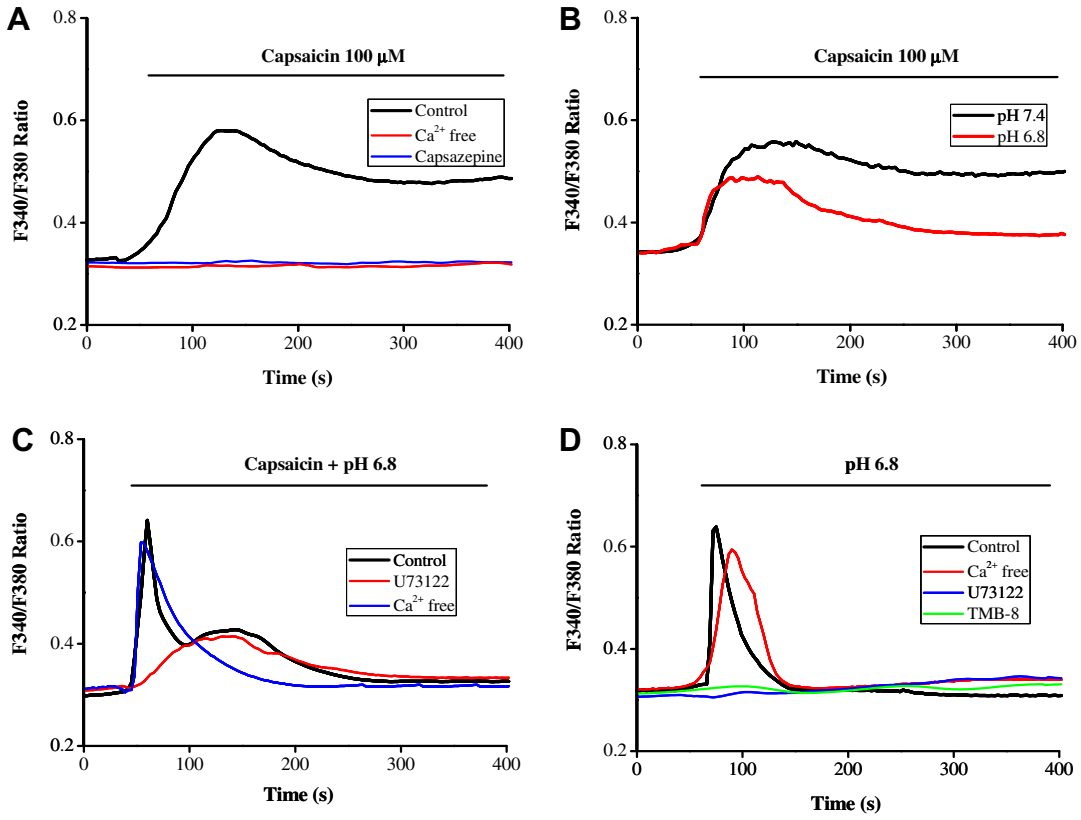

Fig. 1. Acidification to pH 6.8 regulated Ca^{2+} mobilization in rat synoviocytes. (A) Representative $[Ca^{2+}]_c$ traces after stimulating synoviocytes with capsaicin (100 μM) under various conditions. Capsazepine (2 μM) was of capsaicin. (B) Representative traces showing the application of capsaicin (100 μM) at pH 6.8 and pH 7.4 (control). (C) Representative traces showing both the application of capsaicin (100 μM) and acidification to pH 6.8 under different conditions. U73122 (20 μM) was added 15 min before adding capsaicin. (D) Representative traces after decreasing extracellular pH from 7.4 to 6.8 under various conditions. TMB-8 (10 μM) or U73122 (20 μM) was added 15 min before the decrease in pH value.

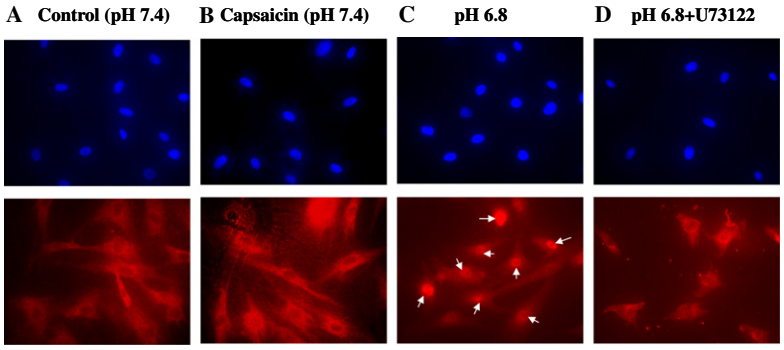

Fig. 2. Acidification to pH 6.8 activated the nuclear translocation of NF-κB. Synoviocytes labeled with NF-κB subunit p65 (red) and DAPI (blue) were imaged simultaneously. (A) Control cells were treated with neither capsaicin nor acidic solution. (B) Cells were treated with capsaicin (100 μM) for 1 h in normal HBSS (pH 7.4). (C and D) Cells were placed in pH 6.8 solution for 1 h in the absence (C) and presence (D) of U73122. U73122 (20 μM) was added 15 min before the decrease in pH. Arrows indicate the localization of NF-κB in the nuclei. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3. Moderate acidification depressed capsaicin-induced ROS production

To investigate signals downstream of $[Ca^{2+}]_c$ elevation and NF-κB translocation, the influence of moderate extracellular acidification on intracellular reactive oxygen species (ROS) was examined. Compared with control (Fig. 3Aa and B), the treatment with a pH 6.8 solution for 1 h induced a relatively slight intracellular ROS generation (Fig. 3Ab and B), but it depressed the massive ROS production evoked by capsaicin (100 μM) (Fig. 3Ac, Ad and B). In the presence of U73122 (20 μM), this depression was reversed (Fig. 3Ae and B), suggesting the contribution of PLC. Moreover, the pretreatment with 20 μM 15dPGJ2, an IκB kinase blocker, also reversed the pH 6.8-induced ROS depression significantly (Fig. 3Af and B), indicating the involvement of NF-κB. These results indicated that the acidification to pH 6.8 depressed the capsaicin-induced ROS production, and this depression was dependent on activation of PLC and NF-κB.

3.4. Moderate acidification inhibited capsaicin-induced synoviocyte death

To investigate the influence of pH 6.8 on capsaicin-induced synoviocyte death, cell viability was assessed using MTT. Capsaicin

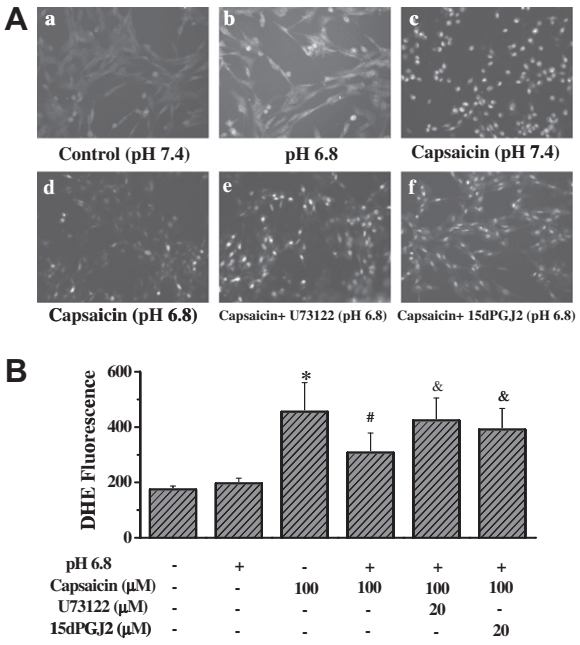

Fig. 3. Acidification to pH 6.8 depressed capsaicin-induced ROS generation. (A) Dihydroethidium (DHE) fluorescence images, captured using a 20× objective, are shown. The fluorescence intensity represents the ROS concentration. (a) Control cells. (b) Cells placed in a pH 6.8 solution for 1 h. (c and d) Cells stimulated with capsaicin (100 μM) in a pH 7.4 (c) and pH 6.8 (d) solution. (e and f) Cells stimulated with capsaicin (100 μM) in a pH 6.8 solution after pre-treatment with 20 μM U73122 (e) or 20 μM 15dPGJ2 (f) for 15 min. (B) Statistic data of florescence intensity ($n = 30$). $^{*}P < 0.05$, compared with control; $^{\#}P < 0.05$, compared with capsaicin or pH 6.8; $^{\&}P < 0.05$, compared with pH 6.8 plus capsaicin.

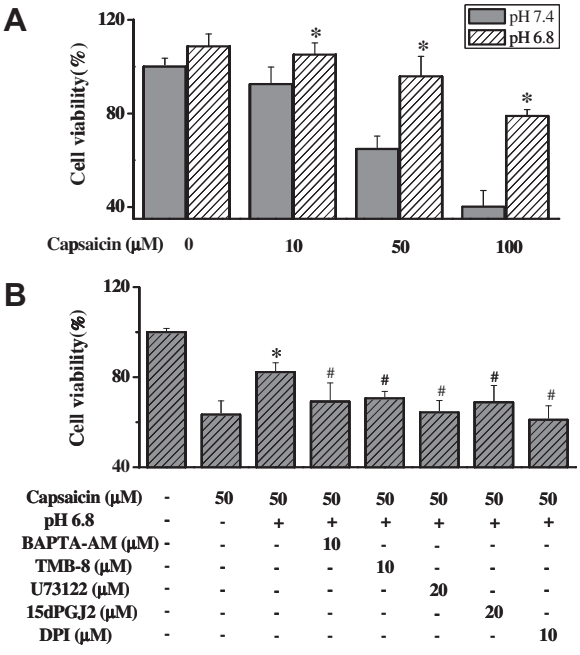

Fig. 4. Acidification to pH 6.8 inhibited capsaicin-induced synoviocyte death. Synoviocyte viability was assessed by a MTT assay. The value for each group was normalized to that of control (without capsaicin, in normal medium). (A) Synoviocytes were incubated for 24 h with capsaicin (0, 10, 50, 100 μM) in a normal medium or in a pH 6.8 medium (mean ± SEM, $n = 6$). $^{*}P < 0.05$, in comparison with the corresponding group at pH 7.4. (B) Synoviocytes were incubated for 24 h in a pH 6.8 medium with capsaicin (50 μM) alone or in the presence of BAPTA-AM (10 μM), TMB-8 (10 μM), U73122 (20 μM), 15dPGJ2 (20 μM) or DPI (10 μM) (mean ± SEM, $n = 6$). $^{*}P < 0.05$, in comparison with capsaicin in normal medium; $^{\#}P < 0.05$, in comparison with capsaicin alone in pH 6.8 medium.

reduced the cell viability in a dose-dependent manner in a normal pH medium, however when treating cells with capsaicin in a pH 6.8 medium, cell viability of each group was significantly higher than that in normal pH medium (Fig. 4A). This indicated that capsaicin-induced cell death was inhibited at pH 6.8. This inhibition of cell death in a pH 6.8 medium was significantly reversed by TMB-8 (10 μM), U73122 (20 μM) or BAPTA-AM (an intracellular calcium chelator, 10 μM) (Fig. 4B), suggesting that the effect of pH 6.8 medium on capsaicin-induced cell death was dependent on PLC-related Ca^{2+} release. Similar effects were observed while pre-treating the cells with 15dPGJ2 (20 μM) or diphenylene iodonium (DPI, 10 μM), a NAD(P)H oxidase inhibitor (Fig. 4B), indicating the involvement of NF-κB and ROS. Taken together, these data suggested that the acidic medium (pH 6.8) inhibited capsaicin-induced synovial cell death, and this effect was dependent on the regulation of $[Ca^{2+}]_c$, NF-κB activation and ROS production.

4. Discussion

The inflammatory process of RA is often accompanied by acidification of the synovial fluid from the physiological pH 7.4 to about pH 6.8 [3,4]. Here, we show that the extracellular acidification to pH 6.8 inhibited the capsaicin-induced sustained Ca^{2+} entry through attenuating the activity of TRPV1. In the mean time, it triggered a PLC-related Ca^{2+} release from intracellular stores. At pH 6.8, the translocation of NF-κB to the nuclei was found, and this depends on PLC activation. Moreover, the capsaicin-evoked massive ROS production and cell death were depressed at pH 6.8, both of which are dependent on the activation of PLC and NF-κB. These effects of moderate acidic pH 6.8 on synovial cells are opposite to those found at the extracellular pH 5.5. This could be explained by the findings that TRPV1 is directly activated at pH < 5.9, and the effects of pH 5.5 were due to TRPV1 activation [9], and that TRPV1 can be regulated but is not directly activated by modest acidification (pH > 6) [11–16]. Our results in this study are consistent with previous findings that moderate extracellular acidosis inhibits the TRPV1 activity in rat dorsal root ganglion neurons [16].

It is noteworthy that acidification to pH 6.8 initiated a PLC-related intracellular Ca^{2+} release. How does this Ca^{2+} release be activated? There are several membrane channels or receptors can response to extracellular protons, including acid-sensing ion channels (ASIC), TRPV1 channels, and proton-sensing G protein coupled receptors (GPCR) [7,8,10,19–21]. TRPV1 channels are activated at pH < 5.9, while ASICs and pH-sensing GPCRs are activated at pH < 7 and pH < 6.8, respectively [7,8,10,19–21]. It has been reported that extracellular acidification (from pH 7.4 to 6.3–6.8) caused intracellular calcium mobilization through activating the proton-sensing GPCRs in synovial cell line SW982 and airway smooth muscle cells [22,23]. Therefore, it is reasonable to speculate that the proton-sensing GPCR may account for the pH 6.8-induced Ca^{2+} release in synoviocytes. Recently, a variety of proton-sensitive GPCRs have been characterized, such as OGR1, G2A, GPR4, and TDAG8 [21,24]. However, which member of proton-sensing GPCRs actually contributes to the pH 6.8-triggered $[Ca^{2+}]_c$ increases needs to be further investigated.

RA is prominently characterized by synoviocyte hyperplasia [25,26]. It is accepted that intracellular calcium signaling is critical in proliferation [27]. In the present study, we demonstrated that the extracellular acidification to pH 6.8 regulated the $[Ca^{2+}]_c$. Thus, this regulation could be involved in synoviocyte proliferation. NF-κB is of central importance in RA and serves its function by activating pro-inflammatory gene expression, inhibiting apoptosis and promoting proliferation [28]. In this study, we found that the nuclear translocation of NF-κB was activated at pH 6.8. Therefore, this activation of NF-κB could also contribute to synoviocyte proliferation.

Moreover, excess intracellular ROS can induce mitochondria oxidative damage and eventually cause cell death [29]. Our data revealed that the pH 6.8 acidification suppressed the massive ROS accumulation induced by capsaicin, and eventually inhibited the cell death. All these data suggested that pH 6.8 inhibited capsaicin-induced synoviocyte death through regulating the $[Ca^{2+}]_c$, NF-κB translocation and ROS production.

In summary, we found, in this study, the moderate extracellular acidification suppressed the capsaicin-evoked Ca^{2+} entry and triggered a PLC-related Ca^{2+} release, activated the NF-κB nuclear translocation, depressed the capsaicin-induced ROS generation, and eventually inhibited the cell death caused by capsaicin in rat synoviocytes. These findings will lead to new insights into the cellular basis for tissue fluid acidosis in development of RA.

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