Biochemical and Biophysical Research Communications 424 (2012) 279–284

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Divalent copper is a potent extracellular blocker for TRPM2 channel

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ARTICLE INFO

Article history:
Received 15 June 2012
Available online 27 June 2012

Keywords:
TRPM2 channel
Calcium channel
Copper
Mercury
Iron
Lead
Selenium

ABSTRACT

Transient receptor potential melastatin 2 (TRPM2) is a Ca^{2+}-permeable cationic channel in the TRP channel family. The channel activity can be regulated by reactive oxygen species (ROS) and cellular acidification, which has been implicated to the pathogenesis of diabetes and some neuronal disorders. However, little is known about the effect of redox-active metal ions, such as copper, on TRPM2 channels. Here we investigated the effect of divalent copper on TRPM2.

TRPM2 channel was over-expressed in HEK-293 cells and the whole-cell current was recorded by patch clamp. We found the whole-cell current evoked by intracellular ADP-ribose was potently inhibited by Cu^{2+} with a half maximal inhibitory concentration (IC_{50}) of 2.59 μM. The inhibitory effect was irreversible. The single channel activity was abolished in the outside-out patches, and intracellular application of Cu^{2+} did not prevent the channel activation, suggesting that the action site of Cu^{2+} is located in the extracellular domains of the channel. TRPM2 current was also blocked by Hg^{2+}, Pb^{2+}, Fe^{2+} and Se^{2+}.

We concluded that Cu^{2+} is a potent TRPM2 channel blocker. The sensitivity of TRPM2 channel to heavy metal ions could be a new mechanism for the pathogenesis of some metal ion–related diseases.

1. Introduction

Transient receptor potential melastatin 2 (TRPM2) is a member of the melastatin subfamily of TRP channels which are permeable to cations such as Ca^{2+} and Na^{+}. TRPM2, previously known as TRPC7 or LTRPC2 [1,2], is abundantly expressed in the brain, and later studies show that TRPM2 is an ubiquitously expressed channel found in many tissues including bone marrow, spleen, heart, liver, lung, pancreatic islets and immunocytes [2,3]. Several important physiological functions of TRPM2 channel have been demonstrated using knockout animals or *in vitro* models including insulin release [3,4], cytokines and reactive oxygen species (ROS) production [5], cell motility and cell death [6], and immune response [7]. The genetic variants of TRPM2 have been linked to the pathogenesis of several neurological diseases like bipolar disorder [8], western pacific amyotrophic lateral sclerosis and parkinsonism-dementia [9], and the regulation of amyloid beta-peptide (Aβ)-induced striatal cell death that involves in the Alzheimer's disease [10]. In addition, a number of endogenous modulators for TRPM2 channel have been identified, among which the most efficient direct channel activator is adenosine diphosphate ribose (ADPR). Free ADPR opens TRPM2 channels by binding to the Nudix-like motif in the C-terminus, and evokes a current with linear current–voltage (*IV*) relationship [1,11]. The activation of TRPM2 channel by ADPR is dependent on the intracellular Ca^{2+} concentration [12] and negatively regulated by adenosine monophosphate (AMP) [13] and acidic pH [14,15]. Hydrogen peroxide (H_2O_2) is another activator for TRPM2 channel, although the mechanism of H_2O_2-induced TRPM2 activation is still unclear [2]. Therefore, TRPM2 is a ROS-sensitive Ca^{2+}-permeable channel, which may play important roles in the oxidative stress-related diseases.

Metal ions are important factors or cofactors in cellular physiology [16]. Among them, copper is a redox-active element and participates in many important cellular functions by binding to a variety of proteins such as ceruloplasmin, cytochrome *c* oxidase and superoxide dismutase [17]. Both deficiency and elevated level of copper can induce oxidative stress that leads to cell or tissue damage [18]. Copper deficiency is seen in infants with Menkes' disease that is caused by genetic mutations in the copper transporter ATP7A [19] and in patients with gastrointestinal surgery, such as gastric bypass surgery [20]. In contrast, patients showing excessive copper accumulation in the body are due to exposure to excess copper in drinking water or other environmental sources [21], or the genetic disorder (Wilson's disease) with copper accumulation in the liver and the basal ganglia of the brain, which is caused by the mutations in ATP7B, a transporter responsible for exporting copper out of the cells [22]. Moreover, the elevated copper level has also been reported in the plasma of diabetic patients [23] and in the brain of patients with neurodegenerative disorders [24]. It is unclear that the increased blood copper concentration

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0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.06.107

is just a consequence of diabetes or the cause of dysfunction of insulin signaling and glucose homeostasis, however, the treatment with Cu^{2+} chelator to reverse diabetic copper overload seems effective in preventing diabetic organ damage [25]. With regard to neurological disorders, copper has been implicated in the pathogenesis of many neurodegenerative disorders including Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease, and prion disease [26,27]. Excess copper can initiate or stimulate the progression of Alzheimer's disease by promoting the aggregation of amyloid-beta peptides to form senile plaques in the brain [28,29] or by oxidative stress-driven cell death [17]. Nevertheless, the effect of copper in Alzheimer's disease is still in much debate. The protective effect against beta-sheet secondary structure formation by copper has also been demonstrated [30,31]. These mixed results could be due to the imbalance of Cu^{2+} in the affected region, rather than a bulk Cu^{2+} accumulation or deficiency [32,33]. Therefore, the identification of potential new Cu^{2+} target is important for understanding the pathogenesis of Cu^{2+}-related disorders.

Given the evidences that TRPM2 is a redox-sensitive channel and mediates cell death, as well as its high expression in the brain and the association with neurodegenerative disorders and diabetes which are oxidative stress related diseases. It is intriguing to suppose that the activity of TRPM2 channels could be modulated by certain pathological factors related to these diseases, such as Cu^{2+} that plays an important role in regulating oxidative status in a cell. Therefore, we investigated the effect of Cu^{2+} on TRPM2 channels in the TRPM2 transfected HEK-293 cells by electrophysiological approach. Other heavy metal ions, such as Hg^{2+}, Pb^{2+}, Fe^{2+} and Gd^{3+}, and divalent selenium (Se^{2+}) were also studied for comparison.

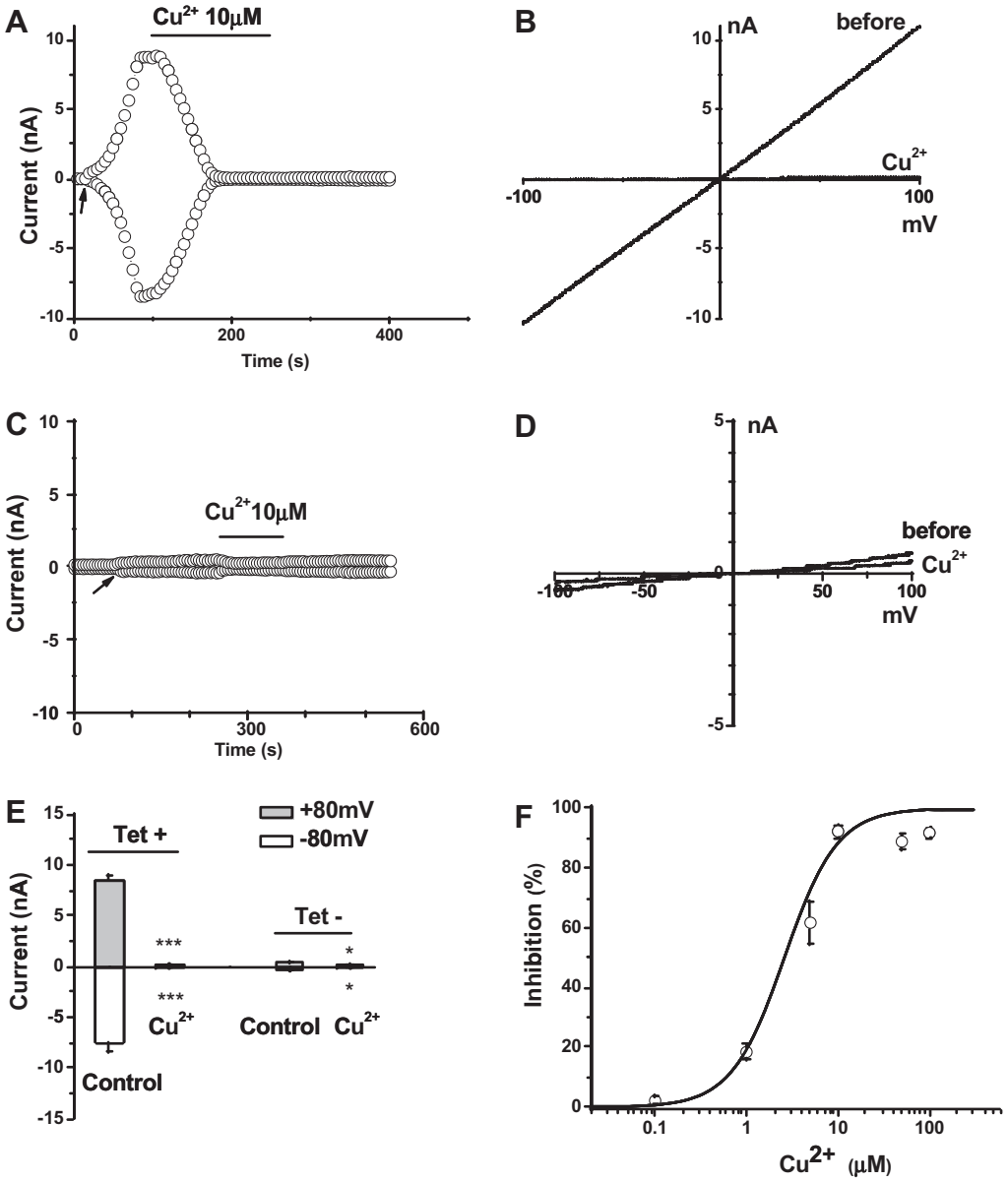

Fig. 1. Effects of Cu^{2+} on the currents of TRPM2-overexpressing HEK-293 cells. (A) The time course of the currents measured at +80 and −80 mV. Cu^{2+} (10 μM) inhibited the whole-cell TRPM2 currents evoked by 500 μM ADPR in the pipette solution. The arrow indicates the time point when the whole-cell configuration was formed. (B) Representative *IV* curves before and after perfusion of Cu^{2+} shown in (A). (C) Cu^{2+} (10 μM) inhibited the endogenous currents in the cell without tetracycline induction. (D) *IV* curves for (C). (E) Mean ± SEM for the inhibition of Cu^{2+} on the current of Tet-induced and non-induced cells. (F) Dose–response curve for TRPM2 inhibition by Cu^{2+} (n = 7–13 for each concentration).

2. Materials and methods

2.1. Cell culture and transfection

A tetracycline-controlled expression system for human TRPM2 channel was generated using pcDNA4/TO vector and stably transfected into the HEK-293 T-REx cells (Invitrogen, Paisley, UK) as previously described [34]. The cells were maintained in DMEM/F-12 medium supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin (Gibco, Paisley, UK) and 10% fetal bovine serum (Sigma–Aldrich, Poole, UK). The expression of TRPM2 was induced by 1 μg/ml tetracycline in the cell culture medium for 24–72 h before patch-clamp recording. Cells without tetracycline induction were used as control.

2.2. Electrophysiology

Whole-cell patch-clamp recording was performed at room temperature (23–26 °C). Briefly, signal was amplified with an Axopatch 200B amplifier and controlled by the software pClamp 10. A 1-s ramp voltage protocol from −100 mV to + 100 mV was applied at a frequency of 0.2 Hz from a holding potential of 0 mV. Signals were sampled at 10 kHz and filtered at 1 kHz. The glass microelectrodes with resistance of 3–5 MΩ were used. The 200 nM Ca^{2+} buffered pipette solution contained (in mM) 115 CsCl, 10 EGTA, 2 $MgCl_2$, 10 HEPES, and 5.7 $CaCl_2$ (pH 7.2 adjusted with CsOH, and osmolarity ~290 mOsm adjusted with mannitol). The calculated free Ca^{2+} is 200 nM. ADP-ribose (0.5 mM) was included in the pipette solution to activate TRPM2 channels in the whole-cell configuration. The standard bath solution contained (mM) 130 NaCl, 5 KCl, 8 D-glucose, 10 HEPES, 1.2 $MgCl_2$ and 1.5 $CaCl_2$. The pH was adjusted to 7.4 with NaOH.

2.3. Chemicals

General salts, ADP-ribose (ADPR), H_2O_2, copper sulphate ($CuSO_4$), gadolinium chloride ($GdCl_3$), lead nitrate ($Pb(NO_3)_2$), selenium dioxide, mercury chloride ($HgCl_2$) ferrous chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$) and 2-aminoethoxydiphenyl borate (2-APB) were purchased from Sigma–Aldrich (Poole, UK).

2.4. Statistics

Data are expressed as mean ± SEM. Data sets were compared using paired *t* test for the results before and after treatment with significance indicated if $P < 0.05$.

3. Results

3.1. TRPM2 channel inhibited by Cu^{2+}

The effect of divalent Cu^{2+} on TRPM2 channel was investigated using whole-cell patch clamp recording in the HEK-293 cells overexpressed with human TRPM2 gene in a tetracycline-regulated expression system [34]. The activity of TRPM2 channel was induced by ADP-ribose (500 μM) in the pipette solution (Fig. 1A) or by bath application of H_2O_2 (500 μM) (data not shown). The current with a typical linear current–voltage (*IV*) relationship was quickly evoked by ADP-ribose in the tetracycline-induced TRPM2 cells (Tet+) and achieved maximum within 1 min after membrane

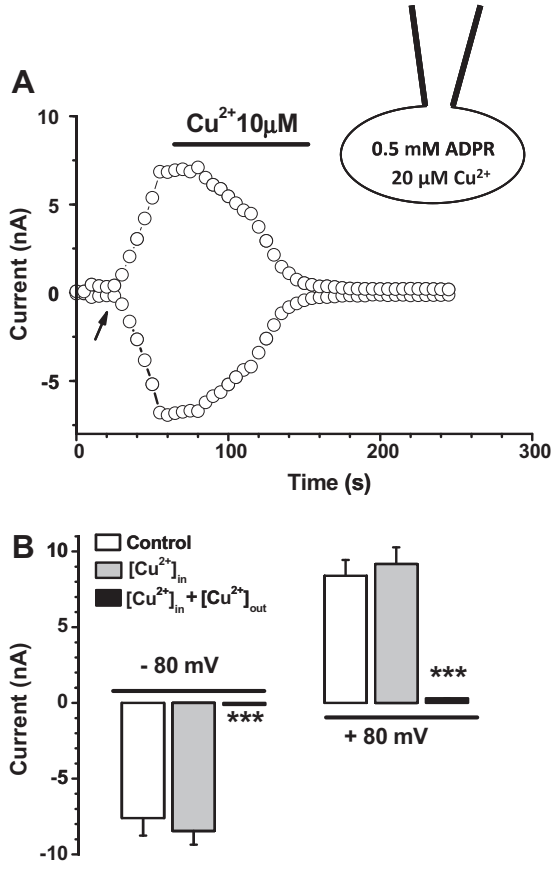

Fig. 2. Cu^{2+} inhibits TRPM2 channels from the cell surface. (A) Addition of Cu^{2+} (20 μM) into the pipette solution did not prevent the channel activation by ADPR, whereas the perfusion of Cu^{2+} in bath solution completely abolished the whole-cell currents. The arrow indicates the time point when the whole-cell configuration was achieved. (B) Comparison of the peak amplitudes of TRPM2 currents measured at +80 and −80 mV in the control group and in the presence of intracellular or extracellular Cu^{2+} ($n = 3$, $^{***}P < 0.001$).

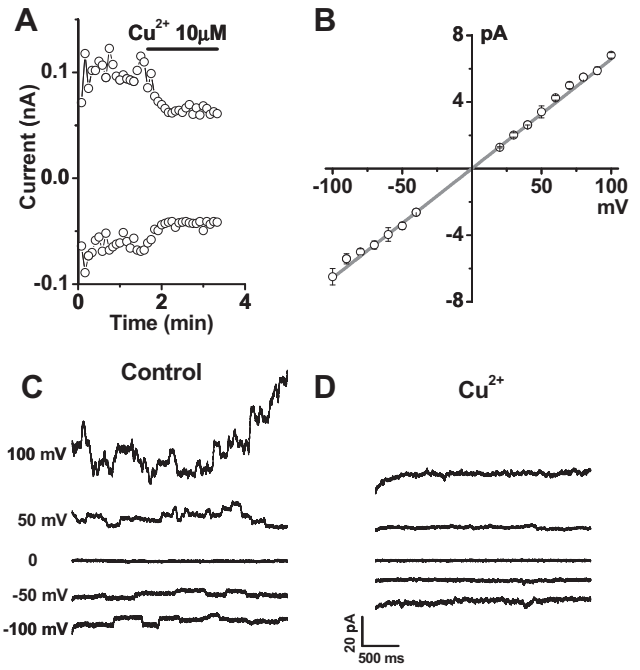

Fig. 3. Outside-out patches showing the effect of Cu^{2+}. (A) Example for the time course of the effect of Cu^{2+}. (B) Mean unitary current sizes for ADPR-induced TRPM2 single channel events plotted against voltages. Straight line was fitted and the mean unitary slope conductance was 66 pS (0.5 mM ADP-ribose). (C) Example of single channel activity of TRPM2 recorded by outside-out patches before perfusion with Cu^{2+}. (D) Cu^{2+} (10 μM).

breakthrough (Fig. 1A and B), which is consistent with our previous reports and others [1,12,35]. Perfusion with 10 μM divalent Cu^{2+} abolished the current of TRPM2. The inhibitory effect of Cu^{2+} seemed to be irreversible on washout, which suggests that Cu^{2+} may form covalent bonds with the channel protein. In the control cells without tetracycline induction (Tet-), the whole-cell current evoked by ADP-ribose was very small, and also inhibited by Cu^{2+} (10 μM), suggesting that there is an endogenous current sensitive to Cu^{2+} in the native cells (Fig. 1C and D). The inhibition of Cu^{2+} on TRPM2 was concentration-dependent with an IC_{50} of 2.59 ± 0.66 μM and a slope factor of 1.50 ± 0.35 (Fig. 1F).

3.2. Extracellular effect of Cu^{2+} on TRPM2

Due to the ubiquitous expression of Cu^{2+} transporters in the plasma membrane and intracellular membranes, and the different concentrations in the extracellular cleft and cytosol [16], we therefore determined the action site of Cu^{2+} on TRPM2 channel. We included higher concentration (20 μM) of Cu^{2+} into the pipette solution to see whether the activation of TRPM2 current can be prevented. We found that intracellular Cu^{2+} application failed to prevent the TRPM2 current induced by ADP-ribose, but the subsequent bath perfusion with 10 μM Cu^{2+} abolished the current (Fig. 2), suggesting that the

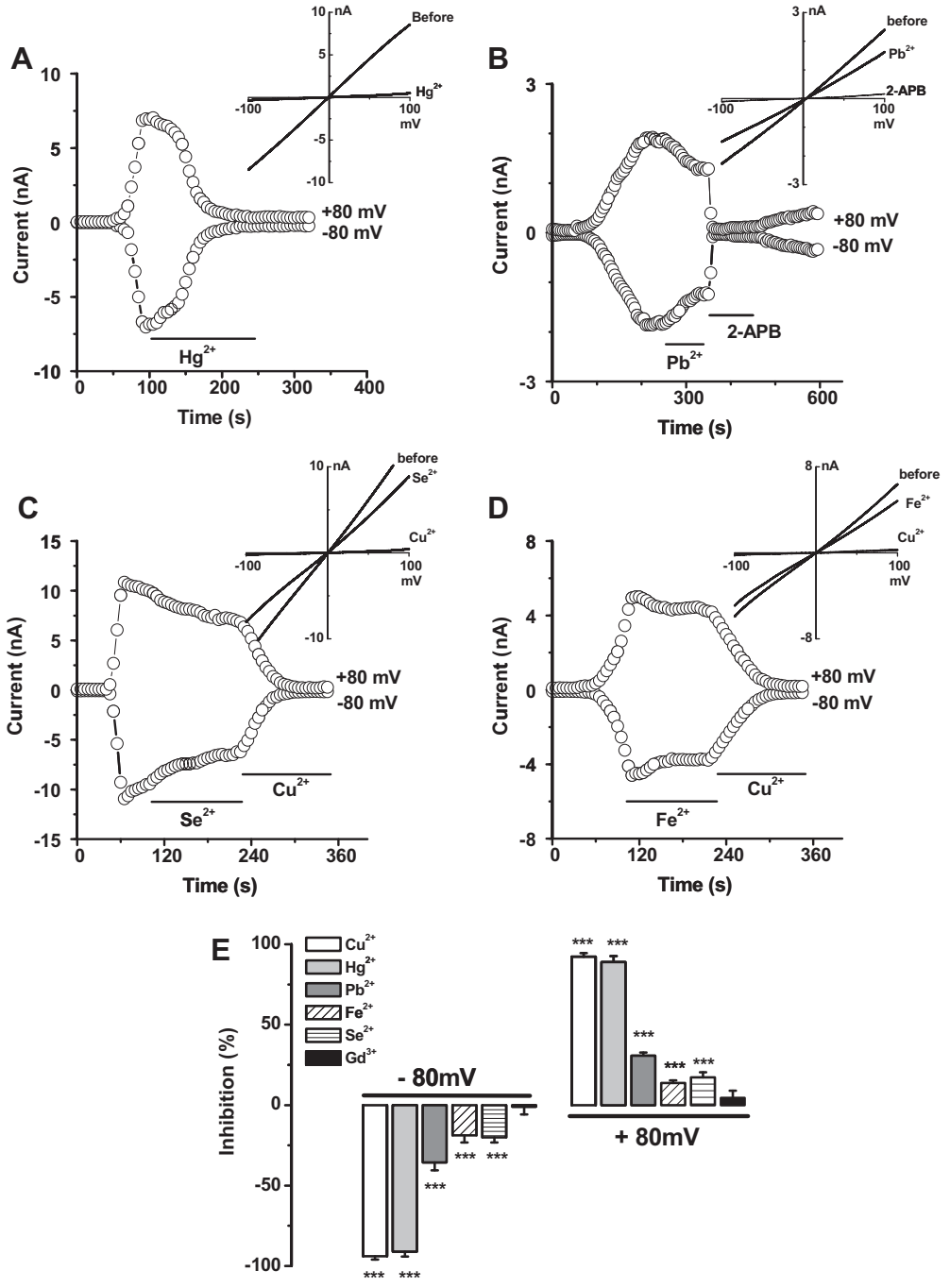

Fig. 4. Effect of Hg^{2+}, Pb^{2+}, Fe^{2+} and Se^{2+} on TRPM2 channels. (A) Hg^{2+} (10 μM) inhibited the whole-cell TRPM2 current activated by ADPR. (B) Pb^{2+} (10 μM) partially inhibited the TRPM2 current, and subsequent application of 2-APB (100 μM) dramatically reduced the currents. (C) Effect of Se^{2+} (10 μM). (D) Effect of Fe^{2+} (10 μM). (E) Comparison of the inhibitory effects of Hg^{2+}, Pb^{2+}, Fe^{2+}, Se^{2+}, Gd^{3+} at 10 μM on TRPM2 currents measured at +80 and −80 mV (n = 4–6 for each group).

action site of Cu^{2+} on TRPM2 channel could be located in the external surface of the transmembrane domains.

To further confirm the extracellular effect, the outside-out patch was performed. Cu^{2+} (10 μM) significantly inhibited the TRPM2 current in the outside-out patches (Fig. 3A). We also recorded the single channel activity of TRPM2. The slope conductance for the TRPM2 induced by ADP-ribose was 65.8 ± 0.23 pS (n = 4), which is similar to 64 pS [15] and close to 60 pS recorded under the conditions of 100 nM Ca^{2+} and 100 μM ADP-ribose [1]. Bath perfusion with 10 μM Cu^{2+} abolished the single channel activity (Fig. 3).

3.3. Comparison with other metal ions on TRPM2 channels

Mercury and lead are important metal ions related to neuronal development and dysfunction, therefore we investigated their effects on TRPM2 channel. Hg^{2+} at 10 μM completely inhibited the TRPM2 current, whilst Pb^{2+}, Fe^{2+}, and Se^{2+} at 10 μM showed a partial inhibition (Fig. 4A and D). The inhibitory effect of Hg^{2+} was difficult to be washed out. Fe^{2+} at 50 μM also showed inhibitory effect and the current was decreased by 25.3 ± 6.6% (n = 3), but higher concentrations of Fe^{2+} ($\geqslant$100 μM) caused a leak current due to cell damage. The percentage of inhibition was compared against the normalized effect (100%) of 2-APB at 100 μM on the same cell. The potency for Hg^{2+} and Cu^{2+} was similar, but stronger than that of Pb^{2+}, Fe^{2+} and Se^{2+} (Fig. 4F). No significant effect was observed for the trivalent cation Gd^{3+}, which is in agreement with our previous report [36].

4. Discussion

In this study, we demonstrate that Cu^{2+} at micromolar concentrations potently inhibits TRPM2 channel activity. The action site of Cu^{2+} is extracellularly located and the inhibitory effect of Cu^{2+} is irreversible. Hg^{2+}, Pb^{2+}, Fe^{2+} and Se^{2+} also show blocking effect on TRPM2 channel, but Pb^{2+}, Fe^{2+} and Se^{2+} are less potent than Cu^{2+} and Hg^{2+}. These findings provide a novel mechanism for the pathophysiology of Cu^{2+} in human diseases.

Copper has been implicated in the pathogenesis of several neurodegenerative disorders including Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, Parkinson's disease, and prion disease [26,27]. Excess of copper is associated with the production of ROS, which in turn triggers a series of events including oxidative stress-induced cell injury, intracellular protein deposits (neurofibrillary tangles), neuronal dysfunction and consequently cell death [37]. On the other hand, ROS activate some Ca^{2+} channels, such as TRPM2, and cause the disruption of cellular Ca^{2+} homeostasis. The common change of Ca^{2+} homeostasis in Alzheimer's disease is an increased intracellular calcium level that could occur either indirectly through Aβ modulating an existing Ca^{2+} channel or directly through cation-selective channels formed by Aβ [38]. In this study, we found that extracellular Cu^{2+} significantly inhibited the TRPM2 channels, which provide a new evidence for the relationship between ROS-sensitive Ca^{2+} channel TRPM2 and Cu^{2+}. The TRPM2 channel activity is activated by ROS or H_2O_2, but inhibited by Cu^{2+}, therefore Cu^{2+} seems to be protective in the Ca^{2+} homeostasis. Because of the extremely high copper levels in senile plaques (393 ± 123 μM) [24], we suppose that the activity of TRPM2 enhanced by H_2O_2 and Aβ in the affected regions of the brain is likely to be completely inhibited by Cu^{2+}. Nevertheless, the physiological significance of TRPM2 channel inhibited by Cu^{2+} still needs to be further investigated, because the inhibition by Cu^{2+} is an irreversible process, which may affect the normal function of TRPM2 in these cells. In primary cultures of rat striatum, the Aβ-induced Ca^{2+} increase and cell death can be prevented by a dominant negative isoform of TRPM2 (TRPM2-S), suggesting that lowering activity of TRPM2 could be protective [10]. Moreover, the genetic variants of TRPM2 have been identified in several neurological diseases, but little is known about the channel functionality in the native cells related to these diseases [9].

Copper concentration in blood plasma is around 15 μM in normal population [16], however, copper accumulates in the brain and displays differential distribution patterns in the central nervous system. Much higher concentration has been estimated in the cerebrospinal fluid (~70 μM). The concentration in the synaptic cleft may reach 200–400 μM in some neuronal diseases, whereas the normal extracellular copper concentration in the brain is of the order of 0.2–1.7 μM [16]. The elevated copper levels have also been reported in patients with type 1 or type 2 diabetes [39], which shares many pathogenetic mechanisms with Alzheimer's disease and vascular dementia. In addition, copper accumulation in the body is caused by genetic variants of Cu^{2+} transporter genes (ATP7A and ATP7B), i.e., Menkes syndrome and Wilson disease. These suggest that the inhibitory mechanism by micromolar Cu^{2+} on TRPM2 channel should happen in normal subjects and under some disease conditions.

Apart from the inhibition on the oxidative stress-sensitive TRPM2 channels, Cu^{2+} also inhibits the voltage-gated Ca^{2+} channels including T-, L-, N-, P-, and Q-type currents [16]. The $Ca_v3.2$ channel is more sensitive to Cu^{2+} than other types of voltage-gated Ca^{2+} channels with an IC_{50} = 0.9 μM [40]. In addition, high concentrations of Cu^{2+} stimulate TRPV1 and TRPA1 channels [41,42], suggesting that the overall effect of Cu^{2+} on intracellular Ca^{2+} level may vary, which depends on the local concentration of Cu^{2+} and the expression of individual Ca^{2+} channels.

We have not examined the molecular targets of Cu^{2+} in this study, however, Cu^{2+} may bind directly to amino acids against most likely cysteine or the hydrophilic-charged amino acids (histidine, lysine, arginine, aspartate, and glutamate) to alter protein function. Indeed, the residue substitution in the outer vestibule of the pore including Lys952, His995 and Asp1002 significantly changed the sensitivity to Zn^{2+} [43]. Further investigation is needed to confirm these binding sites for Cu^{2+}. In addition, the binding to cysteine residues or oxidizing the residues may catalyse the formation of disulphide bonds between physically adjacent cysteine residues, and thereby indirectly change protein structure and function. Moreover, a third more indirect way that copper can modulate protein function is through the generation of free radicals, which can profoundly alter protein and cell function, particularly for the ROS-sensitive channels, such as TRPM2 channels in this study. Unlike the extracellular effect of Cu^{2+}, the action site for ROS or hydroxyl radical that generated by mixing with Fe^{2+} and H_2O_2 has been demonstrated as an intracellular effect, which is mainly related to the C-terminal Nudix-like domain [2,44]. Therefore, the channel appears to be opened by intracellular ROS, and closed by extracellular Cu^{2+}. On the other hand, higher concentrations of extracellular Cu^{2+} may enter cells via Cu^{2+} transporters that in turn regulate the ROS production. Recently, Zn^{2+} has been shown to inhibit TRPM2 channel, however, the potency of Zn^{2+} was much lower than that of Cu^{2+} [43]. The difference in potency for the two ions may have important physiological relevance because the plasma levels of copper and zinc are oppositely correlated in many diseased conditions such as diabetes and hypertension and the ratio of Zn^{2+}/Cu^{2+} have been evaluated in some diseases [16,45,46]. Excessive copper and deficit of zinc would progressively disrupt the cellular Ca^{2+} homeostasis by tuning the activity of a number of TRP and other Ca^{2+} channels [16]. The inhibition of TRPM2 channel by Pb^{2+}, Se^{2+} and Fe^{2+} was mild, suggesting these metal ions could be less important as direct channel regulators.

Besides the neurological disorders, TRPM2 channel is also important for insulin secretion in pancreatic β-cells [3,4]. Copper deficiency enhances the insulin secretion in isolated pancreatic

islets [47], suggesting that copper can regulate the insulin release, which could be related to TRPM2 channels. Taken together, the inhibition of TRPM2 by copper is an important mechanism for the normal physiological function in the body. The imbalance of TRPM2 channel activity caused by excess copper or ROS may be one of the pathophysiological mechanisms for disruption of Ca^{2+} homeostasis in diabetes and neurodegenerative disorders.

Acknowledgments

This work was supported by British Heart Foundation (PG/08/071/25473) (to S.Z.X.). B.Z. was sponsored by China Scholarship Council.

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