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Copper inhibits P2Y₂-dependent Ca²⁺ signaling through the effects on thapsigargin-sensitive Ca²⁺ stores in HTC hepatoma cells

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Abstract

Purinergic P2Y₂ G-protein coupled receptors play a key role in the regulation of hepatic Ca²⁺ signaling by extracellular ATP. The concentration of copper in serum is about 20 μM. Since copper accumulates in the liver in certain disease states, the purpose of these studies was to assess the effects of copper on P2Y₂ receptors in a model liver cell line. Exposure to a P2Y₂ agonist UTP increased [Ca²⁺]_i by stimulating Ca²⁺ release from thapsigargin-sensitive Ca²⁺ stores. Pretreatment of HTC cells for several minutes with copper did not affect cell viability, but potently inhibited increases in [Ca²⁺]_i evoked by UTP and thapsigargin. During this pretreatment, copper was not transported into the cytosol, and inhibited P2Y₂ receptors in a concentration-dependent manner with the IC₅₀ of about 15 μM. These results suggest that copper inhibits P2Y₂ receptors through the effects on thapsigargin-sensitive Ca²⁺ stores by acting from an extracellular side. Further experiments indicated that these effect of copper may lead to inhibition of regulatory volume decrease (RVD) evoked by hypotonic solution. Thus, copper may contribute to defective regulation of purinergic signaling and liver cell volume in diseases associated with the increased serum copper concentration.

Keywords

P2Y₂ receptors; UTP; thapsigargin; intracellular Ca²⁺; cell volume; copper

1. Introduction

Hepatocytes secrete ATP into the extracellular space in a regulated manner [1,2]. Once outside the cell, ATP acts as a potent autocrine/paracrine signal by activating purinergic receptors in the plasma membrane. These receptors regulates a broad range of important cell and organ functions including membrane ion transport, hepatic glucose release and bile secretion [3,4]. Multiple lines of evidence suggest that many effects of extracellular ATP are mediated by activation of purinergic P2Y₂ G-protein coupled receptors that stimulate Ca²⁺ release from the intracellular stores and Ca²⁺ influx through store-operated channels (SOC) in the plasma membrane [5]. The resulting increase in [Ca²⁺]_i stimulates opening of ion

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channels involved in the regulation of cell volume [6,7]. Thus, P2Y₂ receptors may contribute to the modulation of liver cell volume.

Copper is essential for human physiology as a cofactor of many intracellular enzymes that regulate cellular respiration, radical detoxification and metabolism [8]. For example, copper modulates the activity of cytochrome-c oxidase, the terminal enzyme of electron transport and oxidative phosphorylation in the mitochondria. Copper is also a cofactor of superoxide dismutase, the key enzyme of cellular antioxidant defense. Thus, intracellular copper modulates many important cellular functions. However, it is not known whether extracellular copper is also able to modulate cellular functions.

Clinical studies have demonstrated that serum concentration of copper is markedly increased in Wilson disease, primary biliary cirrhosis and many other cholestatic disorders of the liver [9,10]. Consequently, the purpose of these studies was to assess the potential effects of copper on Ca²⁺ signaling regulated by P2Y₂ receptors. Using fluo-3 fluorescence to measure the changes in [Ca²⁺]_i and calcein fluorescence to measure membrane transport of copper, we found that copper inhibits P2Y₂-dependent Ca²⁺ signaling through the effects on thapsigargin-sensitive Ca²⁺ stores by acting from an extracellular side. These previously unknown effects of copper contributed to inhibition of RVD evoked by hypotonic solution. Thus, inhibition of P2Y₂ receptors and RVD by copper may be involved in defective regulation of purinergic signaling and liver cell volume in diseases associated with the increased serum copper concentration.

2. Materials and Methods

2.1. Cell cultures

All studies were performed in rat HTC hepatoma cells. The procedure for cell preparation has been previously described [11,12]. Cells were used within 24 h after plating.

2.2. Solutions for imaging

All imaging experiments were performed after washing culture medium with a standard physiologic solution that contained 142 mM NaCl, 4 mM KCl, 1 mM KH₂PO₄, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM Hepes, 10 mM D-glucose. The pH was 7.25, and osmolarity was about 300 mosmolkg⁻¹. All compounds were purchased from Sigma-Aldrich (St. Louis, MO).

2.3. Imaging and analysis

Cells were plated on coverslips in the recording chambers, and incubated overnight in the media as described above. After washing with a standard physiologic solution, cells were visualized through a 40X oil immersion objective (NA= 1.35) and an Olympus IX71 microscope. All images were acquired with a Sensicam QE camera controlled by a SlideBook 3.0 software (Intelligent Imaging Innovations, Denver, CO). Analysis of the fluorescence images was also performed using SlideBook 3.0. Cellular fluorescence was measured by drawing a region of interest over the entire cell, and background fluorescence was measured from the regions with no cells. Background fluorescence was subtracted from the cellular fluorescence.

2.4. Measurement of [Ca²⁺]_i

[Ca²⁺]_i was measured using a Ca²⁺-sensitive fluorescent dye Fluo-3 AM (Molecular Probes, Eugene OR) as previously described [12]. Fluo-3 AM was dissolved in DMSO with equal amounts of Pluronic solution (10 % in DMSO). Cells were incubated with 20 μM Fluo-3 AM for 30 min. The dye was washed from extracellular solution, and fluo-3 fluorescence

was excited through an excitation filter (peak at 480 nm). The light was collected with an emission filter (peak at 535 nm), and images were taken every 10 sec using light exposures of 50 ms in duration. The changes in fluo-3 fluorescence are directly proportional to the changes $[Ca^{2+}]_i$. The data were expressed as (F/F_0) or $(\Delta F/F_0)$. The F is fluo-3 fluorescence, the F_0 is the baseline fluo-3 fluorescence, and the ΔF is the difference between F and F_0 .

2.5. Analysis of P2Y₂-dependent Ca²⁺ response

All drugs were applied by a gravity-fed flow system that allowed for rapid exchange of extracellular solution. UTP is a preferred agonist for P2Y₂ receptors. The magnitude of Ca²⁺ response evoked by UTP was determined by measuring the peak of $\Delta F/F_0$ after exposure to UTP. The effect of copper on P2Y₂ receptors was assessed by measuring the $\Delta F/F_0$ after exposure to UTP in the presence of different copper concentrations (0 – 100 μ M). The concentration of copper that inhibited 50 % of the UTP response (IC_{50}) and the slope were determined by fitting the data to an inhibition curve using IgorPro 6.01 software. (Wavemetrics, Lake Oswego OR).

2.6. Measurement of cell viability

Cell viability was measured using ethidium bromide (Sigma Aldrich, St Louis MO). Ethidium bromide is a fluorescent dye which enters into the cytosol of dead cells, and stains nucleic acids. For these experiments, cells were exposed to 20 μ M ethidium bromide at the room temperature. Ethidium fluorescence was excited through an excitation filter (peak at 560 nm). The light was collected with an emission filter (peak at 610 nm), and images were taken using light exposures of 200 ms in duration.

2.7. Measurement of membrane copper transport

Membrane copper transport was measured using calcein AM (Molecular Probes, Eugene OR). Calcein AM permeates cell membranes, and is retained by healthy cells. Calcein fluorescence is potently quenched by micromolar concentrations of copper, and a decrease in calcein fluorescence has been used to measure membrane transport of copper [13]. Prior to measurements, cells were incubated with 1 μ M calcein AM dissolved in DMSO for 10 min at 37 deg C. After washing the dye from extracellular solution, calcein fluorescence was excited through an excitation filter (peak at 480 nm). The light was collected with an emission filter (peak at 535 nm), and images were acquired using light exposures of 20 ms in duration.

2.8. Measurement of cell volume

Cell volume was calculated from the bright-field images by measuring area of a cell equatorial cross section. Cell volume ($4\pi R^3/3$), where R is cell radius, is directly proportional to the area of a cell equatorial cross section (πR^2). Bright field images were acquired every 2 min. To minimize errors due to a non-spherical cell shape, cells with a spherical shape were selected.

2.9. Statistics

Data were expressed as mean $\pm$ S.E.M (standard error of mean). Results were compared using a two-tailed Student's t-test on paired and unpaired data, and P values < 0.05 were considered to be statistically significant.

3. Results

3.1. P2Y₂ receptors and thapsigargin-sensitive Ca²⁺ stores

UTP is a preferred agonist for P2Y₂ receptors in native liver cells and cloned expression systems [5,14,15]. To assess whether UTP activates P2Y₂ receptors in HTC cells, fluo-3 fluorescence was measured after exposure to UTP. Representative recording in Fig. 1A shows that UTP rapidly increased [Ca²⁺]_i by ~3-fold above the basal level. This increase was followed by a decrease to a steady-state value above the basal level. Subsequent application of thapsigargin to deplete intracellular Ca²⁺ stores had no effect on [Ca²⁺]_i (Fig. 1A). Figure 1B shows that an exposure to thapsigargin increased [Ca²⁺]_i, and potently inhibited increases in [Ca²⁺]_i evoked by UTP. The peak of $\Delta F/F_0$ in response to UTP decreased from 3.6 ± 0.2 (n= 83) under control conditions to 0.10 ± 0.02 (n= 47, $P < 0.001$) in the presence of thapsigargin. When UTP was co-applied with a competitive purinergic antagonist suramin, the peak of $\Delta F/F_0$ decreased to 0.75 ± 0.33 (n= 9, $P < 0.001$) (Fig. 1C). These data indicate that activation of P2Y₂ receptors leads to stimulation of Ca²⁺ release from thapsigargin-sensitive Ca²⁺ stores in HTC cells.

3.2. The effects of copper on P2Y₂ receptors

To assess whether copper modulates the P2Y₂-dependent Ca²⁺ response, [Ca²⁺]_i was measured after exposure to UTP in the presence of copper. The data in Fig. 2A illustrate that co-application of copper and UTP had no effect on the initial rise of [Ca²⁺]_i. The peak of $\Delta F/F_0$ was 2.93 ± 0.60 (n= 10), and was not different from the value obtained in the absence of copper ($P > 0.3$). After reaching a peak, [Ca²⁺]_i slowly decreased to a steady-state value that was significantly lower than the basal level. When copper was co-applied with thapsigargin, the Ca²⁺ response was completely blocked, and [Ca²⁺]_i also slowly decreased below the basal level (Fig. 2A). The $\Delta F/F_0$ decreased to -0.20 ± 0.07 (n= 9) and -0.60 ± 0.03 (n= 23) for UTP and thapsigargin, respectively. To assess whether copper is able to completely inhibit the UTP response, [Ca²⁺]_i was measured in cells that were pretreated with copper for about 5 min before the exposure to UTP. Representative recordings in Fig. 2B show that copper had no effect on the basal [Ca²⁺]_i, but completely inhibited the Ca²⁺ response evoked by UTP and thapsigargin. Furthermore, [Ca²⁺]_i slowly decreased to a steady value that was significantly lower than the baseline. Under these conditions, the $\Delta F/F_0$ decreased to -0.40 ± 0.06 (n= 11) and -0.80 ± 0.05 (n= 9) for UTP and thapsigargin respectively. These results indicate that copper inhibits P2Y₂-dependent Ca²⁺ signaling through the effects on thapsigargin-sensitive Ca²⁺ stores.

To assess the concentration dependence of inhibition of P2Y₂ receptors by copper, [Ca²⁺]_i was measured in the presence of different copper concentrations after exposure to UTP. Pretreatment with copper inhibited the UTP response in a concentration-dependent manner with the $IC_{50} = 13.6 \pm 4.1$ μ M and the slope= 1.3 ± 0.6 (Fig. 2C).

To further evaluate the effects of copper, calcein fluorescence was measured after exposure to copper. Calcein fluorescence is quenched by micromolar concentrations of copper, and a decrease in calcein fluorescence has been used to measure membrane transport of copper [13]. Figure 2D shows that copper did not change the fluorescence of calcein-loaded HTC cells. Thus, during short-term exposures copper is not transported into the cytosol, and may exert effects on thapsigargin-sensitive Ca²⁺ stores by acting from an extracellular side.

3.3. The effects of copper on cell viability and SOC

Prolonged exposure to the concentrations of copper > 50 μ M has been reported to decrease viability of liver cells [16]. To assess whether copper decreases viability of HTC cells, ethidium fluorescence was measured after exposure to 100 μ M copper. Figure 3A shows that

rupturing the plasma membrane with water potentially increased ethidium fluorescence. An exposure to copper did not significantly change the time course of ethidium fluorescence when compared to control conditions (Fig. 3A). These results indicate that during short-term exposures, copper does not affect viability of HTC cells.

The data in Fig. 2A show that copper blocked increases in $[Ca^{2+}]_i$ after stimulation of intracellular Ca^{2+} release evoked by UTP. Because these changes are mediated in large part by Ca^{2+} influx through SOC, we examined whether copper is also able to inhibit SOC. For these experiments, copper was applied several minutes after the exposure to thapsigargin when SOC are maximally activated. Figure 3B shows that copper decreased the $\Delta F/F_0$ by -1.0 ± 0.2 ($n = 11$), indicating that copper inhibits SOC by about 30% in HTC cells.

3.4. Inhibition of RVD by copper

It has been shown that stimulation of Ca^{2+} release from thapsigargin-sensitive Ca^{2+} stores is required for RVD in HTC cells [7]. To assess whether copper has an effect on RVD, cell volume was measured after exposure to hypotonic solution. The data summarized in Fig. 4 illustrate that pretreatment with copper had no effect on cell volume under isotonic conditions, but completely prevented RVD evoked by hypotonic solution. Similar results were obtained after pretreatment with thapsigargin (Fig. 4). These data indicate that the effects of copper on thapsigargin-sensitive Ca^{2+} stores may lead to inhibition of RVD in HTC cells.

4. Discussion

The major finding of these studies is that copper inhibits of $P2Y_2$ -dependent Ca^{2+} signaling through the effects on thapsigargin-sensitive Ca^{2+} stores by acting from an extracellular side. Because hepatic $P2Y_2$ receptors play a key role in the regulation of Ca^{2+} signaling by extracellular ATP [5], these findings suggest that copper may modulate purinergic signaling in liver cells through the effects on $P2Y_2$ receptors.

The effects of copper on $P2Y_2$ receptors were studied in HTC cells. These cells have $P2Y$ receptors and signaling pathways similar to primary hepatocytes, and have been used as a model to study purinergic signaling and cell volume regulation in the liver [6,17,18]. Exposure to UTP increased $[Ca^{2+}]_i$ by several-fold, consistent with stimulation of Ca^{2+} release from intracellular Ca^{2+} stores. As expected, the UTP response was inhibited by suramin, and by depleting intracellular Ca^{2+} stores with thapsigargin. Previous studies have shown that HTC cells express multiple $P2Y$ receptors including $P2Y_1$, $P2Y_2$ and $P2Y_6$, but not $P2Y_4$ receptors [15,19]. Because UTP is a potent agonist for $P2Y_2$ and $P2Y_4$ receptors, and not $P2Y_1$ and $P2Y_6$ [14], it is likely that UTP preferentially activates $P2Y_2$ and not other $P2Y$ receptors in HTC cells, as previously observed in these cells [15].

The data in Fig. 2 show that inhibition of the UTP response by copper required several minutes preincubation to be effective. This is in contrast to a competitive antagonist suramin, which potentially inhibited the UTP response when co-applied with UTP (compare Figs. 1C and 2A). Thus, copper may inhibit $P2Y_2$ receptors by binding to a site which is different from UTP. It has been shown that zinc also inhibits $P2Y_2$ receptors in a similar manner [20]. However, this inhibition requires longer incubation times (15 min) and higher concentrations (IC_{50} about 200 μM), indicating that copper and zinc may inhibit $P2Y_2$ receptors through different mechanisms.

Copper has been shown to inhibit purinergic $P2X_4$ and $P2X_7$ receptors by binding to histidine and/or aspartic acid residues on an extracellular portion of these receptors [21–23]. Similarly, copper could bind to these residues on an extracellular portion of a $P2Y_2$ receptor,

and prevent activation of signaling pathways that lead to stimulation of Ca^{2+} release from the intracellular Ca^{2+} stores. This explanation implies that thapsigargin would be still able to deplete Ca^{2+} stores and increase $[\text{Ca}^{2+}]_i$ in the presence of copper. However, copper completely inhibited increases in $[\text{Ca}^{2+}]_i$ evoked by thapsigargin (see Figs. 2A and B). These data suggest another mechanism that copper may inhibit P2Y_2 -dependent Ca^{2+} signaling through the effects on thapsigargin-sensitive Ca^{2+} stores. One potential explanation may be that copper enters the cytosol, and directly inhibits Ca^{2+} release at the level of intracellular Ca^{2+} stores. This is unlikely since during short-term exposures copper is not transported into the cytosol (see Fig. 2D), and in principle it should not interfere with depletion of Ca^{2+} stores by thapsigargin, which acts from an intracellular side. Another explanation may be that copper binds to a putative copper-binding receptor on the plasma membrane, and this binding leads to depletion of Ca^{2+} stores since both UTP and thapsigargin failed to increase $[\text{Ca}^{2+}]_i$ after a pretreatment with copper (see Fig. 2B). If copper depletes Ca^{2+} stores, then similar to thapsigargin, an exposure to copper would be expected to increase $[\text{Ca}^{2+}]_i$. However, copper alone did not increase $[\text{Ca}^{2+}]_i$, suggesting that different mechanisms may be involved, and/or copper may also rapidly increase the intracellular Ca^{2+} -buffering capacity that would prevent elevation of $[\text{Ca}^{2+}]_i$. The present study does not provide evidence for any of these possibilities, and the cellular mechanisms responsible for the effects of copper on thapsigargin-sensitive Ca^{2+} stores are not known.

Depletion of intracellular Ca^{2+} stores with thapsigargin potentially inhibited RVD evoked by hypotonic solution (see Fig. 4). This is consistent with the previous studies showing that stimulation of Ca^{2+} release from thapsigargin-sensitive stores is required for RVD in HTC cells [7]. In the present study, we show that pretreatment with copper also inhibits RVD. These results suggest that the effects of copper on thapsigargin-sensitive Ca^{2+} stores may contribute to inhibition of RVD.

Assuming that these findings are relevant for the regulation of hepatocyte volume and purinergic signaling, two additional points merit emphasis. First, serum concentration of copper is about 20 μM [24]. This value is similar to the IC_{50} found here. Thus, it is attractive to speculate that under normal physiologic conditions, copper may exert tonic inhibition of P2Y_2 receptors and the regulatory response of liver cells to increases in cell volume. Second, clinical studies have shown that serum concentration of copper is markedly increased in Wilson disease, primary biliary cirrhosis and many other cholestatic disorders of the liver [9,10]. Many of these patients have impaired liver functions that if not treated lead to the development of more severe liver diseases and neuropsychiatric disorders. One major goal of therapy of Wilson disease is to reduce serum copper concentration, and accumulation of copper in the liver and brain tissue. In light of these studies, high copper concentrations may inhibit P2Y_2 receptors and RVD, and lead to defective regulation of hepatocyte volume and purinergic signaling in patients with Wilson disease.

In summary, these results suggest that copper inhibits P2Y_2 -dependent Ca^{2+} signaling in liver cells through the effects on thapsigargin-sensitive Ca^{2+} stores. Furthermore, these previously unknown effects of copper contributed to inhibition of RVD evoked by hypotonic solution. Accordingly, copper may play an important role in the regulation of purinergic signaling and liver cell volume.

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Fig. 1.

P2Y₂ receptors in HTC cells. (A) Cells were incubated with fluo-3 AM, and $[Ca^{2+}]_i$ was measured in response to 20 μM UTP (open bar). Subsequent exposure to 10 μM thapsigargin (at arrow) had no effect on $[Ca^{2+}]_i$. (B) $[Ca^{2+}]_i$ was measured after depletion of intracellular Ca^{2+} stores with thapsigargin (open bar). In the presence of thapsigargin, exposure to UTP (at arrow) did not further increase $[Ca^{2+}]_i$. (C) Representative recording illustrates that co-application of suramin (100 μM) and UTP evoked only a small increase in $[Ca^{2+}]_i$.

Fig. 2.

The effects of copper on P2Y₂ receptors and membrane transport of copper. (A) $[Ca^{2+}]_i$ was measured after co-application of 100 μ M copper (open bar) and 20 μ M UTP or 10 μ M thapsigargin (both at arrow). Copper inhibited the Ca^{2+} response evoked by thapsigargin, but did not inhibit the initial Ca^{2+} response evoked by UTP. (B) Cells were exposed to 100 μ M copper for 5 min prior to exposure to UTP or thapsigargin (both at arrow). Note that copper pretreatment completely inhibited both responses. (C) Cells were pretreated for 5 min with different copper concentrations, and then exposed to UTP (20 μ M). The UTP response (in %, open circles) was obtained by normalizing the peak of $\Delta F/F_0$ to the value obtained in the absence of copper. The data were fitted to an inhibition curve (solid line), and the number of experiments is indicated in parenthesis. (D) Cells were incubated with 1 μ M calcein AM for 10 min. After washing the dye, calcein fluorescence was measured in response to 100 μ M copper (at arrow, n = 10). Calcein fluorescence was normalized to the initial value obtained at t = 0 min. Copper had no effect on calcein fluorescence.

Fig. 3.

Cell viability and SOC in the presence of copper. (A) Cells were exposed to 20 μM ethidium bromide at $t = 0$ min, and the fluorescence was measured under control conditions ($n = 4$), and after exposure to 100 μM copper or water (at arrow, $n = 8$ for both conditions). Relative ethidium fluorescence was determined by normalizing the data to a value obtained at $t = 0$ min. Standard errors for control experiments and after copper exposure were small. Note that copper had no effect on ethidium fluorescence. (B) $[\text{Ca}^{2+}]_i$ was measured after exposure to 10 μM thapsigargin. Subsequent exposure to copper (100 μM , at arrow) significantly decreased the steady $[\text{Ca}^{2+}]_i$ level.

Fig. 4.

The effects of copper on RVD. Cell volume was determined from the bright-field images as described in Methods. Hypotonic solution (40 %, open bar) increased cell volume that slowly recovered through RVD to a value close to the basal value within about 30 min (open circles, $n = 4$). At arrow, cells were pretreated with 100 μM copper (filled circles, $n = 5$) or 10 μM thapsigargin (open triangles, $n = 6$). Both manipulations markedly inhibited RVD.