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The mechanisms of Zn^{2+} effects on Ca^{2+}-permeable AMPA receptors on carp retinal horizontal cells

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ABSTRACT

It was demonstrated in our recent study that Zn^{2+} had dual effects on Ca^{2+}-permeable AMPA receptors on carp retinal horizontal cells. However, the possible mechanism(s) underlying the Zn^{2+} effects is still unclear. In the present study, we examined the Zn^{2+} effects in the presence of cyclothiazide (CTZ), an allosteric potentiator of AMPA receptors, which could attenuate the receptor desensitization. It was shown that the potentiation effect that low concentration Zn^{2+} (10 μM) exerted on the amplitude of the current mediated by Ca^{2+}-permeable AMPA receptors was more remarkable in the presence of moderate concentration of CTZ (20 μM). Meanwhile, the inhibitory effect induced by high concentration Zn^{2+} (1 mM) was not affected by CTZ. Furthermore, the involvement of extracellular Ca^{2+} in the Zn^{2+} effects was also examined. It was shown that the inhibitory effect induced by high concentration Zn^{2+} (1 mM) was abolished or significantly attenuated in Ca^{2+}-free (0 mM) Ringer's, but significantly enhanced in high-Ca^{2+} (20 mM) Ringer's. However, the Zn^{2+} (10 μM) potentiation effect was not changed either in Ca^{2+}-free (0 mM) or high-Ca^{2+} (20 mM) Ringer's. These results suggest that the Zn^{2+} potentiation effect involves the interaction with CTZ-binding site on the AMPA receptors, while the Zn^{2+} inhibitory effect is related to the extracellular Ca^{2+} concentration. Overall, the dual effects that Zn^{2+} exerts on Ca^{2+}-permeable AMPA receptors on retinal horizontal cells are mediated by separate mechanisms.

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1. Introduction

AMPA receptors (AMPARs), which mediate the majority of fast excitatory neurotransmission at glutamatergic synapses in the central nervous system, are homo- or hetero-tetramers assembled by four types of subunits termed as GluR1–4. The tetramers without GluR2 subunit are highly permeable to Ca^{2+}, which are called Ca^{2+}-permeable AMPARs (CP-AMPARs). CP-AMPARs are expressed on carp retinal horizontal cells (HCs) (Okada et al., 1999; Huang et al., 2004; Huang and Liang, 2005; Sun et al., 2010), which should contribute to regulate the cell's physiological activities and participate in some pathological processes.

Zn^{2+}, as a potential neuronal modulator in vertebrate retinas, is co-located and probably co-released with glutamate in photoreceptor terminals (Wu et al., 1993; Redenti et al., 2007; Lee et al., 2008). It has been well accepted that Zn^{2+} could modulate the AMPA-preferring glutamate receptors in retinal HCs (Schmidt, 1999; Shen and Yang, 1999; Zhang et al., 2002). Our recent work also proved the dual effects that Zn^{2+} exerted on CP-AMPARs on carp retinal H1 HCs (Sun et al., 2010): application of Zn^{2+} at low concentration (10 μM) potentiated the CP-AMPAR-

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mediated current by abolishing the receptor desensitization; but application of Zn^{2+} at high concentrations (100 and 1000 μM) inhibited the current in a dose-dependent manner.

However, the mechanism(s) of Zn^{2+} effects on modulating the AMPARs is not well understood. Given that the potentiation of CP-AMPARs on HCs by low concentration Zn^{2+} is similar to the cyclothiazide (CTZ) effect (Yamada and Tang, 1993; Wong and Mayer, 1993), both of which could block AMPARs' desensitization, we conducted experiments to investigate whether the Zn^{2+} potentiation effect was related to the CTZ-binding site on CP-AMPARs. In addition, given the high Ca^{2+}-permeability of CP-AMPARs, we speculated that extracellular Ca^{2+} might be also involved in the Zn^{2+} effects in modulating these receptors.

In the present study, we first examined the Zn^{2+} effects exerted on CP-AMPARs on carp retinal H1 HCs in the presence of CTZ. The Zn^{2+} (10 μM) potentiation effect was more remarkable in increasing the amplitude of the current mediated by CP-AMPARs in the presence of moderate concentration of CTZ (20 μM). However, the inhibition of CP-AMPAR-mediated current by high concentration Zn^{2+} (1 mM) was not changed in the presence of CTZ. Furthermore, we also investigated the Zn^{2+} effects in Ca^{2+}-free (0 mM) and high-Ca^{2+} (20 mM) Ringer's. It was shown that the Zn^{2+} (1 mM) inhibitory effect was dependent on the extracellular Ca^{2+} concentration, but the Zn^{2+} (10 μM) potentiation effect was not affected by the extracellular Ca^{2+}. All these results suggest that in the CP-AMPARs on retinal H1 HCs, the Zn^{2+} potentiation effect at low concentration is related to the CTZ-binding site; however, the Zn^{2+} inhibitory effect at high concentration is dependent on the extracellular Ca^{2+}.

2. Results

Zn^{2+} has dual effects on CP-AMPAR-mediated current on carp retinal H1 HCs: application of Zn^{2+} at low concentration (10 μM) potentiated the CP-AMPAR-mediated current by abolishing the receptor desensitization without changing the peak current amplitude (see Fig. 1A), but Zn^{2+} of high concentration (1 mM) inhibited this current (see Fig. 2A). These results are consistence with what we recently reported (Sun et al., 2010). In the present work, we first studied the Zn^{2+} effects exerted on CP-AMPAR-mediated current on retinal H1 HCs in the presence of CTZ. Furthermore, the contribution of extracellular Ca^{2+} to the Zn^{2+} effects was also tested. In order to examine the glutamate current mediated by CP-AMPARs, pentobarbital (PB) of 100 μM, a selective blocker of CP-AMPAR, was applied in all the experiments (Taverna et al., 1994; Yamakura et al., 1995; Van Damme et al., 2002; Sun et al., 2010).

2.1. The Zn^{2+} potentiation effect was more remarkable in increasing the current amplitude in the presence of 20 μM CTZ

A typical experimental recording showing the Zn^{2+} potentiation effect in the presence of 20 μM CTZ is plotted in Fig. 1B. In the presence of 20 μM CTZ, glutamate (3 mM) elicited an inward current in an isolated H1 HC with the peak current being 450.3 pA. When 10 μM Zn^{2+} was pre-superfused for 10 s, the peak current elicited by 3 mM glutamate was enhanced to 787.5 pA. After Zn^{2+} was washed out for 1 min, the peak current elicited by 3 mM glutamate recovered to 437.5 pA. Statistical data given in Fig. 1C show that the CP-AMPAR-mediated peak currents were potentiated to 150.6±25.5% of the control level (mean±SD, $p<0.05$, $n=8$) in the presence of 20 μM CTZ. Application of 10 μM Zn^{2+} significantly enhanced the amplitude of the current mediated by CP-AMPARs in the presence of 20 μM CTZ, which is remarkably different from the Zn^{2+} (10 μM) effect observed in the absence of CTZ, which potentiated the CP-AMPAR-mediated current by abolishing the current desensitization but without significant changes (99.5±11.2% of the control level) in the current amplitude (see Fig. 1C).

Moreover, the desensitization process of the CP-AMPAR-mediated current was also slowed down by 10 μM Zn^{2+} in the presence of 20 μM CTZ (see Fig. 1B). In the presence of 20 μM CTZ, the desensitization time constant (τ, estimated by Eq. (1))

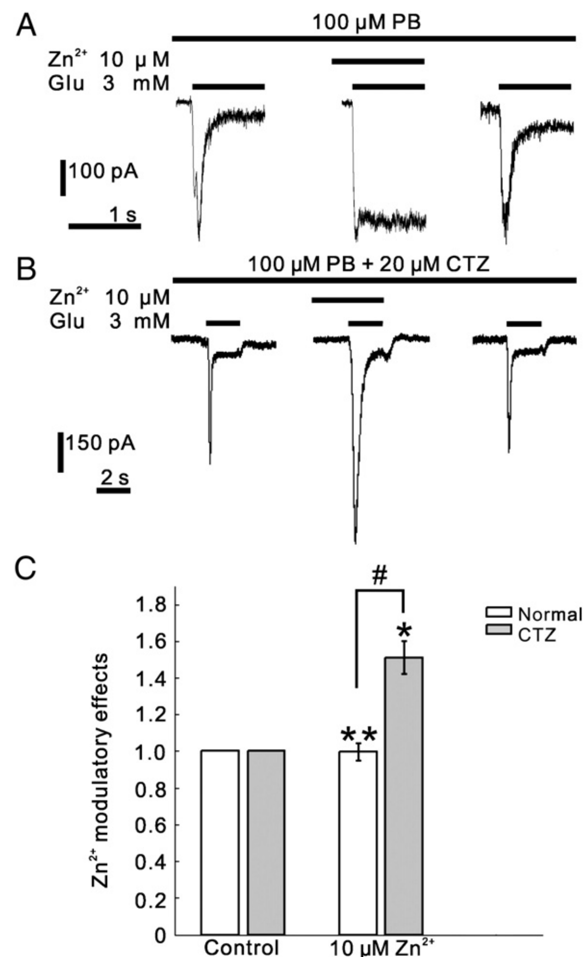

Fig. 1 – Zn^{2+} potentiation effect on the peak level of Glu current on H1 HCs in the presence of cyclothiazide (CTZ) and pentobarbital (PB). (A–B) The Zn^{2+} potentiation of CP-AMPAR response in the absence or presence of 20 μM CTZ. (C): Normalized Zn^{2+} potentiation effect on Glu current (peak current) mediated by CP-AMPARs in the absence ($n=5$, white columns) and presence of CTZ ($n=8$, gray columns) (* $p<0.05$, ** $p>0.05$, paired t-test, as compared to control). # $p<0.05$ (unpaired t-test, comparison between the data as indicated).

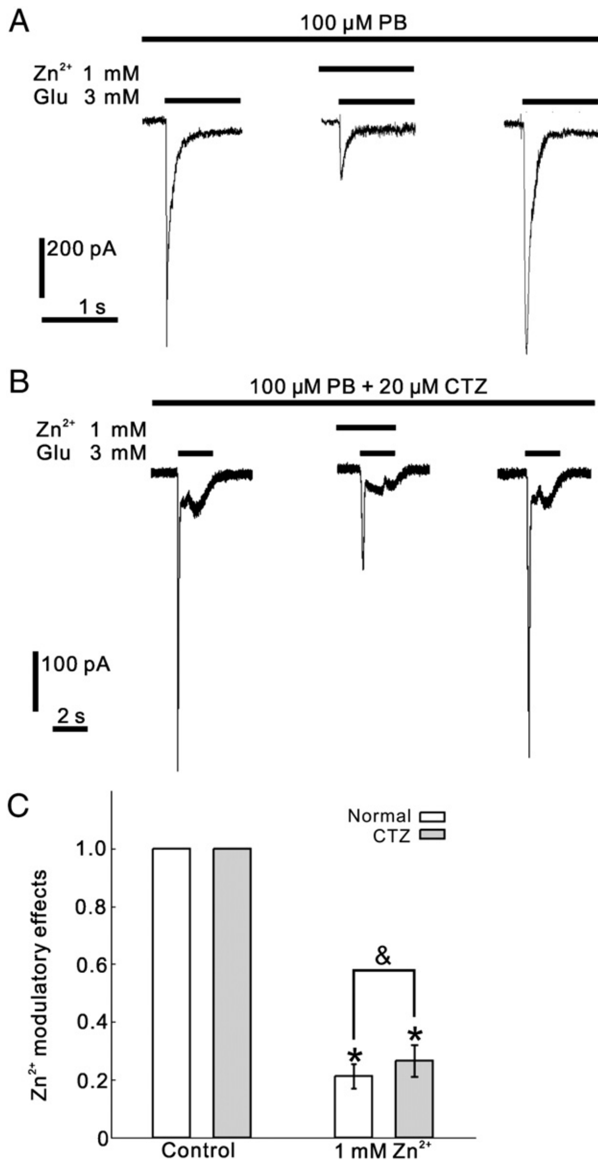

Fig. 2 – Zn^{2+} inhibitory effect on the peak level of Glu current on H1 HCs in the presence of CTZ and PB. (A–B) The Zn^{2+} inhibition of CP-AMPAR response in the absence or presence of 20 μM CTZ. (C) Normalized Zn^{2+} inhibitory effect on Glu current (peak current) mediated by CP-AMPARs in the absence (*n*=5, white columns) and presence of CTZ (*n*=5, gray columns) (* $p<0.05$, paired *t*-test, as compared to control). $^{\&}p>0.05$ (unpaired *t*-test, comparison between the data as indicated).

of the glutamate (3 mM) elicited current was 65.5 ms. When 10 μM Zn^{2+} was pre-superfused for 10 s, the τ value of the inward current was enhanced to 225.4 ms. After Zn^{2+} was washed out for 1 min, the τ value recovered to 57.8 ms. Statistical data show that the desensitization time constant (τ) was increased to 352.4±24.0% of the control level (with 20 μM CTZ) during application of 10 μM Zn^{2+} (mean±SD, $p<0.05$, $n=8$) and recovered to 102.9±11.8% of the control level (mean±SD, $p>0.05$) after Zn^{2+} was washed out.

2.2. The Zn^{2+} inhibitory effect was not affected by CTZ

The inhibition of CP-AMPAR-mediated current by high concentration Zn^{2+} (1 mM) was also tested (see Fig. 2B). In the presence of 20 μM CTZ, glutamate (3 mM) elicited an inward current of 514.3 pA (peak current), which was reduced to 170.4 pA after pre-superfusion with 1 mM Zn^{2+} for 10 s. When Zn^{2+} was washed out for 1 min, the peak current recovered to 493.0 pA. Statistical data given in Fig. 2C show that the CP-AMPAR-mediated currents were decreased to 26.4±12.0% of the control level after application of 1 mM Zn^{2+} in the presence of CTZ (peak current, mean±SD, $p<0.05$, $n=5$). This inhibitory effect was not significantly different ($p>0.05$, unpaired *t*-test) from that observed in the absence of CTZ (21.1±9.7% of the control level, see Fig. 2C).

The dose-dependent curve of Zn^{2+} effects in modulating the CP-AMPAR-mediated current in the absence and presence of CTZ (20 μM) is plotted in Fig. 3. Different levels of Zn^{2+} (1, 10, 30, 100, 300 and 1000 μM) respectively changed the CP-AMPAR-responses to 95.9±17.8%, 99.5±11.2%, 97.0±8.9%, 72.2±4.7%, 53.4±9.3% and 21.1±9.7% (mean±SD) of the control level in the absence of CTZ ($n=5$, black curve), while the same levels of Zn^{2+} respectively changed the CP-AMPAR-responses to 147.0±6.2% ($n=8$), 150.6±25.5% ($n=8$), 103.0±22.4%, 63.4±7.7%, 61.2±5.8% and 26.4±12.0% (mean±SD) of the control level in the presence of 20 μM CTZ ($n=5$ otherwise indicated, gray curve). These results demonstrate that the potentiation effect induced by low concentration Zn^{2+} (1 μM, 10 μM) was enhanced by increasing the peak current amplitude in the presence of 20 μM CTZ, but the inhibitory effect induced by high concentration Zn^{2+} (100 μM, 300 μM, 1 mM) was not affected by 20 μM CTZ.

2.3. The Zn^{2+} inhibitory effect was abolished or attenuated in Ca^{2+}-free Ringer's

Given that the inhibitory effect induced by high concentration Zn^{2+} was not changed in the presence of CTZ, it is suggested that another separate mechanism might be involved. We therefore further examined whether the extracellular Ca^{2+}

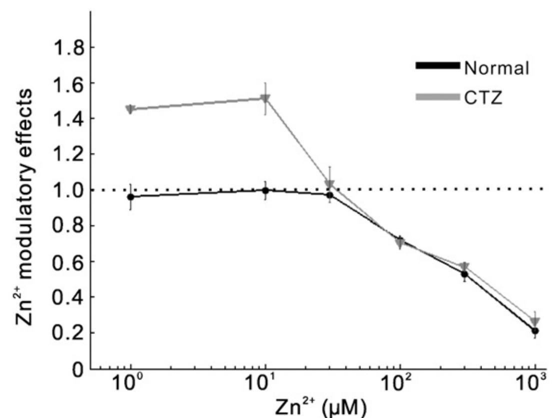

Fig. 3 – The dose-dependent Zn^{2+} effects on the peak level of CP-AMPAR-mediated current elicited by 3 mM Glu in the absence (black curve) and presence (gray curve) of 20 μM CTZ on H1 HCs. The data are plotted in mean±SE (*n*=8 for 1 and 10 μM Zn^{2+}, *n*=5 for others).

concentration could affect the Zn^{2+} inhibitory effect. To avoid the underestimation of the peak value of AMPAR current, CTZ at a concentration of 20 μM, which did not affect the Zn^{2+} inhibition of the CP-AMPAR-mediated current (see Figs. 2 and 3), was applied as the background perfusion. Meanwhile, kainate (KA), a full agonist of AMPARs (Hollmann and Heinemann, 1994), was also applied to elicit the AMPAR current without desensitization.

A typical experimental recording is plotted in Fig. 4A. In Ca^{2+}-free Ringer's containing 20 μM CTZ, glutamate (3 mM) elicited an inward current in an isolated H1 HC with the peak current being 1114.7 pA. When 1 mM Zn^{2+} was pre-superfused for 10 s, the peak current elicited by 3 mM glutamate was enhanced to 1601.3 pA. After Zn^{2+} was washed out for 1 min, the peak current elicited by 3 mM glutamate recovered to 1042.7 pA. Statistical data given in Fig. 4C show that instead of inhibition, application of 1 mM Zn^{2+} potentiated the CP-AMPAR-mediated currents to 144.6±24.7% of the control level (peak current, mean±SD, $p<0.05$, $n=8$) in Ca^{2+}-free Ringer's containing 20 μM CTZ. This is significantly different ($p<0.05$, unpaired t-test) from the inhibitory effect induced by 1 mM Zn^{2+} in normal Ca^{2+} (2.0 mM) Ringer's containing (26.4±12.0% of the control level, see Fig. 4C) or not containing (21.1±9.7% of the control level, see Figs. 2A and C) 20 μM CTZ.

The Zn^{2+} inhibitory effect exerted on CP-AMPAR-mediated current elicited by KA, a full agonist of AMPARs, was further tested in Ca^{2+}-free Ringer's. The results are plotted in Fig. 5A. It is clearly shown that in Ca^{2+}-free Ringer's, application of 100 μM KA elicited a non-desensitizing current of 511.1 pA, which was reduced to 311.7 pA after pre-superfusion with 1 mM Zn^{2+} for 10 s and recovered to 477.8 pA after 1 min Ringer's washout. Statistical data presented in Fig. 5C show that the KA-elicited currents were reduced to 61.8±20.8% of the control level after application of 1 mM Zn^{2+} in Ca^{2+}-free Ringer's (mean±SD, $p<0.05$, $n=8$). The Zn^{2+} inhibition of KA-elicited current mediated by CP-AMPARs in Ca^{2+}-free Ringer's was significantly attenuated ($p<0.05$, unpaired t-test) as compared to that in normal Ca^{2+} (2.0 mM) Ringer's (43.2±7.8% of the control level, see Fig. 5C).

2.4. *The Zn^{2+} inhibitory effect was more remarkable in high-Ca^{2+} Ringer's*

The inhibitory effect of CP-AMPAR-mediated current induced by 1 mM Zn^{2+} was further examined in high-Ca^{2+} (20 mM) Ringer's (see Fig. 4B). In high-Ca^{2+} Ringer's containing 20 μM CTZ, 3 mM glutamate elicited an inward current of 1036.4 pA (peak current), and this current was significantly reduced to 227.3 pA after pre-application of 1 mM Zn^{2+} for 10 s. When Zn^{2+} was washed away for 1 min, the peak current recovered to 918.2 pA. Statistical data given in Fig. 4C show that the CP-AMPAR-mediated currents were reduced to 15.3±8.3% of the control level (peak current, mean±SD, $p<0.05$, $n=8$) by application of 1 mM Zn^{2+} in high-Ca^{2+} Ringer's. The Zn^{2+} inhibitory effect was significantly strengthened in high-Ca^{2+} Ringer's (containing 20 μM CTZ) as compared to the effect in both CTZ-containing normal Ca^{2+} (2.0 mM) and Ca^{2+}-free Ringer's ($p<0.05$, unpaired t-test, see Fig. 4C).

The Zn^{2+} inhibitory effect on the KA-elicited current mediated by CP-AMPARs was also examined in high-Ca^{2+} Ringer's. As shown in Fig. 5B, application of 100 μM KA elicited a non-desensitizing current of 461.1 pA in an isolated H1 HC in high-Ca^{2+} Ringer's. This current was reduced to 138.9 pA after pre-superfusion of 1 mM Zn^{2+} for 10 s. After Zn^{2+} was washed out for 1 min, the KA-elicited current recovered to 433.3 pA. Statistical data presented in Fig. 5C show that the KA-elicited

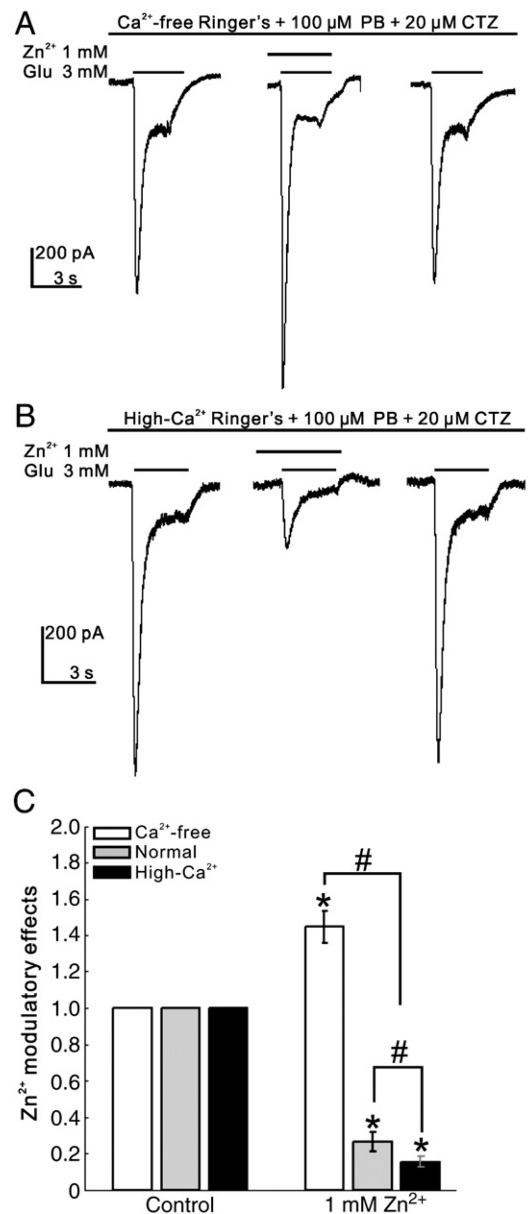

Fig. 4 – Zn^{2+} (1 mM) effects on the peak level of CP-AMPAR-mediated responses on H1 HCs in Ca^{2+}-free and high-Ca^{2+} Ringer's (containing 20 μM CTZ). (A–B) Application of 1 mM Zn^{2+} potentiated the CP-AMPAR-mediated response in Ca^{2+}-free Ringer's but dramatically inhibited the response in high-Ca^{2+} Ringer's. (C): Normalized Zn^{2+} (1 mM) effects on CP-AMPAR-mediated responses (peak currents) in normal Ca^{2+} ($n=5$, gray columns), Ca^{2+}-free ($n=8$, white columns) and high-Ca^{2+} Ringer's ($n=8$, black columns) (* $p<0.05$, paired t-test, as compared to control). # $p<0.05$ (unpaired t-test, comparison between the data as indicated).

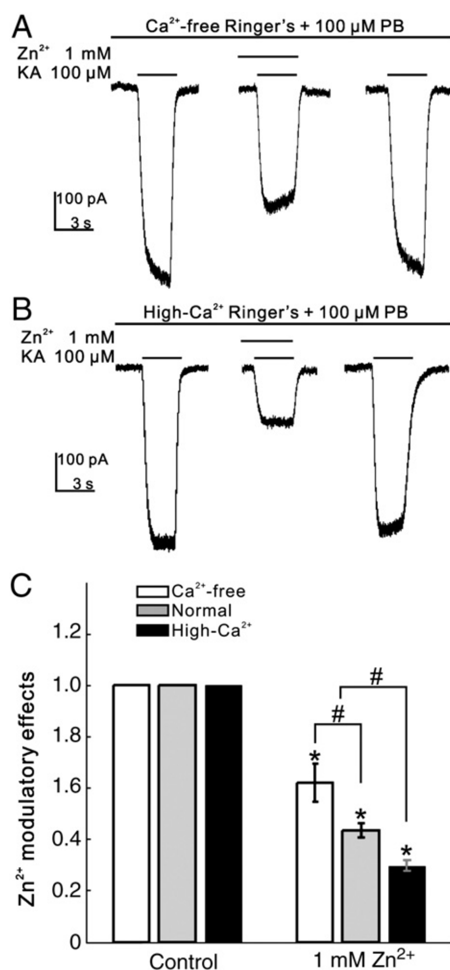

Fig. 5 – Zn^{2+} (1 mM) effects on CP-AMPAR-mediated responses elicited by KA on H1 HCs in Ca^{2+}-free and high-Ca^{2+} Ringer's. (A–B) Application of 1 mM Zn^{2+} reduced the KA-elicited currents both in Ca^{2+}-free and high-Ca^{2+} Ringer's. (C) Normalized Zn^{2+} (1 mM) effects of CP-AMPAR-mediated responses elicited by KA in normal Ca^{2+} ($n=8$, gray columns), Ca^{2+}-free ($n=8$, white columns) and high-Ca^{2+} Ringer's ($n=8$, black columns) (* $p<0.05$, paired *t*-test, as compared to control). # $p<0.05$ (unpaired *t*-test, comparison between the data as indicated).

currents were reduced to 29.6±5.7% (mean±SD, $p<0.05$, $n=8$) of the control level during Zn^{2+} (1 mM) application in high-Ca^{2+} Ringer's. This Zn^{2+} inhibition of KA-elicited current mediated by CP-AMPARs was significantly strengthened as compared to that in normal Ca^{2+} (2.0 mM) and Ca^{2+}-free Ringer's ($p<0.05$, unpaired *t*-test, see Fig. 5C).

These results indicate that the inhibitory effect that high concentration Zn^{2+} exerts on CP-AMPAR-mediated current is related to the extracellular Ca^{2+} concentration.

3. Discussion

In the present study, the mechanisms underlying the dual effects that Zn^{2+} exerts on CP-AMPARs on carp retinal H1 HCs were investigated. The main results are as follows: (1) Zn^{2+} (10 μM) potentiation effect on CP-AMPAR-mediated current is more remarkable in enhancing the peak current amplitude in the presence of moderate concentration of CTZ (20 μM); (2) the inhibitory effect induced by high concentration (1 mM) of Zn^{2+} was not affected by CTZ; (3) Zn^{2+} (1 mM) inhibitory effect on CP-AMPAR-mediated current was abolished or attenuated in Ca^{2+}-free Ringer's but strengthened in high-Ca^{2+} Ringer's. However, the Zn^{2+} potentiation effect was not affected by changing the extracellular Ca^{2+} concentration (data not shown). These results suggest that Zn^{2+} at low concentration is likely to act on the CTZ-binding site of CP-AMPARs. Meanwhile, the inhibitory effect induced by high concentration Zn^{2+} is related to the extracellular Ca^{2+}.

In addition to the amplitude enhancement, the desensitization process of the CP-AMPAR-mediated current was also slowed down by 10 μM Zn^{2+} in the presence of 20 μM CTZ (see Fig. 1B). However, this partial block of the desensitization process differs from that of the Zn^{2+} (10 μM) effect in the absence of CTZ, which completely blocked the desensitization of CP-AMPAR-mediated response (see Fig. 1A). In order to further estimate the interaction between CTZ and Zn^{2+} (10 μM) on CP-AMPARs, CTZ at various concentrations (0.1–300 μM) was applied in our experiments (see Supplementary Fig. I). The results were such that moderate concentrations (5–100 μM) of CTZ could enhance the Zn^{2+} potentiation effect by increasing the peak current amplitude, while lower (<5 μM) or higher (>100 μM) concentrations of CTZ could abolish the Zn^{2+} potentiation effect either by decreasing the peak current amplitude or speeding up the current desensitization process, respectively. Although the difference between the mechanisms underlying the Zn^{2+} potentiation effects in the absence and presence of CTZ is still not clear, it can almost be certain that the Zn^{2+} potentiation effect is related to the CTZ-binding site of CP-AMPARs on H1 HCs. Since the potentiation of CP-AMPAR-mediated current by low concentration Zn^{2+} may be involved in the regulation of the physiological activities of retinal HCs, the modulation of CTZ-binding site on CP-AMPARs might affect the Zn^{2+} potentiation effect and further influence the activities of retinal HCs in physiological conditions.

On the other hand, in bass (*Roccus chrysops*) retinal HCs, the Zn^{2+} (3–300 μM) inhibitory effect on AMPAR current was not affected by CTZ, which suggests that the Zn^{2+} inhibitory effect is not related to the CTZ-binding site on AMPARs (Zhang et al., 2002). Our present data also demonstrate that the Zn^{2+} (1 mM) inhibition of CP-AMPAR-mediated current on carp retinal H1 HCs is not changed in the presence of CTZ, which suggests that a separate mechanism should be involved in the Zn^{2+} inhibitory effect. It was previously reported that in two homo-assembled AMPARs (GluR1 and GluR3) expressed on *Xenopus oocytes*, the Zn^{2+} potentiation effect was enhanced in Ca^{2+}-free Ringer's, while in high-Ca^{2+} Ringer's, application of Zn^{2+} was inclined to inhibit the receptors (Dreixler and Leonard, 1997). However, our results demonstrate that the Zn^{2+} potentiation of CP-AMPAR response on retinal H1 HCs was not affected by the extracellular Ca^{2+}. On the other hand, the Zn^{2+} inhibition of the response was abolished (turned into a potentiation effect) or attenuated in Ca^{2+}-free Ringer's but strengthened in high-Ca^{2+} Ringer's.

In the experiments investigating the involvement of extracellular Ca^{2+} in Zn^{2+} inhibitory effect, "glutamate+CTZ" and KA were used respectively to elicit the CP-AMPAR-

mediated current on H1 HCs to avoid the underestimation of the peak current. The results obtained from these two sets of parallel experiments were basically compatible. In Ca^{2+}-free Ringer's, Zn^{2+} (1 mM) effect changed to potentiation of the response elicited by "glutamate + CTZ" instead of inhibition (see Figs. 4A and C), while the Zn^{2+} inhibition of the response elicited by KA was significantly attenuated (see Figs. 5A and C). Moreover, in high-Ca^{2+} Ringer's, both sets of the results show that high-Ca^{2+} condition facilitated the Zn^{2+} (1 mM) inhibitory effect on CP-AMPAR-responses elicited by either "glutamate + CTZ" or KA (see Figs. 4B, C and 5B, C).

Given the high Ca^{2+}-permeability of CP-AMPARs, the involvement of extracellular Ca^{2+} in Zn^{2+} effects seems readily acceptable. The interaction between the endogenous Zn^{2+} and Ca^{2+} may occur at the agonist-binding site, the receptor channel pore or other functional site(s) inside the channel. Our recent study has shown that the Zn^{2+} effects did not change the glutamate-affinity of CP-AMPARs on retinal H1 HCs (see Fig. 6 of Sun et al., 2010), which suggests that the agonist-binding site is not involved. Moreover, it was reported that the Zn^{2+} inhibitory effect of AMPARs on bass retinal HCs was not changed in the outside-out patch–clamp recording as compared to that in the whole-cell recording, which supports the non-involvement of the internal binding site(s) of AMPARs (Zhang et al., 2002). Given the above evidence and the passage of Ca^{2+} through the CP-AMPARs, the likely interaction site of the two ions might be at the receptor channel pore. However, this interaction between the endogenous Ca^{2+} and Zn^{2+} still needs further investigation.

Furthermore, the involvement of endogenous Ca^{2+} in Zn^{2+} inhibitory effects of CP-AMPARs on retinal HCs might be important in protecting the neurons from excitotoxicity. In pathological conditions, the CP-AMPARs on the postsynaptic membrane are over-activated by glutamate released from presynaptic neurons, which in turn leads to excessive influx of Ca^{2+} into the postsynaptic neurons and, finally, induces cell-death (for reviews, see Ugarte and Osborne, 2001; Kwak and Weiss, 2006). Meanwhile, the co-released Zn^{2+} with glutamate, accumulated in high concentration in the synaptic cleft, might protect the neurons from further Ca^{2+} influx by inhibiting the CP-AMPARs on these neurons, and this Zn^{2+} protection effect may be further regulated by interaction with the endogenous Ca^{2+}.

In summary, the dual effects that Zn^{2+} exerts on CP-AMPARs on retinal H1 HCs might be related to the CTZ-binding site on these receptors and the extracellular concentration of Ca^{2+}, respectively. Therefore, the modulation of CTZ-binding site on CP-AMPARs and/or changing the extracellular Ca^{2+} concentration might regulate the Zn^{2+} dual effects on these receptors and further influence the physiological and/or pathological role(s) of CP-AMPARs on retinal HCs.

4. Experimental procedures

4.1. *Preparation*

The experiments were performed on H1 HCs isolated from adult carp (*Carassius auratus*, 15–20 cm body length) retinas, following the method previously described (Jiang et al., 2008), which strictly conformed to the humane treatment and use of animals as prescribed by the Association for Research in Vision and Ophthalmology. In brief, retina isolated from an eyeball was cut into 8–12 pieces, and the retinal pieces were incubated in 4 ml Hank's solution (in mM: 120.0 NaCl, 3.0 KCl, 0.5 $CaCl_2$, 1.0 $MgSO_4$, 1.0 Na-pyruvate, 1.0 NaH_2PO_4, 0.5 $NaHCO_3$, 20.0 HEPES and 16.0 glucose) with 25 U/ml papain and 4 mg L-cysteine for 30 min at 25 °C . The retinal pieces were rinsed and stored in the Hank's solution at 4 °C. Cells were freshly dissociated from the retinal pieces by gentle mechanical trituration in Ringer's solution (normal Ringer's (in mM): 120.0 NaCl, 5.0 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 10.0 HEPES and 16.0 glucose) and then the cell suspension was placed onto a plastic dish. The H1 HC was easily distinguished by its characteristic morphology (Jiang et al., 2008). Glutamate (Glu), kainate (KA), pentobarbital (PB) and $ZnCl_2$ (Zn^{2+}) were dissolved in the Ringer's solution. Cyclothiazide (CTZ) was prepared in dimethyl sulfoxide (DMSO) and diluted to the final concentration in Ringer's solution (DMSO < 0.5%). Ca^{2+}-free Ringer's contains (in mM) 120.0 NaCl, 5.0 KCl, 1.0 $MgCl_2$, 10.0 HEPES and 22.0 glucose; high-Ca^{2+} Ringer's contains (in mM) 120.0 NaCl, 5.0 KCl, 20.0 $CaCl_2$, 1.0 $MgCl_2$ and 10.0 HEPES. The pH value for all the solutions was adjusted to 7.4 with NaOH. All the drugs were purchased from Sigma (St Louis, MO, USA).

4.2. *Whole-cell recording and drug application*

Cells were voltage-clamped at −60 mV, and whole-cell recordings were achieved by 5–8 MΩ patch pipette pulled from borosilicate glass (Sutter Instrument Inc., USA) using a horizontal puller (P87, Sutter Instrument Inc.). The pipette filled with intracellular solution (in mM: 140.0 CsCl, 0.05 $CaCl_2$, 1.0 $MgSO_4$, 0.5 EGTA, 10.0 HEPES, the pH value was adjusted to 7.3 with CsOH) was mounted on a motor-driven micromanipulator (MC1000e, SD Instrument Inc., USA) and was connected to a patch amplifier (Axopatch 200B, Axon Instrument Inc., USA). An Ag/AgCl wire was used as a reference electrode. The liquid junction potential was compensated online. Fast capacitance, cell capacitance transients and 70% of the series resistance of the recording electrode were compensated. Data acquisition was performed using AxoScope software (Axon Instrument Inc.), with sample rate being 1 kHz and was low-pass filtered (0–1 kHz). The recorded data were analyzed by Clampfit 9.2 software (Axon Instrument Inc.). The time constant (τ) of AMPAR desensitization was estimated by the monoexponential fitting to the decay of the current:

$$A(t) = A \times \exp[-(t-k)/\tau] + c \quad (1)$$

where t is the time variant, $A(t)$ is the current amplitude at time t, A is the peak current amplitude, k is the starting time of the fitting, τ is the time constant and c is a displacement constant.

The statistical data are all presented in the form of mean ± SD in the text and mean ± SE in the figure illustrations. Paired *t*-test was performed for statistical analysis unless otherwise stated.

All the drugs were applied using the superfusion system (DAD-12, ALA Scientific Instruments, USA).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.brainres.2010.05.033.

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