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# Lasting inhibition of receptor-mediated calcium oscillations in pancreatic acini by neutrophil respiratory burst – A novel mechanism for secretory blockade in acute pancreatitis?

Hui Yuan Liang, Zhi Min Song, Zong Jie Cui *

*Institute of Cell Biology, Beijing Normal University, Beijing 100875, China*

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ABSTRACT

Although overwhelming evidence indicates that neutrophil infiltration is an early event in acute pancreatitis, the effect of neutrophil respiratory burst on pancreatic acini has not been investigated. In the present work, effect of fMLP-induced neutrophil respiratory burst on pancreatic acini was examined. It was found that neutrophil respiratory burst blocked calcium oscillations induced by cholecystokinin or by acetylcholine. Such lasting inhibition was dependent on the density of bursting neutrophils and could be overcome by increased agonist concentration. Inhibition of cholecystokinin stimulation was also observed in AR4-2J cells. In sharp contrast, neutrophil respiratory burst had no effect on calcium oscillations induced by phenylephrine (PE), vasopressin, or by ATP in rat hepatocytes. These data together suggest that inhibition of receptor-mediated calcium oscillations in pancreatic acini by neutrophil respiratory burst would lead to secretory blockade, which is a hallmark of acute pancreatitis. The present work has important implications for clinical treatment and management of acute pancreatitis.

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## 1. Introduction

Neutrophil is an essential component of innate immune system [1]. It has been reported that neutrophil content in blood increased in patients with inflammation, with concurrent neutrophil infiltration of inflamed tissues [2–4]. Respiratory burst of neutrophils frequently leads to damage to surrounding tissues. Neutrophil contents both in the whole blood and in inflamed tissues could be used for diagnosis of inflammatory diseases [5–8]. Reports have suggested that neutrophil infiltration is a key event in the initiation and development of acute pancreatitis [3,9,10].

Reports both on pancreatitic patients [5–7] and on animal models have suggested that neutrophils play a pivotal role in the pathogenesis of acute pancreatitis [3,11,12]. The activity of neutrophil myeloperoxidase and elastase in the blood has been used as makers of acute pancreatitis [5,7]. Myeloperoxidase and neutrophil elastase contents were at peak values on day 1 of acute pancreatitis [5–7], but the blood contents of other markers such as C-active protein did not reach peak levels until on days 2–4 or even later [5–7]. It is possible to measure neutrophil infiltration in greater temporal detail in animal models of acute pancreatitis. Different methods to measure neutrophil content in inflamed pancreas all suggested that neutrophil infiltration start within 1 h of the onset of acute pancreatitis [3,12]. Chemiluminescent measurements of superoxide anion on the surface of pancreas further confirmed that neutrophil infiltration started early in acute pancreatitis [11,13]. Therefore abundant evidence points to early neutrophil infiltration in acute pancreatitis. These understandings notwithstanding, how neutrophils might regulate pancreatic acinar cell function is not known.

With the above in mind, we have investigated the effect of respiratory burst of neutrophils on pancreatic acinar cell function in a co-incubation system. Real time functional changes in pancreatic acini after neutrophil respiratory burst were examined by monitoring calcium concentration in perifused pancreatic acini. It was found that neutrophil respiratory burst blocked calcium oscillations induced by physiological concentrations of CCK and ACh. The inhibition of CCK1 receptor in pancreatic acini by neutrophil respiratory burst was reproduced in pancreatic tumor cell AR4-2J. Such inhibition of receptor-mediated cytosolic calcium oscillations by neutrophil respiratory burst was not seen in isolated rat hepatocytes. Calcium oscillations induced by activation of $\alpha$1 adrenergic receptors, V1a vasopressin receptors, or P2Y purinergic receptors were not inhibited by neutrophil respiratory burst. To the best of our knowledge, this is the first report to show that neutrophil respiratory burst blocks cell surface receptors in pancreatic acini. This has important implications for the understanding of pathogenesis of acute pancreatitis and may provide guidelines for the clinical treatment of acute pancreatitis.

* Corresponding author.
*E-mail address:* zjcui@bnu.edu.cn (Z.J. Cui).

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## 2. Materials and methods

### 2.1. Reagents

Cholecystokinin octapeptide (CCK), bethanechol (Beth), acetylcholine chloride (ACh), soybean trypsin inhibitor, arginine vasopressin (VP), L-phenylephrine (PE), L-glutamine, N-formyl-Met-Leu-Phe (fMLP), ATP and heparin sodium were from Sigma–Aldrich (St. Louis, MO, USA). Collagenase P and collagenase H were from Roche (Mannheim, Germany). Bovine serum albumin (BSA), 4-(2-Hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES) were bought from Calbiochem (Darmstadt, Germany). Fura-2 AM was from AAT Bioquest Inc. (Sunnyvale, CA, USA). Dextran T-500 was from Fluka. Concentrated MEM amino acids mixture, DMEM/F12 (1:1) medium, 0.25% trypsin (with EDTA) were from Invitrogen. Cell-Tak was from BD Biosciences (Bedford, MA, USA).

### 2.2. Isolation of rat pancreatic acini

Male Sprague-Dawley rats (250 ± 50 g) were used for pancreatic acini isolation [14]. Excised pancreas was digested by collagenase P (2 g $L^{-1}$) at 37 °C in a shaking water bath for 30 min (120 cycles per min) then dispersed with a plastic pipette. Isolated acini were filtered and washed before use.

Buffer for acini isolation had the following composition (in mM): 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.13 $MgCl_2$, 1.0 $NaH_2PO_4$, 5.5 D-glucose, 10 HEPES, 2.0 L-glutamine, and 2% BSA, 2% minimum essential medium amino acids mixture, 0.1 g $L^{-1}$ soybean trypsin inhibitor (SBTI). Buffer pH was adjusted to 7.4 with 4 M NaOH, and buffer was oxygenated with $O_2$. Buffer for acini perfusion had the same composition, but SBTI, amino acids mixture, glutamine and BSA were omitted.

### 2.3. Isolation of rat neutrophils

Rat neutrophils were isolated from heparinized blood [15]. Blood was collected from Sprague-Dawley rats (250–400 g). Neutrophils were dextran sedimented (4.5% dextran in 0.9% NaCl solution in 40 min at 4 °C), centrifuged through a solution for lymphocyte separation (500 g, 15 min). Residual erythrocytes were broken by hypotonic lysis. Isolated neutrophils had a purity of >95% as determined by Wright's stain. Cell viability was >95% as checked by trypan blue exclusion.

### 2.4. AR4-2J cell culture

AR4-2J cells were cultured in a mixed medium (DMEM/F12 by 1:1) containing FBS (20% v/v) and antibiotics (100 μg/ml streptomycin, 100 unit/ml penicillin) as reported [16].

### 2.5. Measurement of cytosolic calcium concentration

Cells were loaded with Fura-2 AM (at 10 μM) for 30 min (60 min for AR4-2J) in a shaking water bath (37 °C, 50 cycles per min). Fura-2 AM-loaded cells were attached to Cell-Tak-coated cover-slips, which formed the bottom of Sykes-Moor perfusion chambers. Cells were perfused at a rate of 1 mL $min^{-1}$, and stimulants were introduced by changing buffers. Calcium concentration was measured on an inverted fluorescence microscope (Nikon TE 2000U), which was coupled to a calcium imaging system (EasyRatioPro, Photon Technology International, New Jersey, USA). Fura-2 was excited alternately at 340/380 nm (monochromator slit width 1 nm), fluorescence images were captured (after passing through a band pass filter 510 ± 40 nm) on a CCD camera (QuantEM 512SC, Roper Scientific) (Figs. 1–4, S1 and S2). Data in Figs. 3D, 4D, S3 were obtained in a PMT-based system [14]. Fluorescence ratios (F340/F380) were taken as indicative of calcium concentration [17,18]. Calcium oscillatory frequencies induced by the 1st and 2nd agonist stimulations in *N* experiments were analyzed and presented as mean ± SEM next to the calcium traces. Asterisk (*) indicates $P < 0.05$.

## 3. Results

### 3.1. Neutrophil respiratory burst blocked CCK-induced calcium oscillations in rat pancreatic acini

Bacterial peptide fMLP triggers respiratory burst [19]. fMLP ($10^{-8} - 10^{-4}$ M) induced respiratory burst dose-dependently in isolated rat neutrophils, with maximum effect at $10^{-5}$ M (data not shown). fMLP 10 μM was therefore used to trigger respiratory burst. Neutrophils (at a density of $5 \times 10^5$/ml) alone or after incubation with fMLP had no effect on basal calcium in isolated rat pancreatic acini (Fig. 1A). Two sequential doses of CCK (10 pM) induced reproducible calcium oscillations (Fig. 1B). fMLP (10 μM) alone had no effect on either basal calcium or on the second dose of CCK stimulation (Fig. 1C). After simultaneously additions (with a 10 s gap) of neutrophils ($5 \times 10^5$/ml) and fMLP (10 μM), however, a second dose of CCK (10 pM) failed to induce any calcium oscillations (Fig. 1D). In this latter experiment (Fig. 1D), to ensure full contact between stimulated neutrophils and pancreatic acin, perfusion was stopped for 4 min immediately after the addition of neutrophils. Perfusion was re-started 4 min later. The multiple color-coded calcium traces in each panel (Fig. 1A–D) indicate calcium changes in individual pancreatic acinar cells in one typical experiment, the *N* numbers in each figure indicate number of rats used to perform identical experiments.

Time-matched parallel experiments were done as shown in Fig. 2A–C. Two sequential doses of CCK induced reproducible calcium oscillations (Fig. 2A). After respiratory burst (neutrophils, $5 \times 10^5$/ml plus fMLP 10 μM), a second CCK dose no longer induced any calcium oscillations (Fig. 2B). Pancreatic acini exposed to respiratory burst remained perfectly healthy, because a subsequent dose of bethanechol (Beth, 5 μM) induced a robust calcium increase (Fig. 2B). Respiratory burst from a reduced amount of neutrophils ($1.67 \times 10^5$/ml, 1/3 of that in Fig. 1D and Fig. 2B) did not block calcium oscillations induced by a second CCK dose (Fig. 2C). Non-bursting neutrophils ($10^6$/ml, double density of Fig. 1D and Fig. 2B) alone, however, had no effect on calcium oscillations induced by a second dose of CCK 10 pM (Fig. 2D). Therefore inhibition exerted by respiratory burst was dependent on the density of bursting neutrophils.

### 3.2. Neutrophil respiratory burst blocked ACh-induced calcium oscillations in rat pancreatic acini

Similarly, ACh-induced calcium oscillations were also inhibited by neutrophil respiratory burst (Fig. 3). In these experiments, sequential doses of ACh (20 nM) induced reproducible calcium oscillations in isolated rat pancreatic acini (Fig. 3A). The pattern of calcium oscillations induced by ACh was slightly different from CCK (compare Fig. 3 with Figs. 1 and 2). A higher ACh dose (100 nM) induced plateau calcium increase (Fig. 3A). fMLP 10 μM alone had no effect on either basal calcium or calcium oscillations induced by a second dose of ACh 20 nM(Fig. 3B); ACh 100 nM still induced a long-lasting calcium plateau (Fig. 3B). After neutrophil respiratory burst, the second dose of ACh 20 nM failed to induce any calcium oscillations; even ACh 100 nM had no effect (Fig. 3C). An ACh dose of 200 nM was required to induce regular calcium oscillations (Fig. 3C). Therefore inhibition of calcium

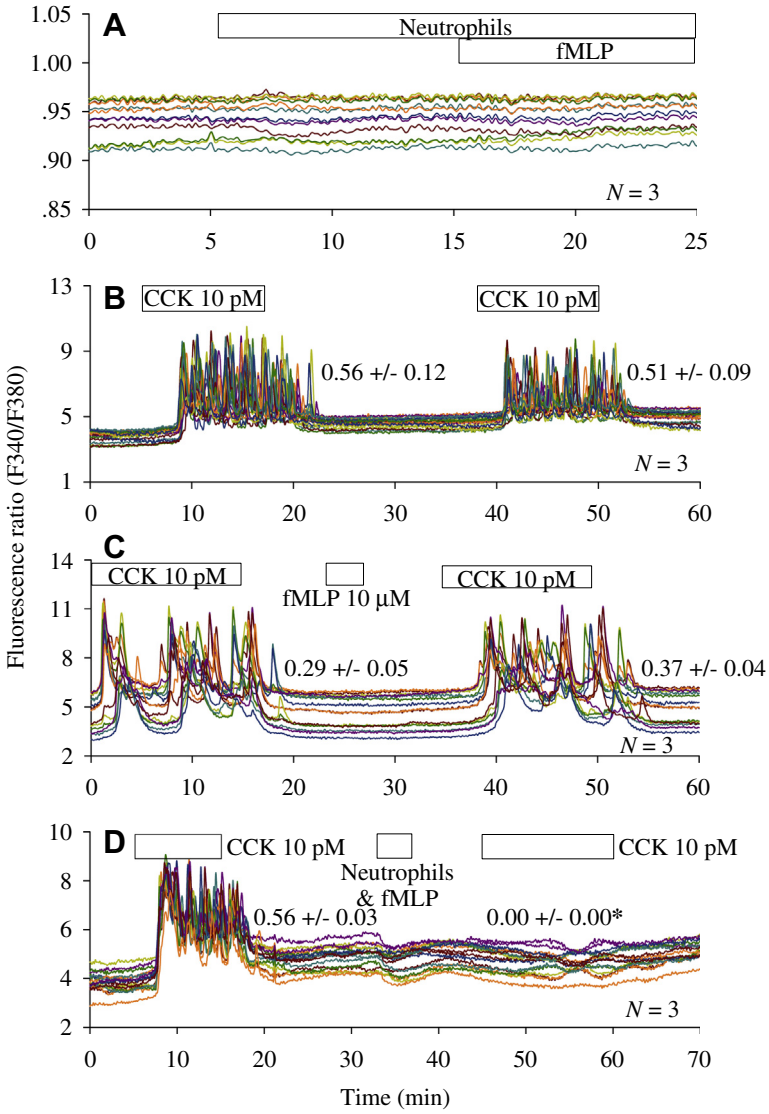


**Fig. 1.** Rat neutrophil respiratory burst blocked calcium oscillations induced by CCK in pancreatic acini. Fura-2-loaded rat pancreatic acini were perifused and neutrophils, fMLP, CCK were added as indicated by the horizontal bars. (A) Neutrophils alone, or simultaneous presence of neutrophils ($5 \times 10^5$/ml) plus fMLP (10 µM) ($N = 3$). (B) Two sequential doses of CCK (10 pM) were added ($N = 3$). (C) fMLP (10 µM, 4 min) and 2 sequential doses of CCK (10 pM) were added ($N = 3$). (D) Simultaneous addition (for 4 min) of neutrophils ($5 \times 10^5$/ml) and fMLP (10 µM) in between 2 tandem doses of CCK (10 pM). Color-coded calcium traces in each panel were from individual pancreatic acinar cell in a single experiment. These are representative of $N$ separate experiments. Averaged oscillatory frequencies are presented as calcium spikes per min.

oscillations by neutrophil respiratory burst could be overcome by increased agonist (ACh in this case) concentration (Fig. 3C). Similar to CCK stimulation, non-bursting neutrophils ($10^6$/ml, double density of Fig. 3C) alone also had no effect on calcium oscillations induced by a second dose of ACh 20 nM (Fig. 3D).

### *3.3. Neutrophil respiratory burst blocked calcium increases induced by CCK in AR4-2J cells*

AR4-2J cells were derived from an exocrine pancreatic tumor in rat [20]. CCK (10 pM) induced fluctuating calcium increases in AR4-2J cells, these calcium increases were reproduced by a second CCK dose (Fig. 4A). fMLP 10 µM alone had no effect on CCK-induced calcium increases (Fig. 4B). But neutrophil respiratory burst (neutrophils $5 \times 10^5$/ml activated by fMLP 10 µM) blocked calcium increases stimulated by the second dose of CCK 10 pM (Fig. 4C). Neutrophils alone even at higher density ($10^6$/ml) had no effect (Fig. 4D).

## 4. Discussion

It has been found in this work that neutrophil respiratory burst blocked calcium oscillations triggered by CCK and ACh in rat pancreatic acini (Figs. 1–3), and blocked calcium increases triggered by CCK in AR4-2J cells (Fig. 4). In sharp contrast, neutrophil respiratory burst had no effect on calcium oscillations induced by PE, ATP or by vasopressin in isolated rat hepatocytes (Figs. S1–S3).

CCK and ACh are physiological secretagogues for rodent pancreatic acini, they activate CCK1 receptors and M3 cholinergic receptors, respectively [21,22]. In the present work, CCK (10 pM) and ACh (20 nM) induced calcium oscillations in isolated rat pancreatic acini (Figs. 1–3). To our great surprise, it was found that fMLP-activated neutrophils blocked CCK- and ACh-induced calcium oscillations (Figs. 1–3). Inhibition of CCK response was also found in AR4-2J cells (Fig. 4). Total inhibition of calcium oscillations in pancreatic acini by neutrophil respiratory burst has important implications for the pathogenesis of acute pancreatitis.

Neutrophil infiltration is pivotal in the pathogenesis of acute pancreatitis [12,23,24]. It has been reported that neutrophil

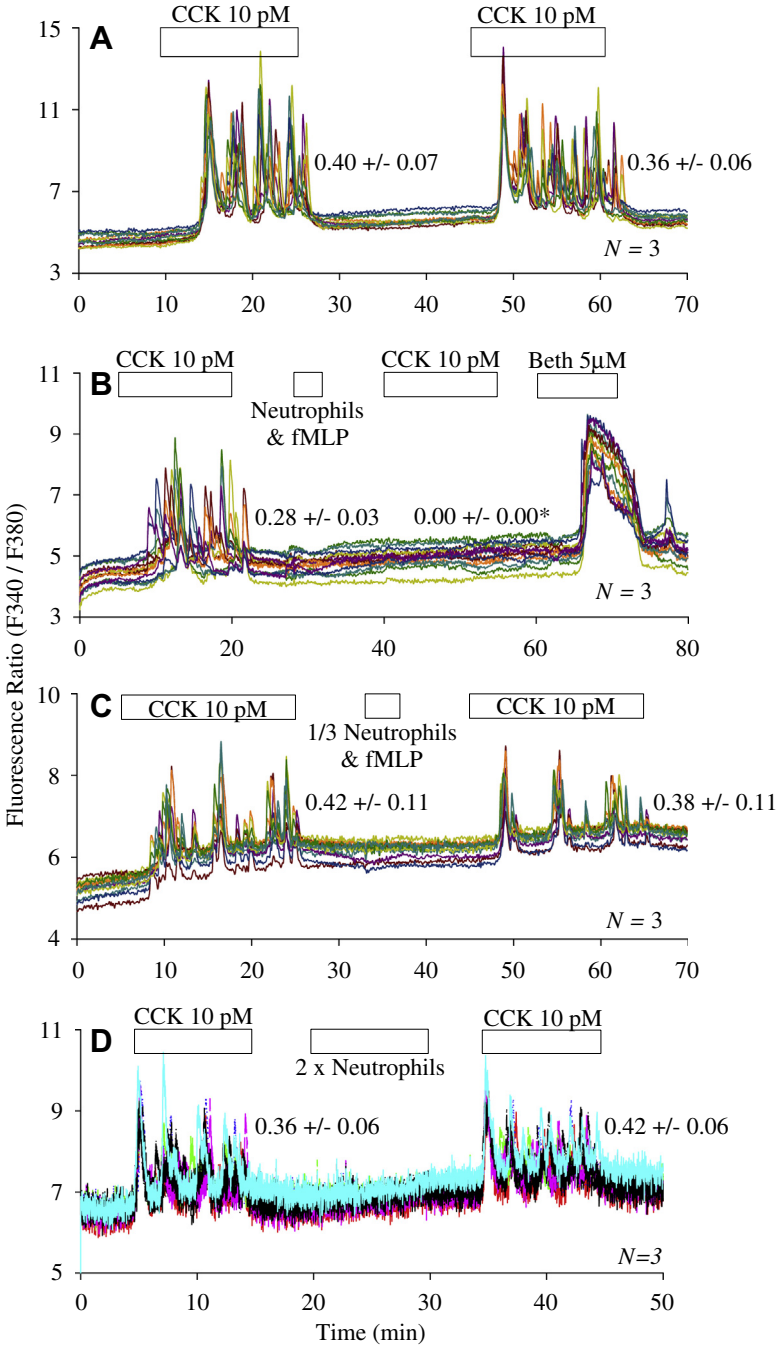


**Fig. 2.** Neutrophil respiratory burst blocked calcium oscillations induced by CCK in pancreatic acini. Dependence on neutrophil density. Fura-2-loaded rat pancreatic acini were perifused, CCK, neutrophils, fMLP and bethanechol (Beth) were added as indicated by the horizontal bars. (A) Two tandem doses of CCK (10 pM) were added ($N = 3$). (B) Simultaneous addition (4 min) of neutrophils ($5 \times 10^5$/ml) and fMLP (10 µM) in between 2 tandem doses of CCK (10 pM), followed by bethanechol (Beth, 5 µM) ($N = 3$). (C) Simultaneous addition (4 min) of neutrophils ($1.67 \times 10^5$/ml, 1/3 of neutrophil density in panel B) and fMLP (10 µM) in between 2 tandem doses of CCK (10 pM)($N = 3$). (D) Neutrophils ($10^6$/ml, double of panel B) and two tandem doses of CCK (10 pM) were added ($N = 3$). Color-coded calcium traces in each panel were from individual pancreatic acinar cells in a single experiment. These are representative of $N$ separate experiments. Experiments in panels ***A-C*** were done in time-matched parallel. Averaged oscillatory frequencies are presented as calcium spikes per min.

infiltration started 2–3 h after induction of acute pancreatitis, as indicated by measurements of superoxide generation [11,13]. Pancreatic neutrophil infiltration started 1 h after injection of 5% sodium taurocholate, as indicated by measurements of leukotriene generation [25]. Measurements in pancreatic myeloperoxidase indicated that neutrophil infiltration started as early as half an hour after sodium taurocholate infusion in rats [26]. In human patients, neutrophil infiltration increased significantly on the first day of acute pancreatitis as indicated by plasma myeloperoxidase levels [5]. Neutrophil elastase has been accepted as a reliable marker for the early phase of acute pancreatitis [6,7].

The present work reports on the regulation of pancreatic acini by activated neutrophils and its implications for acute pancreatitis. A widely recognized feature of acute pancreatitis is inhibited pancreatic secretion [27]. Previous studies have suggested that at least three factors contribute to secretory blockade: reduced apical secretion, enhanced basolateral secretion, disruption of paracellular barrier [27–29]. Our data presented here indicate that

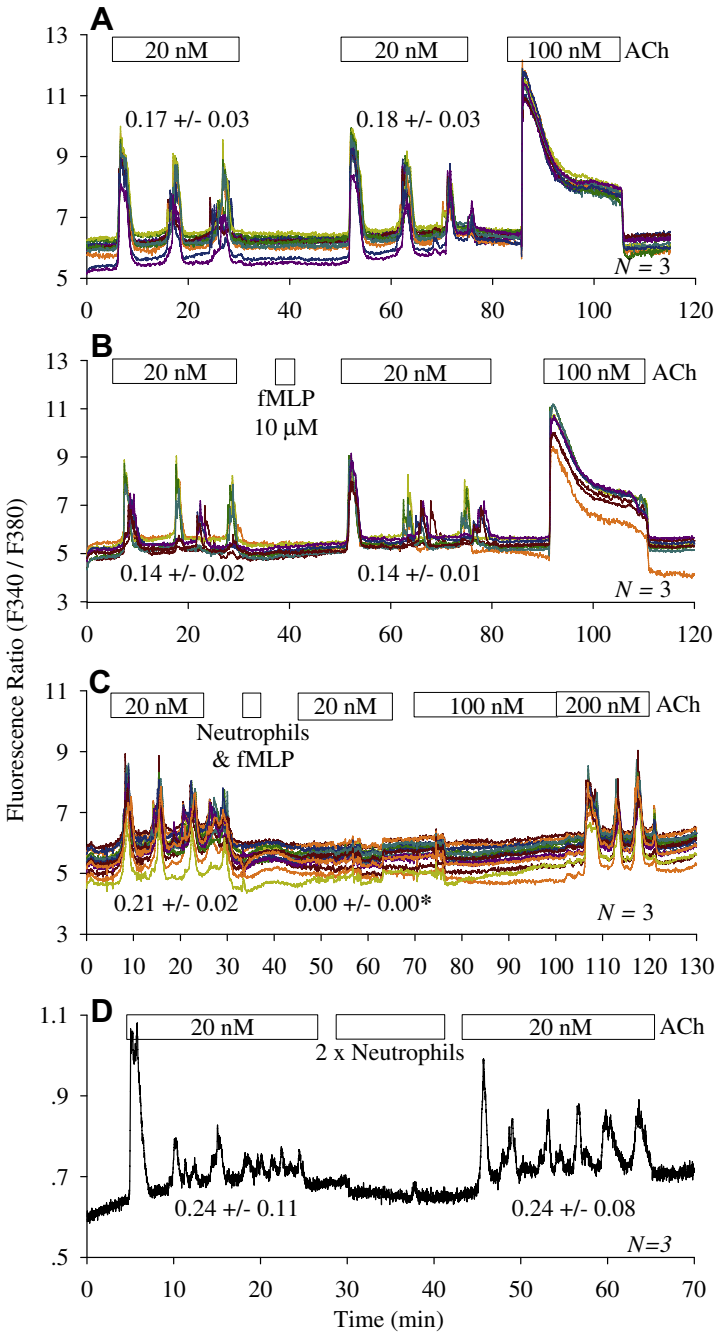


**Fig. 3.** Rat neutrophil respiratory burst blocked calcium oscillations induced by ACh in pancreatic acini. Fura-2-loaded rat pancreatic acini were perifused, ACh, fMLP and neutrophils were added as indicated by the horizontal bars. (A) Two tandem doses of ACh 20 nM followed by ACh 100 nM ($N = 3$). (B) fMLP (10 µM, 4 min) was added in between 2 tandem doses of ACh 20 nM, followed by ACh 100 nM ($N = 3$); (C) Simultaneous addition (4 min) of neutrophils ($5 \times 10^5$/ml) and fMLP (10 µM) in between 2 tandem doses of ACh 20 nM, followed by ACh at 100, 200 nM ($N = 3$). (D) Neutrophils ($10^6$/ml, double of panel C) and two sequential doses of ACh (20 nM) were added ($N = 3$). Color-coded (A–C) or black (D) calcium traces in each panel were from individual pancreatic acinar cells in a single experiment. These are representative of $N$ identical experiments. Averaged oscillatory frequencies are presented as calcium spikes per min.

neutrophils could inhibit pancreatic secretion by blocking calcium oscillations which were mediated by CCK1 and M3 receptors (Figs. 1–4). In other words, blocking calcium oscillations would lead to secretory blockade and zymogen retention, because pancreatic acinar cell secretion is calcium-dependent [21,22].

Indeed retention of activated zymogens in pancreatic acinar cells is the major cause for auto-digestion. Zymogen activation is also related to neutrophil contents and to respiratory burst activity. Neutrophil depletion reduced trypsinogen activation in mouse pancreatic acini [23]. Trypsinogen activation was also lower in NADPH oxidase (NOX)-knockout mice than in wild-type littermates [23]. Secretagogue receptors also seem correlated with neutrophil infiltration. Antagonist inhibition of CCK1 receptors *before* pancreatitis induction in rat enhanced neutrophil infiltration and aggravated acute pancreatitis [30]. Receptor inhibition may stimulate CCK or ACh release through feedback regulation, which would result in subsequent over-stimulation and intracellular digestive enzyme activation, leading to acute pancreatitis [31].

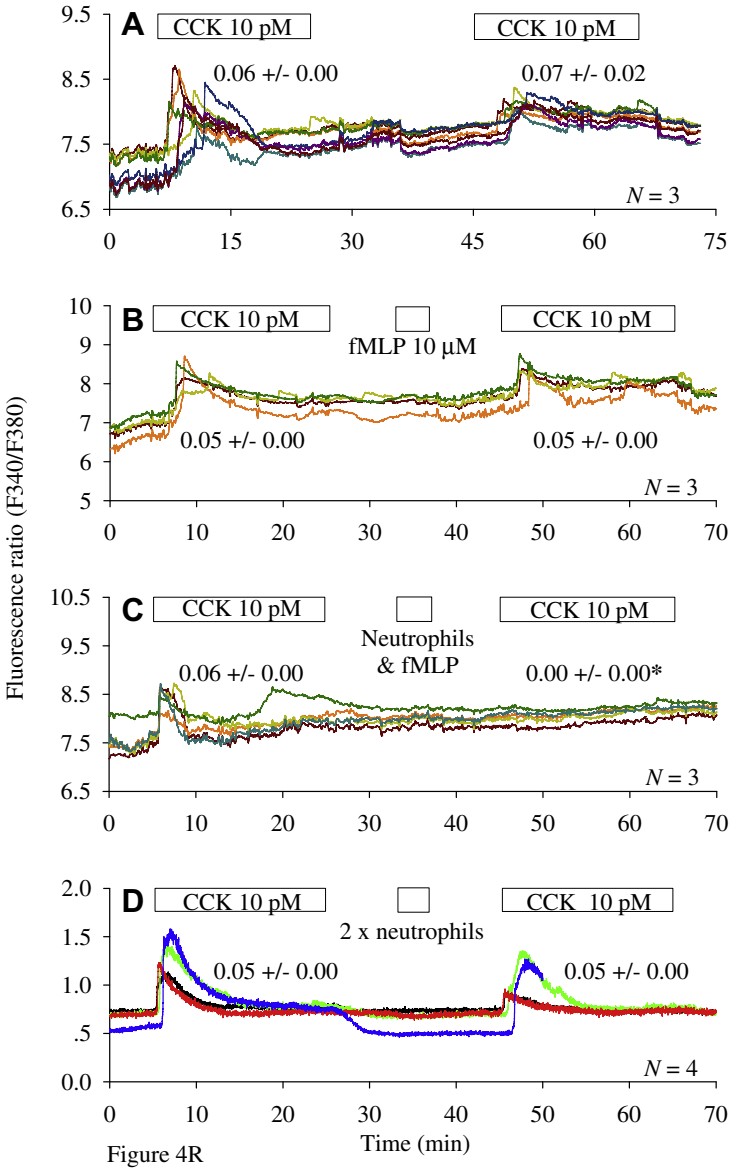


**Fig. 4.** Neutrophil respiratory burst blocked calcium increases induced by CCK in pancreatic tumor cell AR4-2J. Fura-2-loaded AR4-2J cells were perifused, CCK, fMLP and neutrophils were added as indicated by the horizontal bars. (A) Two sequential doses of CCK (10 pM) were added ($N = 3$). (B) fMLP (10 µM, 4 min) was added in between 2 tandem doses of CCK 10 pM ($N = 3$). (C) Simultaneous addition (4 min) of neutrophil ($5 \times 10^5$/ml) and fMLP (10 µM) in between 2 tandem doses of CCK 10 pM ($N = 3$). (D) Neutrophils ($10^6$/ml, double of panel C) and two tandem doses of CCK (10 pM) were added ($N = 4$). Color-coded calcium traces in each panel were from individual AR4-2J cells from a single experiment and are representative of $N$ identical experiments (A–C) or from different experiments (D). Averaged oscillatory frequencies are presented as calcium spikes per min.

Effects of activated neutrophils on endothelial cells and on lung tissues have been investigated [4,32]. Zhu et al. found that fMLP-stimulated neutrophils at a density of $2 \times 10^7$/ml, 40-fold higher than used in the present work ($5 \times 10^5$/ml), induced calcium transients in blood vessel endothelial cells, but neutrophils at a density $<2 \times 10^6$ ml$^{-1}$ was without any effect [33]. The neutrophil density used in the present work ($5 \times 10^5$/ml) would lead to a relatively mild oxidative stress in comparison. A mild oxidative stress, however, seemed sufficient to exert inhibition on CCK1 and $M_3$ receptor-mediated calcium oscillations (Figs. 1–4). The detailed molecular mechanisms involved in such inhibition are not presently known. The products of respiratory burst ($O_2^-$, $H_2O_2$, $OH^{\cdot}$, HOCl, $^1O_2$) may exert such inhibitory effect by oxidizing sulfur-containing residues in CCK1 and M3 receptors. Methionine residues, for example, have been found to be a redox switch in some signaling proteins [34]. Indeed recent works indicate that oxidation-prone sulfur-containing residues in G protein-coupled receptors are likely redox gears for pharmacology and function [35].

Although neutrophil respiratory burst blocked calcium oscillations in rat pancreatic acini, the burst had no effect on calcium oscillations in rat hepatocytes (Figs. S1–S3). The basis for this difference is unclear, but may be related to difference in total cellular antioxidant capacity. The liver provides the first line of defense against microbes and toxins which have crossed the intestinal barrier [2]. Kupffer cells are resident macrophages in liver, and they are critical for rapid clearance of microbes from systemic circulation [36]. Kupffer cells can also undergo respiratory burst. In addition, hydroxyl radicals could be generated in liver through Fenton reaction, due to significant storage of copper and iron [37]. Therefore, it is possible that hepatocytes *in situ* are under more severe oxidative stress than pancreatic acini.

In the present work, the oxidative stress applied was relatively mild, because neutrophils were used at a low density ($5 \times 10^5$/mL), 40-fold lower than used by others [33]. It has been reported that exposure to moderate oxidative stress induces stress resistance [38]. Thus, daily mild oxidative stress may protect hepatocytes from further oxidative insult. Furthermore, antioxidant capacity in liver is higher than in other organs [39,40]. The level of antioxidant enzymes – SOD, catalase and glutathione peroxidase in liver was found to be 3.8, 1.5, 4.6 times of that in the pancreas, respectively [41]. Thus compared to liver, the pancreas is more vulnerable to oxidative stress. Hepatocytes could tolerate oxidative stress better than pancreatic acini.

In conclusion, fMLP-triggered rat neutrophil respiratory burst had an inhibitory effect on CCK1 and $M_3$ receptors in freshly isolated rat pancreatic acini, and on CCK1 receptors in pancreatic carcinoma cell AR4-2J. P2Y purinergic receptors, V1a vasaopressin receptors and α1-adrenergic receptors in rat hepatocytes were not susceptible to inhibition by neutrophil respiratory burst. Our findings point to a novel possibility that infiltrating neutrophils inhibit secretagogue receptors in pancreatic acini to exert secretory blockade. This may have special significance in understanding the pathogenesis of acute pancreatitis.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.081.

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