Effects of ORAI calcium release-activated calcium modulator 1 (ORAI1) on neutrophil activity in dairy cows with subclinical hypocalcemia¹

Bingbing Zhang,^{†,2} Han Guo,^{†,2} Wei Yang,^{†,2} Ming Li,[†] Ying Zou,[†] Juan J. Loor,^{‡,§} Cheng Xia,[†] and Chuang Xu^{†,3}

[†]College of Life Science and Technology, Heilongjiang Bayi Agricultural University, Xinyang Rd. 5, 163319 Daqing, China; [‡]College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, Xinyang Rd. 5, 163319 Daqing, China; and [§]Department of Animal Sciences and Division of Nutritional Sciences, Mammalian NutriPhysioGenomics, University of Illinois, Urbana, IL 61801

ABSTRACT: Hypocalcemia in dairy cows is often associated with inflammation-related disorders such as metritis and mastitis. The protein encoded by the Ca^{2+} release-activated calcium modulator 1 (ORAI1) gene is a membrane Ca^{2+} channel subunit that is activated when Ca^{2+} stores are depleted. Polymorphonuclear neutrophils (PMNL) have a crucial role in the defense against infection through migration, adhesion, chemotaxis, phagocytosis, and reactive oxygen species (ROS) production in response to pathogens. Whether hypocalcemia affects the activity of PMNL and if ORAI1 is involved remains unknown. To address this, PMNL were isolated at 3 d of calving from dairy cows diagnosed as clinically healthy ($n = 20$, CONTROL) or with plasma concentration of calcium < 2.0 mmol/L as a criterion for diagnosis of subclinical hypocalcemia ($n = 20$, HYPOCAL). PMNL isolated from both groups of cows were treated with or without the sarcoendoplasmic Ca^{2+} ATPase inhibitor thapsigargin, Ca^{2+} ionophore Ionomycin, and ORAI1 blocker 2APB. The intracellular Ca^{2+} concentration, ORAI1 abundance, ROS, phagocytosis rate, migration, and adhering capacity of treated PMNL were evaluated. Some of the in vitro assays also included use of small interfering ORAI1 RNA (siORAI1),

100 nM $1,25(\text{OH})_2\text{D}_3$, or 100 nM parathyroid hormone (PTH). Intracellular Ca^{2+} concentration was markedly lower in HYPOCAL. In addition, ORAI1 was detected in PMNL plasma membrane via FACS and was markedly lower in cows with HYPOCAL. Migration, adhesion capacity, and phagocytosis rate of PMNL were lower in response to HYPOCAL. Furthermore, plasma and PMNL concentration of nucleosome assembly protein (NAP2) and pro-platelet basic protein (CXCL7) was markedly lower with HYPOCAL. All these changes were associated with lower ROS production by PMNL. Thapsigargin and ionomycin treatment in vitro increased ORAI1 expression, migration of PMNL, adhering capacity, phagocytosis rate, and ROS production; conversely, those effects were abrogated by siORAI1 and ORAI1 inhibitor 2APB treatment. Also cytosolic Ca^{2+} concentration and ORAI1 abundance were increased by $1,25(\text{OH})_2\text{D}_3$ and PTH supplementation. Overall, the data indicate that failure of PMNL to uptake Ca^{2+} due to downregulation of ORAI1 during subclinical hypocalcemia is a factor contributing to impaired PMNL function. In addition, plasma PTH or $1,25(\text{OH})_2\text{D}_3$ could regulate ORAI1 and also participate in the regulation of PMNL activity.

Key words: cattle, hypocalcemia, PMNL, immune function

¹The study was supported by grants from National Program on Key Research Project of China (Project 2016YFD0501206), Heilongjiang Province General Undergraduate Colleges and Universities Young Innovative Talents Training Program (UNPYSCT-2018081), the National Natural Science Foundation of China (grant 31702308 and 31672622).

²These authors contributed equally to this work.

³Corresponding author: xuchuang7175@163.com

Received April 4, 2019.

Accepted June 15, 2019.

© The Author(s) 2019. Published by Oxford University Press on behalf of the American Society of Animal Science. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com.

J. Anim. Sci. 2019.97:3326–3336

doi: 10.1093/jas/skz209

INTRODUCTION

Hypocalcemia (HYPOCAL) in dairy cows is caused by an imbalance in Ca^{2+} metabolism at the onset of lactation (Neves et al., 2018). In high-risk dairy cows, the incidence of subclinical HYPOCAL is as high as 30% to 45% within 3 to 14 d postpartum (Thilsing-Hansen et al., 2002; Goff, 2008; Jawor et al., 2012). Hypocalcemia impairs normal smooth and skeletal muscle function, and can increase incidence of dystocia, retained placenta, and displaced abomasum (Jawor et al., 2012; Rodríguez et al., 2017). In addition, there is a significant association between HYPOCAL early postpartum and inflammation-related disorders such as metritis and mastitis, all of which result in economic losses to the dairy industry (Martinez et al., 2014; Wang et al., 2016).

Polymorphonuclear neutrophils (PMNL) play a crucial role in the defense against infection through migration, chemotaxis, adhesion, and phagocytosis (Herrmann and Meyle, 2015). During the beginning stages of inflammation, PMNL are one of the first-responders and migrate towards the affected sites through the blood vessel wall (Wang et al., 2017). By releasing a large number of inflammatory factors, antibacterial substances, and phagocytizing pathogens, the invading pathogens are contained (de Oliveira et al., 2016; Rosales, 2018). At least in nonruminants, the neutrophil recruitment cascade and its underlying molecular mechanisms are associated with intracellular Ca^{2+} concentrations (Immler et al., 2018). Martinez et al. (2014) reported that blood neutrophil of dairy cows with subclinical HYPOCAL had lower concentrations of cytosolic Ca^{2+} and phagocytosis capacity (Martinez et al., 2014). The actual mechanisms associated with these events in dairy cows are not well known.

It has been well-established in nonruminants that intracellular Ca^{2+} plays important roles in signal transduction in various cell types. For instance, in nonactivated cells, the protein store-operated Ca^{2+} entry (SOCE) that is stimulated by depletion of endoplasmic reticulum Ca^{2+} stores (Parekh, 2010) could regulate various cellular functions including excitation–contraction coupling (Griffiths and MacLeod, 2003), exocytosis (Weiss and Zamponi, 2013; Baydyuk et al., 2015), migration (Bose et al., 2015), and cell proliferation and cell death (Lang et al., 2007). The calcium release-activated calcium

(CRAC) modulator ORAI is a plasma membrane Ca^{2+} influx protein that is associated with store-operated Ca^{2+} entry (Immler et al., 2018), and ORAI regulator stromal interaction molecule (STIM) could sense Ca^{2+} content in the endoplasmic reticulum. STIM can handle ORAI-mediated calcium entry after internal stores are depleted. When STIM1 detects the depletion of Ca^{2+} levels in the ER, STIM1 aggregates and translocates to areas of the ER that are close to the plasma membrane, where they activate Orai channels and facilitate calcium entry (Wang et al., 2008; Giachini et al., 2011). The entry of Ca^{2+} through Orai1 increases intracellular Ca^{2+} levels and activates calcineurin to maintain the calcium balance (Soboloff et al., 2012; Shambharkar et al., 2015).

Because of recent reports demonstrating that ORAI1 regulates intracellular Ca^{2+} , and arrest and shape polarization during neutrophil recruitment (Schaff et al., 2010; Demaurex and Nunes, 2016; Putney et al., 2017), we hypothesized that hypocalcemia downregulates ORAI1 to decrease PMNL functions. To address that hypothesis, the present study focused on in vivo and in vitro analyses of the role of intracellular Ca^{2+} and ORAI1 on PMNL function in the context of subclinical hypocalcemia early postpartum.

MATERIALS AND METHODS

The ethics Committee on the Use and Care of Animals at Heilongjiang Bayi Agricultural University (Daqing, China) approved the study protocol.

Animals

Healthy (CONTROL) and HYPOCAL dairy cows were selected from an 8,000-cow dairy farm located in Daqing City (Heilongjiang Province, China). All cows were fed the same diets received a routine physical examination to ensure absence of other co-morbidities. We chose lactating Holstein cows at 3 d in milk (DIM = 3) with similar numbers of lactations (median = 3, range = 2–4). According to blood calcium concentration and clinical signs, we preselected 60 cows suspected of being hypocalcemia and 80 healthy cows. Subsequently, the concentration of calcium in plasma was measured

by Semi-automatic biochemical analyzer (RT-9600 Rayto, USA). According to clinical symptoms and plasma calcium concentration, 20 subclinically hypocalcemic cows with plasma calcium concentration of 1.38 to 2.00 mmol/L, 20 control cows with greater than 2.10 mmol/L were randomly selected (Mulligan et al., 2006; Reinhardt et al., 2011). The CONTROL group had an average milk yield of 25.43 ± 3.64 kg/d, average body weight of 603 ± 21.4 kg, and average body condition score of 2.91 ± 0.13 . Cows in the HYPOCAL group had an average milk yield of 26.08 ± 3.07 kg/d, average body weight of 612 ± 28.5 kg, and average body condition score of 2.95 ± 0.1 (Rasby et al., 1990).

Isolation and Culture of PMNL

Blood samples from CONTROL and HYPOCAL dairy cows (DIM 3) were collected via venipuncture in a 50-mL centrifuge tube (Nunc, Roskilde, Denmark) containing 0.1 mL Sodium citrate (Solarbio, Beijing, China) as an anticoagulant. Blood was diluted in an equal amount of phosphate-buffered saline containing 0.02% EDTA (Sigma-Aldrich), layered on Biocoll Separating Solution (Biochrom AG, Berlin, Germany), and centrifuged at $800 \times g$ for 30 min. After removal of plasma, lymphocytes, and monocytes, the pellet was suspended in distilled water and shaken for 40 s to lyse erythrocytes. Osmolarity was readjusted immediately by adding an appropriate amount of Hanks' Salt Solution ($10\times$; Biochrom AG). Neutrophils were washed twice with RPMI 1640 medium, resuspended in RPMI 1640 medium (2.0×10^6 cells/mL).

RNA Silencing and Transfection

For silencing, 2×10^6 cells (6-well plate) were seeded 1 h before the experiment and then transfected with 40 pM cow ORAI1 siRNA (Shanghai GenePharma Co., Ltd, Shanghai, China) or nontarget siRNA (Shanghai GenePharma Co., Ltd) using siRNA-Mate transfection reagent (Shanghai GenePharma Co., Ltd) according to the manufacturer's protocol (<http://www.genepharma.com/show.php?ctype=0&coupid=568&catid=111>). PMNL were treated with siOrai1 for 24 h.

Inhibitor and Ca^{2+} Ionophore Treatments

The PMNL from both groups of cows were seeded in 6-wells plates at a density of 5×10^5 cells/cm² (2 mL

per well) with RPMI 1640 medium at 37 °C with 5% CO₂ for 1 h. Then, cells were divided into 8 groups: CONTROL, HYPOCAL, 50 μM ORAI1 inhibitor 2APB treated CONTROL, 50 μM 2APB treated HYPOCAL (2APB could block extracellular Ca^{2+} entry into the cell), 1 μM sarcoendoplasmatic Ca^{2+} ATPase inhibitor thapsigargin treated CONTROL, 1 μM thapsigargin treated HYPOCAL (thapsigargin prevents the uptake of Ca^{2+} back into the intracellular organelles that should deplete ER Ca^{2+} more quickly and therefore prolong or enhance the response of ORAI1 expression by the neutrophil), 100 nM Ca^{2+} ionophore ionomycin (ionomycin could raise the intracellular level of calcium) treated CONTROL, and 100 nM ionomycin treated HYPOCAL (ionomycin could raise the intracellular level of calcium) for 2 min.

Cytosolic Calcium

For the measurement of the cytosolic Ca^{2+} concentration, the PMNL preparation was washed once in Tyrode buffer (pH 7.4), stained with 3 μM Fluo-3AM (Biotinium, USA) in the same buffer, and incubated at 37 °C for 30 min. Relative fluorescence was measured utilizing a Beckman CytoFLEX FCM (Beckman Coulter, USA).

ORAI1 Surface Abundance

Orai1 surface expression in neutrophils was analyzed by flow cytometry. Neutrophils were washed twice with ice-cold PBS and resuspended in PBS containing 5% FBS, and subsequently fixed with 1% paraformaldehyde for 30 min on ice. After rinsing 3 times, neutrophils were incubated for 30 min with ORAI1 rabbit polyclonal antibody (Proteintech, USA), washed twice in Tyrode buffer, and stained in rabbit anti-goat IgG-PE (Santa Cruz Biotechnology, USA) for 20 min. Samples were washed twice in Tyrode buffer and immediately analyzed on a Beckman CytoFLEX FCM (Beckman Coulter, USA).

Quantification of mRNA Expression

Total RNA was isolated with Trizol RNA extraction reagent (Invitrogen Corporation, Carlsbad, CA). mRNA was transcribed with Reverse Transcriptase M-MLV (RNase H-; Takara Bio, Inc., Otsu, Japan) using an oligo dT primer. Quantitative RT-PCR was performed on a BioRad iCycler iQTM Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA). The PCR reaction mixture contained 2 μL of

cDNA, 1 μ M of each primer, 10 μ L of Taq DNA Polymerase (CWBIO, Beijing, China), and sterile water for a final volume of 20 μ L; PCR conditions were 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 s and 56 °C for 30 s. The following primers for ORAI1 analysis were used forward (5'-3'): CGTCCACAACCTCAACTCC and reverse (5'-3'): AACTGTCCGGTCCGTCTTAT.

Measurement of Reactive Oxygen Species

A reactive oxygen species assay kit (APPLYGEN, Beijing, China) was used to determine the intracellular change in reactive oxygen species (ROS) generation. 1×10^5 cells were treated with 10 μ M carboxy-2',7'-dichloro-dihydro-fluorescein diacetate (DCFHDA) probe to detect intracellular reactive oxygen species in PBS for 15 min at 37 °C. Fluorescence was measured at each time period at 488 nm (excitation) and 525 nm (emission; <http://www.applygen.com/product/shenghuaceding/qita/657.html>) by Beckman CytoFLEX FCM (Beckman Coulter, USA).

Immunofluorescence

The PMNL were fixed with 4% paraformaldehyde for 30 min at room temperature. For blocking unspecific binding, PMNL were incubated with 3% Albumin Bovine V (Biosharp, Hefei, China), 5% normal goat serum (Boster, Wuhan, China), and 0.5% Triton (BioFROXX, Guangzhou, China) in PBS (Biosharp, Hefei, China) for 30 min at room temperature. Then, cells were incubated with rabbit anti-ORAI1 (1:500, CST) at 4 °C in a humidified chamber overnight. The cells were rinsed 4 times with PBS and incubated with Cy3 goat anti-rabbit IgG (1:1000, Beyotime Shanghai, China) for 1 h at room temperature. After 4 washing steps, the nuclei were stained with Hoechst 33342 dye (Beyotime, Shanghai, China) for 8 min at room temperature. Images were obtained with a confocal laser-scanning microscope (Leica TCS SP8; Leica, Wetzlar, Germany) with 40 $\times$ /1.3 NA differential interference contrast and analyzed with the instrument's software.

In Vitro Migration Assay

For transwell migration assays, 1×10^6 cells were placed in the top chamber with a noncoated membrane (24-well insert; 8 μ m pore size, Corning, USA) in serum-free medium, whereas the lower chamber of the transwell system contained medium with 10% fetal bovine serum as chemoattractant at 37 °C incubator for 1 h. The PMNL that migrated

(the lower chamber) were measured by fluorescence-activated cell sorting (FACS).

Adhesion Assay

Twenty-four well plates were precoated with 200 μ L FBS for 2 h. For each group, a total of 10^6 PMNL in 200 μ L were added and incubated at 37 °C/5% CO₂ for 1 h. Then PMNL were fixed with 2% paraformaldehyde for 15 min at room temperature. Unbound cells were then removed by washing and vigorous shaking. Hoechst 33342 was used to stain PMNL and observed by fluorescence microscopy. Mean fluorescence intensity was detected.

Phagocytosis Assay

Staphylococcus aureus was labeled with fluorescein isothiocyanate (FITC). The efficiency rate of labeling was assessed by flow cytometry. The PMNL were incubated with FITC-labeled bacteria at 37 °C for 1 h. Then, phagocytosis was terminated while samples were on ice and centrifuged at 350 $\times$ g/min, followed by washing twice in Tyrode's Solution. The fluorescent intensity of neutrophils was measured by FACS to determine phagocytosis rate.

Neutrophil Chemotactic Protein 2 (NAP-2/CXCL7) for Chemotactic Assay

Neutrophil chemotactic protein 2 (NAP-2/CXCL7) was measured via ELISA (Langton, Shanghai, China) in plasma obtained in tubes containing sodium citrate anticoagulant. The isolated PMNL were collected in PBS, freeze-thawed, and centrifuged 350 $\times$ g/min. The supernatant was used to determine the concentration of NAP-2/CXCL7.

1,25(OH)₂D₃ and Parathyroid Hormone Treatments

The concentrations of 1,25(OH)₂D₃ and parathyroid hormone (PTH) in plasma were measured using bovine-specific ELISA kits according to the manufacturer's protocols (Westang Biotechnology Co., Ltd, Shanghai, China). The PMNL were treated with 100 nM 1,25(OH)₂D₃ or 100 nM PTH, respectively, incubated in RPMI 1640 medium at 37 °C with 5% CO₂ for 24 h to measure ORAI1 surface abundance and intracellular Ca²⁺ concentration.

Statistical Analysis

Data are presented as the means $\pm$ standard error of the means (SEM) with *n* representing the number

of independent experiments. All data were tested for significance with the unpaired Student's *t*-test or one-way analysis of variance (ANOVA) followed by a Tukey post hoc test. A probability (*P*) value of <0.05 was considered statistically significant.

RESULTS

The PMNL count was lower in cows with HYPOCAL ($1.7 \pm 0.19 \times 10^6$ cells/mL, *n* = 18) compared with CONTROL ($4.71 \pm 0.53 \times 10^6$ cells/mL, *n* = 22), *P* < 0.05. As depicted in Figure 1A and B, cytosolic Ca^{2+} concentration of PMNL also was lower with HYPOCAL. These data were associated with lower protein expression of ORAI1 (Figure 2A and B) in the membrane of neutrophils (Figure 2D). In addition, abundance of ORAI1 mRNA was detectable in the PMNL of dairy cows (Figure 2C).

Functionality of the isolated PMNL was evaluated through production of ROS and adhesion, migration, phagocytosis, and chemotaxis. As illustrated in Figure 3A and B, the abundance of ROS determined via DCFH-DA fluorescence was lower in cows with HYPOCAL. Furthermore, activity of migration (as shown in Figure 4), adhesion (as shown in the control of Figure 5), phagocytosis (as shown in the first line of Figure 6), and chemotaxis (Figure 7) in PMNL also was lower in HYPOCAL.

Further in vitro experiments explored whether the SOCE moiety protein ORAI1 modified the function of PMNL in dairy cows with HYPOCAL. The PMNL were treated with ORAI1 inhibitor 2APB, sarcoendoplasmic Ca^{2+} ATPase inhibitor thapsigargin, and Ca^{2+} ionophore ionomycin. As reported in Figure 2A and B, ORAI1 expression was decreased by incubation with 2APB, but increased markedly by thapsigargin and ionomycin treatment

in both CONTROL and HYPOCAL cultures. In addition, PMNL migration (Figure 4), adhesion (Figure 5), and phagocytosis (Figure 6) in response to culture with 2APB decreased in CONTROL and HYPOCAL PMNL. In contrast, there was an opposite response when cultures were incubated with thapsigargin and ionomycin.

The following, we used small interfering RNA technology to silence ORAI1 and evaluate the effect on function of the PMNL. The PMNL treated with siORAI1 had lower ORAI1 expression (Figure 8), along with lower production of ROS (Figure 9A and B) and reduced migration (Figure 9C), adhesion (Figure 9D), and phagocytosis (Figure 9E) capacity.

Lastly, the effect of $1,25(\text{OH})_2\text{D}_3$ and PTH on ORAI1 surface abundance and intracellular Ca^{2+} concentration of the PMNL were evaluated. As depicted in Supplementary Figure 1, the plasma concentrations of $1,25(\text{OH})_2\text{D}_3$ were lower in HYPOCAL. Furthermore, isolated PMNL were treated $1,25(\text{OH})_2\text{D}_3$ and PTH; the result showed that $1,25(\text{OH})_2\text{D}_3$ and PTH could increase cytosolic Ca^{2+} concentration (Supplementary Figure 2) and ORAI1 abundance (Supplementary Figure 3).

DISCUSSION

The present study revealed a number of unique effects of intracellular Ca^{2+} on the biological activity of PMNL in dairy cows with subclinical hypocalcemia. Dairy cows with subclinical hypocalcemia are more susceptible to inflammatory-related disorders such as metritis and mastitis (Curtis et al., 1983; Melendez et al., 2004). Neutrophils have major roles in the innate immune response of the organisms against pathogens. The majority of PMNL-activating receptors induce extracellular

Figure 1. Cytosolic Ca^{2+} concentration in neutrophils (PMNL) in dairy cows with subclinical hypocalcemia and normal. (A) Original histogram overlays of Fluo-3 fluorescence reflecting cytosolic Ca^{2+} activity in neutrophils in CONTROL (first) and HYPOCAL (second); (B) Arithmetic means $\pm$ SEM (*n* = 20) of Fluo-3 fluorescence reflecting cytosolic Ca^{2+} activity in neutrophils in CONTROL (black bar) and HYPOCAL (white bar). * (*P* < 0.05) indicates difference from CONTROL (*t*-test).

Figure 2. The abundance of ORAI calcium release-activated calcium modulator 1 (ORAI1) protein in the neutrophils membrane. (A) Original histogram overlays of ORAI1 protein abundance in neutrophils (PMNL) in CONTROL (upper images) and HYPOCAL (lower images) with and without 2APB, ionomycin, and thapsigargin. (B) Arithmetic means $\pm$ SEM ($n = 20$) of ORAI1 protein abundance in PMNL in CONTROL (black bar) and HYPOCAL (white bar) with and without 2APB, ionomycin, and thapsigargin. (C) Original 2% agarose gel showing specific amplification of Orail (289 bp) cDNA in neutrophils of HYPOCAL and CONTROL. (D) Original immunofluorescence images demonstrating nuclear staining (Hoechst 33342 dye; blue left images), ORAI1-specific staining (Orail; red middle images), and an overlay of both nuclear and ORAI1-specific staining (right images) in neutrophils with CONTROL (upper images) and HYPOCAL (lower images). * ($P < 0.05$) and ** ($P < 0.01$) indicate difference from CONTROL. # ($P < 0.05$) and ## ($P < 0.01$) indicate difference from HYPOCAL alone (one-way ANOVA).

Figure 3. The production of reactive oxygen species (ROS) in neutrophils (PMNL) of dairy cows. (A) Original histogram overlays of ROS production in PMNL in CONTROL (upper images) and HYPOCAL (lower images) with and without 2APB, ionomycin, and thapsigargin. (B) Arithmetic means $\pm$ SEM ($n = 20$) of ROS production in PMNL in CONTROL (black bar) and HYPOCAL (white bar) with and without 2APB, ionomycin, and thapsigargin. * ($P < 0.05$) and ** ($P < 0.01$) indicate difference from CONTROL. # ($P < 0.05$) and ## ($P < 0.01$) indicate difference from HYPOCAL alone (one-way ANOVA).

Ca^{2+} entry as an early signaling response (Zhang et al., 2014). Data from the present study confirmed that HYPOCAL is associated with lower intracellular Ca^{2+} in PMNL and this is coupled with a

decrease in activity, migration, adhesion, phagocytosis, and chemotaxis. There is clear evidence that Ca^{2+} influx is required for various key functions of the PMNL including phagocytosis, phagosome

maturation, phagosome ROS production, degranulation, and chemotaxis (Elling et al., 2016; Immler et al., 2018). A decrease in intracellular Ca^{2+} reduces ROS production (Brchard and Tschirhart, 2008). In turn, an impairment of ROS production in response to an immune challenge could hamper the activity of PMNL signaling and production and release of pro-inflammatory factors, thereby delaying clearance of pathogens (Noubade et al., 2014; Zhang et al., 2018). Thus, Ca^{2+} homeostasis within immune cells clearly is a factor rendering cows with subclinical hypocalcemia more susceptible to other disorders (Martinez et al., 2012).

Figure 4. Migration of neutrophils (PMNL) in dairy cows. Arithmetic means $\pm$ SEM ($n = 20$) of migration in PMNL in CONTROL (black bar) and HYPOCAL (white bar) with and without 2APB, ionomycin, and thapsigargin. * ($P < 0.05$) indicates difference from CONTROL. # ($P < 0.05$) and ## ($P < 0.01$) indicate difference from HYPOCAL alone (one-way ANOVA).

It is well-known that many critical processes and functions in PMNL appear to involve Ca^{2+} signaling. Antigen receptors are triggered to induce Ca^{2+} flux from the extracellular space to intracellular space through Ca^{2+} release-activated Ca^{2+} channel (Vig and Kinet, 2009). Depletion of Ca^{2+} stores activates the plasma membrane-localized Ca^{2+} channel moiety ORAI1. Lower intracellular Ca^{2+} stores in PMNL of periparturient cows induce immune suppression (Kimura et al., 2006). From a mechanistic standpoint, expression of ORAI1 in PMNL is crucial for intracellular Ca^{2+} homeostasis. The increase in ORAI1 expression along with increases in migration, adherence and phagocytosis in response to ionomycin and thapsigargin, coupled with the abrogation of these effects by 2APB and siOrail clearly established an important role for ORAI1. Lower plasma Ca^{2+} concentrations could not replenish intracellular Ca^{2+} , meanwhile less intracellular Ca^{2+} in the ER affect cytosolic Ca^{2+} concentrations, which was required to initiate phagocytosis and ROS production in PMNL, thereby compromising PMNL activity.

The availability of Ca^{2+} is regulated by PTH (Nagai et al., 2011), $1,25(\text{OH})_2\text{D}_3$, calcitonin, and Ca^{2+} itself to maintain homeostasis (Honda et al., 2007). PTH can increase Ca^{2+} levels by acting on bone to release Ca^{2+} and increase Ca^{2+} absorption in the kidney (Loupy et al., 2012). PTH is secreted in response to decreasing extracellular Ca^{2+} concentrations. In spite of hypocalcemia,

Figure 5. Adhesion of neutrophils (PMNL) in dairy cows. Original histogram overlays of adhesion in PMNL in CONTROL (upper images) and HYPOCAL (lower images) with and without 2APB, ionomycin, and thapsigargin.

Figure 6. Phagocytosis of neutrophils (PMNL) in dairy cows. Original histogram overlays of phagocytosis in PMNL in CONTROL (upper images) and HYPOCAL (lower images) with and without 2APB, ionomycin, and thapsigargin.

Figure 7. Chemotaxis of neutrophils (PMNL) in dairy cows. (A) Arithmetic means $\pm$ SEM ($n = 20$) of concentration of plasma NAP-2/CXCL7 in neutrophils in CONTROL (black bar) and HYPOCAL (white bar). (B) Arithmetic means $\pm$ SEM ($n = 20$) of concentration of cellular NAP-2/CXCL7 in neutrophils in CONTROL (black bar) and HYPOCAL (white bar). * ($P < 0.05$) and ** ($P < 0.01$) indicate difference from CONTROL (t -test).

Figure 8. Effects of siOrail on the abundance of ORAI1 calcium release-activated calcium modulator 1 (ORAI1) protein in the neutrophils membrane. (A) Original histogram overlays of Orail protein abundance in neutrophils (PMNL) in si-neg (control images) and siOrail (siOrail images). (B) Arithmetic means $\pm$ SEM ($n = 5$) of Orail protein abundance in PMNL in si-neg (black bar) and siOrail (white bar). ** ($P < 0.01$) indicates difference from si-neg (t -test).

Figure 9. Effects of siOrai1 on reactive oxygen species (ROS), migration, adhesion, and phagocytosis of neutrophils (PMNL) in dairy cows. (A) Original histogram overlays of ROS production in PMNL in CONTROL (upper images) and HYPOCAL (lower images) with and without 2APB, ionomycin, and thapsigargin. (B) Arithmetic means $\pm$ SEM ($n = 20$) of ROS production in PMNL in CONTROL (black bar) and HYPOCAL (white bar) with and without 2APB, ionomycin, and thapsigargin. (C) Arithmetic means $\pm$ SEM ($n = 20$) of migration in PMNL in CONTROL (black bar) and HYPOCAL (white bar) with and without 2APB, ionomycin, and thapsigargin. (D) Original histogram overlays of adhesion in PMNL in CONTROL (upper images) and HYPOCAL (lower images) with and without 2APB, ionomycin, and thapsigargin. (E) Original histogram overlays of phagocytosis in PMNL in CONTROL (upper images) and HYPOCAL (lower images) with and without 2APB, ionomycin, and thapsigargin. $^*(P < 0.05)$ and $^{**}(P < 0.01)$ indicate difference from siNEG (t -test).

ORAI1-mutated tubular aggregate myopathy (TAM) individuals have markedly low PTH (Endo et al., 2015). Lower $1,25(\text{OH})_2\text{D}_3$ concentration during hypocalcemia has also been detected (Vieira-Neto et al., 2017), hence, contributing to the reduction in phosphate and calcium transport in renal and intestinal tissue (Kägi et al., 2018; Lang et al., 2018). In the recent, lower $1,25(\text{OH})_2\text{D}_3$ concentrations were detected in dairy cows with hypocalcemia. Moreover, $1,25(\text{OH})_2\text{D}_3$ and PTH treated in PMNL could increase cytosolic Ca^{2+} concentrations and ORAI1 abundance in dairy cows with hypocalcemia. In conclusion, plasma PTH or $1,25(\text{OH})_2\text{D}_3$ could regulate ORAI1 and also participate in the regulation of PMNL activity. In addition, the inability of PMNL from cows with HYPOCAL to increase Ca^{2+} uptake to normal levels in spite of activation of the Ca^{2+} entry pathway needs to be studied further.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Animal Science* online.

Conflict of interest statement. None declared.

LITERATURE CITED

- Baydyuk, M., X. S. Wu, L. He, and L. G. Wu. 2015. Brain-derived neurotrophic factor inhibits calcium channel activation, exocytosis, and endocytosis at a central nerve terminal. *J. Neurosci.* 35:4676–4682. doi:10.1523/JNEUROSCI.2695-14.2015.
- Bose, T., A. Cieřlar-Pobuda, and E. Wiechec. 2015. Role of ion channels in regulating Ca^{2+} homeostasis during the interplay between immune and cancer cells. *Cell Death Dis.* 6:e1648. doi:10.1038/cddis.2015.23.
- Bréchar, S., and E. J. Tschirhart. 2008. Regulation of superoxide production in neutrophils: role of calcium influx. *J. Leukoc. Biol.* 84:1223–1237. doi:10.1189/jlb.0807553.
- Curtis, C. R., H. N. Erb, C. J. Sniffen, R. D. Smith, P. A. Powers, M. C. Smith, M. E. White, R. B. Hillman, and E. J. Pearson. 1983. Association of parturient hypocalcemia with eight periparturient disorders in holstein cows. *J. Am. Vet. Med. Assoc.* 183:559–561.
- Demaurex, N., and P. Nunes. 2016. The role of STIM and ORAI proteins in phagocytic immune cells. *Am. J. Physiol. Cell Physiol.* 310:C496–C508. doi:10.1152/ajpcell.00360.2015.
- Elling, R., B. Keller, C. Weidinger, M. Häffner, S. D. Deshmukh, I. Zee, C. Speckmann, S. Ehl, K. Schwarz, S. Feske, et al. 2016. Preserved effector functions of human ORAI1- and STIM1-deficient neutrophils. *J. Allergy Clin. Immunol.* 137:1587–1591.e7. doi:10.1016/j.jaci.2015.09.047.
- Endo, Y., S. Noguchi, Y. Hara, Y. K. Hayashi, K. Motomura, S. Miyatake, N. Murakami, S. Tanaka, S. Yamashita, R.

- Kizu, et al. 2015. Dominant mutations in ORAI1 cause tubular aggregate myopathy with hypocalcemia via constitutive activation of store-operated Ca^{2+} channels. *Hum. Mol. Genet.* 24:637–648. doi:10.1093/hmg/ddu477.
- Giachini, F. R., V. V. Lima, J. L. Hannan, F. S. Carneiro, R. C. Webb, and R. C. Tostes. 2011. STIM1/orai1-mediated store-operated Ca^{2+} entry: the tip of the iceberg. *Braz. J. Med. Biol. Res.* 44:1080–1087. doi:10.1590/s0100-879x2011007500133.
- Goff, J. P. 2008. The monitoring, prevention, and treatment of milk fever and subclinical hypocalcemia in dairy cows. *Vet. J.* 176:50–57. doi:10.1016/j.tvjl.2007.12.020.
- Griffiths, H., and K. T. MacLeod. 2003. The voltage-sensitive release mechanism of excitation contraction coupling in rabbit cardiac muscle is explained by calcium-induced calcium release. *J. Gen. Physiol.* 121:353–373. doi:10.1085/jgp.200208764.
- Herrmann, J. M., and J. Meyle. 2015. Neutrophil activation and periodontal tissue injury. *Periodontol.* 2000 69:111–127. doi:10.1111/prd.12088.
- Honda, H., N. Hosaka, and T. Akizawa. 2007. [Kidney as a regulating organ for calcium and phosphate homeostasis]. *Clin. Calcium* 17:654–658. doi:CliCa0705654658.
- Immler, R., S. I. Simon, and M. Sperandio. 2018. Calcium signalling and related ion channels in neutrophil recruitment and function. *Eur. J. Clin. Invest.* 48(Suppl 2):e12964. doi:10.1111/eci.12964.
- Jawor, P. E., J. M. Huzzey, S. J. LeBlanc, and M. A. von Keyserlingk. 2012. Associations of subclinical hypocalcemia at calving with milk yield, and feeding, drinking, and standing behaviors around parturition in holstein cows. *J. Dairy Sci.* 95:1240–1248. doi:10.3168/jds.2011-4586.
- Kägi, L., C. Bettoni, E. M. Pastor-Arroyo, U. Schnitzbauer, N. Hernando, and C. A. Wagner. 2018. Regulation of vitamin D metabolizing enzymes in murine renal and extrarenal tissues by dietary phosphate, FGF23, and 1,25(OH)2D3. *Plos One* 13:e0195427. doi:10.1371/journal.pone.0195427.
- Kimura, K., T. A. Reinhardt, and J. P. Goff. 2006. Parturition and hypocalcemia blunts calcium signals in immune cells of dairy cattle. *J. Dairy Sci.* 89:2588–2595. doi:10.3168/jds.S0022-0302(06)72335-9.
- Lang, F., M. Föller, K. Lang, P. Lang, M. Ritter, A. Vereninov, I. Szabo, S. M. Huber, and E. Gulbins. 2007. Cell volume regulatory ion channels in cell proliferation and cell death. *Methods Enzymol.* 428:209–225. doi:10.1016/S0076-6879(07)28011-5.
- Lang, F., C. Leibrock, A. A. Pandya, C. Stournaras, C. A. Wagner, and M. Föller. 2018. Phosphate homeostasis, inflammation and the regulation of FGF-23. *Kidney Blood Press. Res.* 43:1742–1748. doi:10.1159/000495393.
- Loupy, A., S. K. Ramakrishnan, B. Wootla, R. Chambrey, R. de la Faille, S. Bourgeois, P. Bruneval, C. Mandet, E. I. Christensen, H. Faure, et al. 2012. PTH-independent regulation of blood calcium concentration by the calcium-sensing receptor. *J. Clin. Invest.* 122:3355–3367. doi:10.1172/JCI57407.
- Martinez, N., C. A. Risco, F. S. Lima, R. S. Bisinotto, L. F. Greco, E. S. Ribeiro, F. Maunsell, K. Galvão, and J. E. Santos. 2012. Evaluation of periparturient calcium status, energetic profile, and neutrophil function in dairy cows at low or high risk of developing uterine disease. *J. Dairy Sci.* 95:7158–7172. doi:10.3168/jds.2012-5812.
- Martinez, N., L. D. Sinedino, R. S. Bisinotto, E. S. Ribeiro, G. C. Gomes, F. S. Lima, L. F. Greco, C. A. Risco, K. N. Galvão, D. Taylor-Rodriguez, et al. 2014. Effect of induced subclinical hypocalcemia on physiological responses and neutrophil function in dairy cows. *J. Dairy Sci.* 97:874–887. doi:10.3168/jds.2013-7408.
- Melendez, P., G. A. Donovan, C. A. Risco, and J. P. Goff. 2004. Plasma mineral and energy metabolite concentrations in dairy cows fed an anionic prepartum diet that did or did not have retained fetal membranes after parturition. *Am. J. Vet. Res.* 65:1071–1076. doi:10.2460/ajvr.2004.65.1071.
- Mulligan, F. J., L. O'Grady, D. A. Rice, and M. L. Doherty. 2006. A herd health approach to dairy cow nutrition and production diseases of the transition cow. *Anim. Reprod. Sci.* 96:331–353. doi:10.1016/j.anireprosci.2006.08.011.
- Nagai, S., M. Okazaki, H. Segawa, C. Bergwitz, T. Dean, J. T. Potts, Jr, M. J. Mahon, T. J. Gardella, and H. Jüppner. 2011. Acute down-regulation of sodium-dependent phosphate transporter NPT2A involves predominantly the camp/PKA pathway as revealed by signaling-selective parathyroid hormone analogs. *J. Biol. Chem.* 286:1618–1626. doi:10.1074/jbc.M110.198416.
- Neves, R. C., B. M. Leno, K. D. Bach, and J. A. A. McArt. 2018. Epidemiology of subclinical hypocalcemia in early-lactation holstein dairy cows: the temporal associations of plasma calcium concentration in the first 4 days in milk with disease and milk production. *J. Dairy Sci.* 101:9321–9331. doi:10.3168/jds.2018-14587.
- Noubade, R., K. Wong, N. Ota, S. Rutz, C. Eidenschenk, P. A. Valdez, J. Ding, I. Peng, A. Sebrell, P. Caplazi, et al. 2014. NRROS negatively regulates reactive oxygen species during host defence and autoimmunity. *Nature* 509:235–239. doi:10.1038/nature13152.
- de Oliveira, S., E. E. Rosowski, and A. Huttenlocher. 2016. Neutrophil migration in infection and wound repair: going forward in reverse. *Nat. Rev. Immunol.* 16:378–391. doi:10.1038/nri.2016.49.
- Parekh, A. B. 2010. Store-operated CRAC channels: function in health and disease. *Nat. Rev. Drug Discov.* 9:399–410. doi:10.1038/nrd3136.
- Putney, J. W., N. Steinckwich-Besançon, T. Numaga-Tomita, F. M. Davis, P. N. Desai, D. M. D'Agostin, S. Wu, and G. S. Bird. 2017. The functions of store-operated calcium channels. *Biochim. Biophys. Acta. Mol. Cell Res.* 1864:900–906. doi:10.1016/j.bbamer.2016.11.028.
- Rasby, R. J., R. P. Wettemann, R. D. Geisert, L. E. Rice, and C. R. Wallace. 1990. Nutrition, body condition and reproduction in beef cows: fetal and placental development, and estrogens and progesterone in plasma. *J. Anim. Sci.* 68:4267–4276. doi:10.2527/1990.68124267x.
- Reinhardt, T. A., J. D. Lippolis, B. J. McCluskey, J. P. Goff, and R. L. Horst. 2011. Prevalence of subclinical hypocalcemia in dairy herds. *Vet. J.* 188:122–124. doi:10.1016/j.tvjl.2010.03.025.
- Rodríguez, E. M., A. Arís, and A. Bach. 2017. Associations between subclinical hypocalcemia and postparturient diseases in dairy cows. *J. Dairy Sci.* 100:7427–7434. doi:10.3168/jds.2016-12210.
- Rosales, C. 2018. Neutrophil: a cell with many roles in inflammation or several cell types? *Front. Physiol.* 9:113. doi:10.3389/fphys.2018.00113.

- Schaff, U. Y., N. Dixit, E. Procyk, I. Yamayoshi, T. Tse, and S. I. Simon. 2010. Orai1 regulates intracellular calcium, arrest, and shape polarization during neutrophil recruitment in shear flow. *Blood* 115:657–666. doi:10.1182/blood-2009-05-224659.
- Shambharkar, P. B., M. Bittinger, B. Latario, Z. Xiong, S. Bandyopadhyay, V. Davis, V. Lin, Y. Yang, R. Valdez, and M. A. Labow. 2015. TMEM203 is a novel regulator of intracellular calcium homeostasis and is required for spermatogenesis. *Plos One* 10:e0127480. doi:10.1371/journal.pone.0127480.
- Soboloff, J., B. S. Rothberg, M. Madesh, and D. L. Gill. 2012. STIM proteins: dynamic calcium signal transducers. *Nat. Rev. Mol. Cell Biol.* 13:549–565. doi:10.1038/nrm3414.
- Thilising-Hansen, T., R. J. Jørgensen, and S. Østergaard. 2002. Milk fever control principles: a review. *Acta Vet. Scand.* 43:1–19.
- Vieira-Neto, A., I. R. P. Lima, F. Lopes, Jr, C. Lopera, R. Zimpel, L. D. P. Sinedino, K. C. Jeong, K. Galvão, W. W. Thatcher, C. D. Nelson, et al. 2017. Use of calcitriol to maintain postpartum blood calcium and improve immune function in dairy cows. *J. Dairy Sci.* 100:5805–5823. doi:10.3168/jds.2016-12506.
- Vig, M., and J. P. Kinet. 2009. Calcium signaling in immune cells. *Nat. Immunol.* 10:21–27. doi:10.1038/ni.f.220.
- Wang, Y., X. Deng, T. Hewavitharana, J. Soboloff, and D. L. Gill. 2008. Stim, ORAI and TRPC channels in the control of calcium entry signals in smooth muscle. *Clin. Exp. Pharmacol. Physiol.* 35:1127–1133. doi:10.1111/j.1440-1681.2008.05018.x.
- Wang, P. X., S. Shu, C. Xia, Z. Wang, L. Wu, B. Wang, C. C. Xu, and J. Liu. 2016. Protein expression in dairy cows with and without subclinical hypocalcaemia. *N. Z. Vet. J.* 64:101–106. doi:10.1080/00480169.2015.1100970.
- Wang, X., W. Qin, X. Xu, Y. Xiong, Y. Zhang, H. Zhang, and B. Sun. 2017. Endotoxin-induced autocrine ATP signaling inhibits neutrophil chemotaxis through enhancing myosin light chain phosphorylation. *Proc. Natl. Acad. Sci. U S A.* 114: 4483–4488. doi:10.1073/pnas.1616752114.
- Weiss, N., and G. W. Zamponi. 2013. Control of low-threshold exocytosis by T-type calcium channels. *Biochim. Biophys. Acta* 1828:1579–1586. doi:10.1016/j.bbame.2012.07.031.
- Zhang, H., R. A. Clemens, F. Liu, Y. Hu, Y. Baba, P. Theodore, T. Kurosaki, and C. A. Lowell. 2014. STIM1 calcium sensor is required for activation of the phagocyte oxidase during inflammation and host defense. *Blood* 123:2238–2249. doi:10.1182/blood-2012-08-450403.
- Zhang, Y., X. Li, H. Zhang, Z. Zhao, Z. Peng, Z. Wang, G. Liu, and X. Li. 2018. Non-esterified fatty acids over-activate the TLR2/4-NF- κ b signaling pathway to increase inflammatory cytokine synthesis in neutrophils from ketotic cows. *Cell. Physiol. Biochem.* 48:827–837. doi:10.1159/000491913.