BIOCHEMISTRY
including biophysical chemistry & molecular biology
Rapid Report

Biochemistry **2009,** *48,* 12059–12061 12059
DOI: 10.1021/bi901762y

Rapid and Reversible Inhibition of Aquaporin-4 by Zinc[†]

Yoshinori Yukutake,[‡,§] Yoshinori Hirano,[‡] Makoto Suematsu,[§] and Masato Yasui*[,‡]

[‡]*Department of Pharmacology and* [§]*Department of Biochemistry and Integrative Medical Biology, School of Medicine, Keio University, Shinanomachi, Shinjyuku-ku, Tokyo 160-8582, Japan*

Received October 13, 2009; Revised Manuscript Received November 21, 2009

ABSTRACT: Aquaporin-4 (AQP4) is the predominant water channel in the brain. Although AQP4 plays an important role in brain water homeostasis, the molecular mechanisms of AQP4 regulation are not fully understood. In this report, we show how Zn^{2+} rapidly and reversibly decreases the water permeability of AQP4 when it is reconstituted into proteoliposomes. Mutagenesis analysis identified Cys178, located in cytoplasmic loop D, as a target residue of $ZnCl_2$ inhibition. Moreover, treatment with diamide enhanced the inhibitory effects of $ZnCl_2$. These results suggest that the water permeability of AQP4 may be regulated by dynamic changes in intracellular Zn^{2+} concentration linked to the cellular redox state.

Aquaporin-4 (AQP4)[1] is the most abundant water channel protein in the mammalian brain. There are two isoforms, which differ in the length of their N-terminal regions (M1, long and cysteine-rich; M23, short). AQP4 had been considered a mercury-insensitive water channel because it does not contain a cysteine residue at the position corresponding to Cys189 in AQP1 (*1*, *2*). This conclusion is supported by measurements of the osmotic water permeability of AQP4 when expressed in oocytes and various other types of cells (*1*−*4*) and by stopped-flow analysis using AQP4 M1-reconstituted proteoliposomes (*5*). However, we recently showed using a proteoliposome reconstitution system that mercury decreases the osmotic water permeability of AQP4 M23 via Cys178, which is located in loop D of the cytoplasmic region (*6*). In this system, analysis of the function of AQP4's intracellular region is possible because AQP4 is reconstituted into liposomes in bidirectional orientations. These results suggest that AQP4 is regulated via Cys178 in response to intracellular signaling. Thus, in this study, we investigated the effects of biotrace elements or redox reagents, which can modify cysteine residues of proteins, on the water permeability of AQP4 M23. We found that AQP4 M23 is inhibited by $ZnCl_2$ via Cys178, but not by redox reagents. We also show that the inhibitory effect of $ZnCl_2$ is rapid and reversible. To the best of our knowledge, this is the first example of AQP4-dependent osmotic water permeability regulation by a biotrace element. Taken together, these findings indicate that AQP4 may be regulated by intracellular Zn^{2+}.

Previously, we showed that the osmotic water permeability of AQP4 is inhibited by mercury chloride through interaction with the internal cysteine residue Cys178. To test if biotrace elements regulate AQP4 activity, the osmotic water permeability of 10His-ratAQP4 M23-reconstituted proteoliposomes (AQP4 proteoliposomes) was measured with metal or cationic chloride compounds in a stopped-flow apparatus using the carboxyfluorescein quenching method. The osmotic water permeability of AQP4 proteoliposomes [P_f = 89.9 ± 6.7 (SD) μm/s; n = 3] was significantly inhibited by treatment with 1000 μM $ZnCl_2$ (P_f = 62.8 ± 5.8 μm/s; n = 10) (Figure 1A). The osmotic water permeability of AQP4 proteoliposomes was also inhibited by 100 μM $CuCl_2$ (P_f = 58.2 ± 4.1 μm/s; n = 3), while other chloride compounds of biotrace elements ($MgCl_2$, $CaCl_2$, $MnCl_2$, LiCl, $CdCl_2$, and $FeCl_3$) did not significantly alter permeability. Application of either 1000 μM $ZnCl_2$ or 100 μM $CuCl_2$ did not decrease the osmotic water permeability of AQP1 proteoliposomes (Figure 1B). To rule out inhibition caused by binding of $ZnCl_2$ or $CuCl_2$ to the 10-His tag, purified 10His-ratAQP4 M23 was digested with factor Xa to release ratAQP4 M23 from its 10-His tag. Digested AQP4 [10His(−)AQP4] was reconstituted into liposomes, and osmotic water permeability was measured with and without $ZnCl_2$ or $CuCl_2$. The osmotic water permeability of 10His(−)AQP4 is inhibited by $ZnCl_2$ and $CuCl_2$ in manner similar to that of 10His-(+)AQP4, indicating that inhibition is not an artifact of the 10 histidines in the tag (Figure S1 of the Supporting Information).

To investigate dose-dependent inhibition of Zn^{2+} or Cu^{2+}, AQP4 proteoliposomes were pretreated with various concentrations of $ZnCl_2$ or $CuCl_2$ for 5 min, and osmotic water permeability was measured (Figure 1C). The data were fit to the Hill equation to determine half-maximal inhibitory concentrations and apparent Hill coefficients (see the Supporting Information). The half-maximal inhibitory concentration of Zn was 287 ± 58.5 μM, while that of Cu was 7.7 ± 2.5 μM, suggesting that the affinity of Zn^{2+} for AQP4 is much lower than that of Cu^{2+}. Moreover, the apparent Hill coefficients were 2.44 ± 1.00 for Zn^{2+} and 1.26 ± 0.44 for Cu^{2+}.

To test the reversibility of the effects of Zn^{2+} or Cu^{2+}, AQP4 proteoliposomes were treated with $ZnCl_2$ or $CuCl_2$ and osmotic water permeability was measured after DTT treatment or removal of metal chlorides. For DTT treatment, AQP4 proteoliposomes were pretreated with $ZnCl_2$ at 1000 μM or $CuCl_2$ at 100 μM for 5 min and then incubated with 10 mM DTT for 10 min. As shown Figure 2A, permeability was fully restored from Zn^{2+} inhibition by treatment with DTT (P_f = 96.8 ± 6.7 μm/s; n = 3), whereas it was only partly rescued with 100 μM $CuCl_2$ (P_f = 73.6 ± 3.1 μm/s; n = 3). It appears that the inhibitory effect

[†]This work was supported by a Grant-in-Aid for a Leading Project in Biosimulation, in part by Creative Scientific Research Grant GS00419, by the Global Center of Excellence Program for Humanoid Metabolomic Systems Biology, and by Specially Promoted Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT). Y.Y. is a postdoctoral fellow supported by the Global Center-of-Excellence Program for Human Metabolomic Systems Biology of the Ministry of Education, Culture, Sports, Science and Technology (MEXT).

*To whom correspondence should be addressed. Telephone: 81-3-5363-3751. Fax: 81-3-3359-8889. E-mail: myasui@sc.itc.keio.ac.jp.

[1]Abbreviations: AQP, aquaporin; AQP4, aquaporin-4; DTT, dithiothreitol; OG, *N*-octyl β-D-glucoside; P_f, osmotic water permeability; SD, standard deviation.

Published on Web 11/24/2009

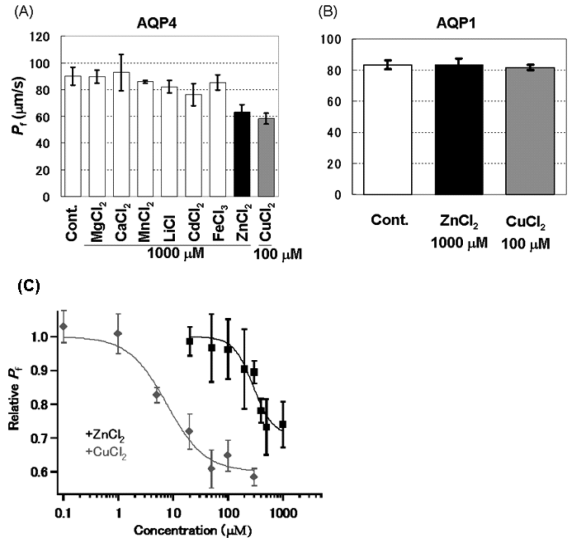

FIGURE 1: Osmotic water permeability of AQP4 proteoliposomes decreases upon treatment with $ZnCl_2$ or $CuCl_2$. (A) Calculated osmotic water permeabilities (P_f) of AQP4 proteoliposomes, which were pretreated with $MgCl_2$, $CaCl_2$, $MnCl_2$, LiCl, $CdCl_2$, $FeCl_3$, and $ZnCl_2$ at 1000 μM and with $CuCl_2$ at 100 μM (n = 3−10) for 5 min. Results are displayed as the mean ± SD. (B) P_f values of AQP1 proteoliposomes with $ZnCl_2$ at 1000 μM and $CuCl_2$ at 100 μM (n = 3). Results are displayed as the mean ± SD. (C) Dose−response effects of Zn^{2+} (■) or Cu^{2+} (◆) on AQP4 proteoliposomes. Hill plots were generated with the aid of IGOR Pro version 5.03J. Results are displayed as the mean ± SD (n = 3−10).

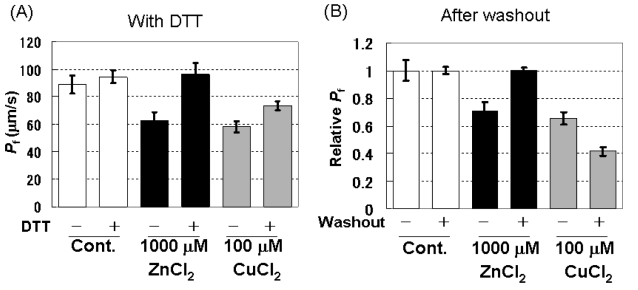

FIGURE 2: Reversibility of Zn^{2+} and Cu^{2+} inhibitory effects on AQP4 water permeability. (A) Reduction effects of AQP4 proteoliposomes with Zn^{2+} or Cu^{2+}. AQP4 proteoliposomes were treated with $ZnCl_2$ at 1000 μM or $CuCl_2$ at 100 μM followed by addition of 10 mM DTT. Results are displayed as the mean ± SD (n = 3). (B) Washout effects of AQP4 proteoliposomes with Zn^{2+} or Cu^{2+}. After AQP4 proteoliposomes were incubated with 1000 μM $ZnCl_2$ or 100 μM $CuCl_2$, the ions were removed by washing (detailed methods provided as Supporting Information). Results are displayed as the mean ± SD (n = 3).

of Zn^{2+} is reversed to a greater extent by DTT than is Cu^{2+}-mediated inhibition.

To test the reversibility via removal of metal chlorides, 6 mL of an AQP4 proteoliposome suspension pretreated with $ZnCl_2$ at 1000 μM or $CuCl_2$ at 100 μM for 5 min was washed three times according to an ultracentrifugation protocol and suspended in 5 mL of 100 mM MOPS buffer (pH 7.5). Interestingly, the osmotic water permeability of AQP4 proteoliposomes treated with $ZnCl_2$ (relative P_f = 0.70 ± 0.06; n = 3) but subsequently washed (relative P_f = 1.00 ± 0.01; n = 3) was fully restored to that of untreated AQP4 proteoliposomes, yet the permeability of AQP4 proteoliposomes treated with $CuCl_2$ (relative P_f = 0.65 ± 0.05; n = 3) and washed (relative P_f = 0.42 ± 0.03; n = 3) was not restored (Figure 2B). These results suggest that the inhibitory effect of Zn^{2+} can be reversed with cysteine residues with

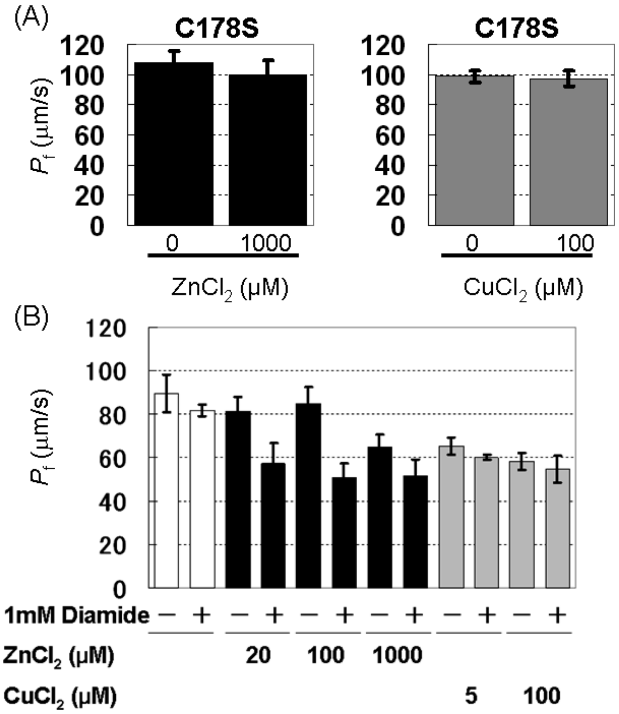

FIGURE 3: Characterization of Zn^{2+} inhibition of AQP4 water permeability. (A) The thiol group of Cys178 in loop D of AQP4 might be the target of Zn^{2+} and Cu^{2+} inihibition. The osmotic water permeability of the C178S mutant of AQP4 was not inhibited by $ZnCl_2$ or $CuCl_2$. (B) Treatment with 1 mM diamide enhanced the Zn^{2+} inhibition to an extent observed for copper alone. AQP4 proteoliposomes were pretreated with 1 mM diamide for 15 min and then incubated with $ZnCl_2$ (20, 100, or 1000 μM) or $CuCl_2$ (5 or 100 μM).

the weak interaction, while the Cu^{2+} effect is partially reversible with strong interaction such as a thiolate bond and/or thiol oxidation.

The osmotic water permeability of the mutant AQP4 C178S, which is mercury-insensitive, was measured in the presence of $ZnCl_2$ given that Cys178 is required for mercurial inhibition. The osmotic water permeability of C178S proteoliposomes was not affected by $ZnCl_2$ (Figure 3A). Surprisingly, Cys178 does mediate Cu^{2+} inhibition (Figure 3A).

To understand the difference between Zn^{2+} and Cu^{2+} with respect to AQP4 sensitivity, we added diamide (1 mM) to the reaction mix, which is a thiol oxidizing agent that enhances the disulfide bond (Figure 3B). Addition of diamide enhanced the inhibitory effect of Zn^{2+} (at 20 μM) on AQP4 (from 81.5 ± 6.6 to 57.2 ± 9.6 μm/s). On the other hand, diamide had no effect on Cu^{2+}-mediated inhibition of AQP4. These results indicated that Zn^{2+} can strongly inhibit the water permeability of AQP4 with thiol oxidants.

We next examined whether oxidizing or reducing agents would alter the osmotic water permeability of AQP4 because cysteine modifications by redox agents often change the functions of proteins. The osmotic water permeability of AQP4 proteoliposomes was measured in the presence and absence of 1 mM diamide, *S*-nitrosoglutathione (GSNO), peroxynitrite ($ONOO^-$), hydrogen peroxide (H_2O_2), and reduced glutathione (GSH) (Figure S2 of the Supporting Information). None of the treatments significantly changed the osmotic water permeability of AQP4, suggesting that the single application of redox agents does not regulate AQP4 function. Additionally, alkylation of AQP4 by iodoacetic acid or *N*-ethylmaleimide did not inhibit

the osmotic water permeability of AQP4 (data not shown). Therefore, we believe that metal ions play a primary role in AQP4 inhibition. Of note, the effect of diamide on Zn^{2+}-mediated inhibition strongly suggests that thiol oxidants enhance the inhibitory effect of Zn^{2+} on AQP4.

Overall, we found that the osmotic water permeability of AQP4 was inhibited by $ZnCl_2$ and $CuCl_2$ via Cys178. However, there were some differences between Zn^{2+}- and Cu^{2+}-mediated inhibition. Zn^{2+} forms the thiolate with the cysteine thiol group, and the thiolate can be reversed by treatment with a thiol reducing agent such as DTT. In this report, the reversibility of Zn^{2+} effects was shown by not only treatment with DTT but also washout, suggesting the existence of the thiolate bond and/or a weaker bond such as a coordination bond. Moreover, diamide enhanced the inhibitory effects of Zn^{2+}, suggesting that Zn^{2+} enhanced the disulfide bond (see the Supporting Information). Cu^{2+} effects are more difficult to predict because the Cu^{2+}-mediated thiolate is susceptible to oxidation and may yield disulfides, sulfenic acids, sulfinic acids, or sulfonic acids. The reduction of oxidized thiol is often tricky, since the thiol reducing agent could reverse sulfides or sulfenic acids, but not sulfinic acids or sulfonic acids. In this report, Cu^{2+}-mediated inhibition was partially restored with DTT but not with washout. Therefore, Cu^{2+} has caused the modification of Cys178 with the thiolate bond and/or some thiol oxidations.

Zn^{2+} is one of the most abundant biotrace ions in the brain (see the Supporting Information) and is important for the activities of many enzymes and membrane proteins, including AQP0, NMDA receptors, AMPA receptors, $GABA_A$ receptors, P2X receptors, and voltage-gated-like ion channels (*7*−*12*). AQP0 is a lens water channel. In the AQP0-expressing oocyte, zinc increases the osmotic water permeability via His40 and His122 in extracellular loops, while for AQP4, zinc decreases the water permeability via intracellular Cys178. Interestingly, in the same monomer, the distance between His40 and His122 in AQP0 is 11 Å. Although the distance is too great for the formation of simultaneous bonds with zinc, it seems to form an ordered water chain, which could connect the histidine side chain with zinc along with the corresponding cooperative conformational change. In AQP4, the distance [10 Å (Figure S3B of the Supporting Information)] between Cys178 and Cys253 is also too great for the formation of a simultaneous bond with zinc. Water might play a similar role in AQP4. The channel regulation by intracellular zinc has rarely been evidenced. Wang et al. described the role for intracellular Zn^{2+} in the Kv4 channel complex. The Kv4 channel complex, a member of the superfamily of voltage-gated K^+ channels, contains a Zn^{2+} binding site ($HX_5CX_{20}CC$) in its intracellular T1 domain (*13*, *14*). Kv4 function is inhibited by the intracellular application of nitric oxide, but the inhibition is suppressed by intracellular Zn^{2+}. Incidentally, the concentration of intracellular Zn^{2+} is closely linked to the cellular redox state. Nitric oxide resulting from oxidative stress enhances the release of intracellular Zn^{2+} from Zn^{2+}-binding proteins such as metallothionein. It has been suggested that nitric oxide increases the rate of intracellular Zn^{2+} release in cultured astrocytes under hypoosmotic conditions (*15*). Under conditions of oxidative stress, AQP4 function may be regulated by low Zn^{2+} concentrations in the brain. Further studies are necessary to investigate the physiological relevance of the effects of Zn on AQP4 in astrocytes.

SUPPORTING INFORMATION AVAILABLE

Detailed discussions, experimental procedures, and Figure S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Jung, J. S., Bhat, R. V., Preston, G. M., Guggino, W. B., Baraban, J. M., and Agre, P. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 13052–13056.
2. Jung, J. S., Preston, G. M., Smith, B. L., Guggino, W. B., and Agre, P. (1994) The hourglass model. *J. Biol. Chem. 269*, 14648–14654.
3. Hasegawa, H., Zhang, R., Dohrman, A., and Verkman, A. S. (1993) *Am. J. Physiol. 264*, C237–C245.
4. Nicchia, G. P., Frigeri, A., Liuzzi, G. M., Santacroce, M. P., Nico, B., Procino, G., Quondamatteo, F., Herken, R., Roncali, L., and Svelto, M. (2000) *Glia 31*, 29–38.
5. Yang, B., van Hoek, A. N., and Verkman, A. S. (1997) *Biochemistry 36*, 7625–7632.
6. Yukutake, Y., Tsuji, S., Hirano, Y., Adachi, T., Takahashi, T., Fujihara, K., Agre, P., Yasui, M., and Suematsu, M. (2008) *Biol. Cell 100*, 355–363.
7. Maret, W. (2009) *BioMetals 22*, 149–157.
8. Mocchegiani, E., Bertoni-Freddari, C., Marcellini, F., and Malavolta, M. (2005) *Prog. Neurobiol. 75*, 367–390.
9. Smart, T. G., Hosie, A. M., and Miller, P. S. (2004) *Neuroscientist 10*, 432–442.
10. Nemeth-Cahalan, K. L., Kalman, K., Froger, A., and Hall, J. E. (2007) *J. Gen. Physiol. 130*, 457–464.
11. Mathie, A., Sutton, G. L., Clarke, C. E., and Veale, E. L. (2006) *Pharmacol. Ther. 111*, 567–583.
12. Huidobro-Toro, J. P., Lorca, R. A., and Coddou, C. (2008) *Eur. Biophys. J. 37*, 301–314.
13. Covarrubias, M., Bhattacharji, A., De Santiago-Castillo, J. A., Dougherty, K., Kaulin, Y. A., Na-Phuket, T. R., and Wang, G. (2008) *Neurochem. Res. 33*, 1558–1567.
14. Wang, G., Strang, C., Pfaffinger, P. J., and Covarrubias, M. (2007) *J. Biol. Chem. 282*, 13637–13647.
15. Kruczek, C., Gorg, B., Keitel, V., Pirev, E., Kroncke, K. D., Schliess, F., and Haussinger, D. (2009) *Glia 57*, 79–92.