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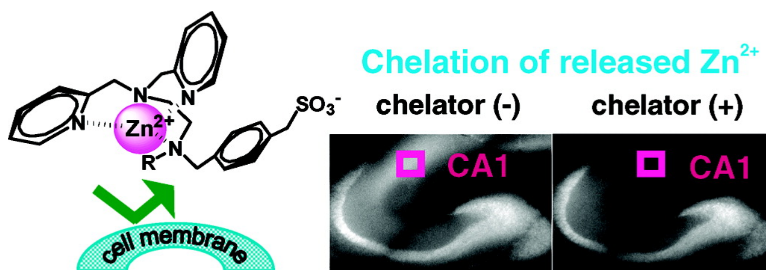

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Design and Synthesis of Zinc-Selective Chelators for Extracellular Applications

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Zinc (Zn^{2+}) is found in every cell in the human body and is mostly tightly bound to proteins as a key component of numerous enzymes and transcription factors.[1,2] Chelatable Zn^{2+} co-localizes with glutamate in the synaptic vesicles of certain glutamatergic vesicles in the mammalian brain, including the hippocampus, amygdala, and neocortex.[3] Free Zn^{2+} exists at a concentration of a few millimolar in the vesicles of presynaptic neurons and is released during synaptic activity or depolarization, modulating the function of certain ion channels and receptors.[4−7] Many reports describe the significance of Zn^{2+} in biological systems,[8−13] but its mechanisms of action are poorly understood.

Although various chemical tools for measuring Zn^{2+} in biological samples, such as fluorescence probes for Zn^{2+}, have been developed,[14−23] better Zn^{2+}-selective chelators are still needed. Research on Zn^{2+} signals in the brain has traditionally employed several chelators, though they have various shortcomings for biological applications. Use of *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN), a classical membrane-permeable Zn^{2+} chelator, does not allow selective manipulation of extracellular or intracellular Zn^{2+}. On the other hand, calcium ethylenediamine-tetraacetic acid (CaEDTA), an extracellular (membrane-impermeable) Zn^{2+} chelator, has the disadvantage of slow kinetics of Zn^{2+} binding. Concentrations of CaEDTA that are sufficient to chelate synaptic Zn^{2+} at equilibrium do not effectively chelate Zn^{2+} within the period of tens to hundreds of microseconds that it takes Zn^{2+} to cross the synapse and interact with various postsynaptic sites.[24] In contrast, a higher concentration of CaEDTA markedly reduces the Zn^{2+} and Ca^{2+} signals.[25] This phenomenon makes it difficult to interpret the action mechanism of Zn^{2+} under physiological conditions, where change of Ca^{2+} concentration can also induce various responses. Especially in electrophysiological studies of ion channels and neuronal activities, reduction of extracellular Ca^{2+} can result in neurological side effects. The development of new, more rapid Zn^{2+} chelators which have low affinity for Ca^{2+} is required for the clarification of the mechanism of synaptic release of Zn^{2+}. We report here the design, synthesis, and properties of new Zn^{2+} chelators, and we describe biological application in hippocampal slices.

We designed (4-{[2-(bis-pyridin-2-ylmethylamino)ethylamino]-methyl}phenyl)methanesulfonic acid, sodium salt (DPESA), and [4-({[2-(bis-pyridin-2-ylmethylamino)ethyl]pyridin-2-ylmethylamino}-methyl)phenyl]methanesulfonic acid, sodium salt (TPESA) utilizing TPEN structure for high Zn^{2+} selectivity and a sulfonic acid moiety for hydrophilicity (Figure 1). The acid dissociation constants and metal chelate stability constants were determined by potentiometric methods (Table 1 and Table S1, Supporting Information). The log *K* values of DPESA and TPESA for Zn^{2+} are 11.8 and 12.3, respectively, showing that these two compounds have high affinity for Zn^{2+}. The log *K* values for Ca^{2+} are 4.20 and 2.47, and those for Mg^{2+} are 3.97 and 2.66, respectively, indicating low affinity for these metal ions.

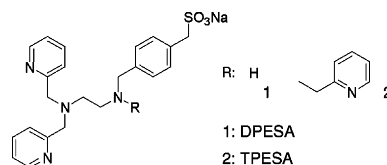

Figure 1. Structures of new zinc-selective chelators.

Table 1. Stability Constants of Chelators

	log *K*			
M^{n+}	DPESA[a]	TPESA[a]	TPEN[b]	EDTA[b]
Ca^{2+}	4.20 ± 0.11	2.47 ± 0.10	4.40	10.7
Mg^{2+}	3.97 ± 0.10	2.66 ± 0.10	1.70	8.64
Zn^{2+}	11.8 ± 0.01	12.3 ± 0.03	15.4	16.3

[a] Stability constants of DPESA and TPESA were determined at 25 °C, $I = 0.1$. The determined value ± SD is given. [b] From the SCDatabase (IUPAC and Academic Software): 25 °C, $I = 0.1$.

To compare the relative association rate constants of the new chelators for Zn^{2+} with those of traditional chelators, competition analysis was performed between Zn^{2+}-fluorescence probe complex and Zn^{2+}-selective chelators (Figure S3, Supporting Information). ZnAF-2[14a] was used as a fluorescence probe for Zn^{2+}; its log K_{obs} value (K_{obs} is the apparent association constant at pH 7.4, $I = 0.10$ M $NaNO_3$) has been reported as 8.57. The fluorescence intensity of ZnAF-2 (1.0 μM) linearly increased up to a 1:1 [ZnAF-2]/[Zn^{2+}] ratio, and the maximum fluorescence was obtained with 1.0 μM Zn^{2+} addition. Then 10 μM chelator was added and the time course of fluorescence decrease was compared among DPESA, TPESA, TPEN, and CaEDTA. The addition of 10 μM TPESA rapidly decreased the fluorescence with a half-life of 18.6 s, which is comparable to that of TPEN (12.2 s). This result suggested that TPESA can reduce the concentration of synaptically released Zn^{2+} rapidly, without changing the extracellular Ca^{2+} concentration. On the other hand, DPESA reduced the fluorescence with a half-life of 67.0 s, which is similar to the value of 65.4 s obtained for CaEDTA. Considering that DPESA may have the same affinity for Zn^{2+} as ZnAF-2, whereas the other chelators have higher affinities (Table 1), the above result suggests rapid Zn^{2+} chelation.

To examine the membrane permeability of the new chelators, we applied them to hippocampal slices (Figure S4, Supporting Information). Acute rat hippocampal slices were incubated with 10 μM ZnAF-2 DA[14a] for 1.5 h at room temperature for dye loading.

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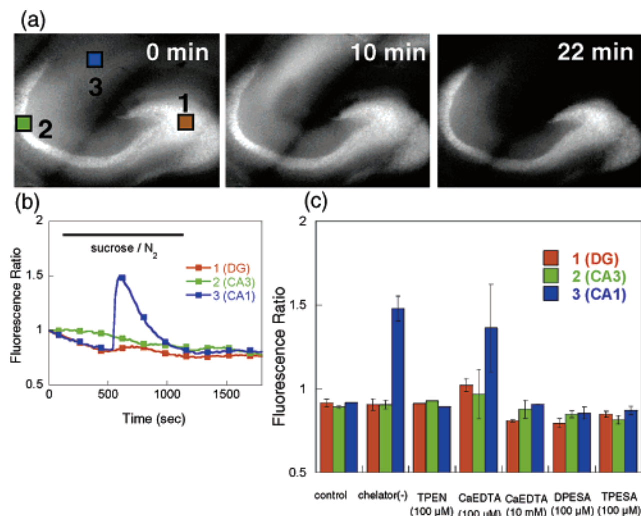

Figure 2. Application of the chelators under ischemic conditions. Dye-loaded slices were exposed to anoxic-aglycemiic ACSF for 17 min (from 2 to 19 min). (a) Fluorescence images at 0, 10, and 22 min after the measurement. The fluorescence ratio in the plot (b) is the ratio of the fluorescence intensity to the initial intensity of the corresponding area in the image at 0 min. (c) Histogram showing the effects of the chelators on the fluorescence change of ZnAF-2 DA-loaded slices under anoxia-aglycemia. Fluorescence ratio in the histogram is the ratio of the fluorescence intensity at 10 min after the start of the measurement to the initial intensity of the corresponding area in the image at 0 min. Each column, except for TPEN, shows the mean ± SE.

ZnAF-2 DA is expected to permeate well through the cell membrane and then to be transformed to ZnAF-2 by esterase in the cytosol, where the dye would be retained for a long time. It interacts with intracellular Zn^{2+} to generate strong fluorescence. The fluorescence was intense in the hilus and the stratum lucidum of the CA3 region, where Zn^{2+} is concentrated in the vesicles.[6] The fluorescence was decreased by extracellular addition of the membrane-permeable chelator TPEN (100 μM) for 30 min, whereas it was scarcely altered by addition of CaEDTA, DPESA, or TPESA. These results support the view that DPESA and TPESA are membrane-impermeable chelators.

It has been suggested that the concentration of intracellular Zn^{2+} at the hippocampal CA1 region transiently increases in response to an ischemic insult, which is content with a relationship between Zn^{2+} and apoptosis.[14b] This increase might be derived from influx of extracellular Zn^{2+} released from the presynaptic terminals, or from the release of Zn^{2+} from intracellular vesicles. To investigate the second possibility, we used DPESA and TPESA (Figure 2).

Acute rat hippocampal slices which had been loaded with ZnAF-2 DA were exposed to anoxic-aglycemic ACSF for 17 min. The fluorescence in the CA1 region increased transiently, but this was not observed when TPEN (100 μM) was added extracellularly (30-min preperfusion with 100 μM TPEN followed by anoxic-aglycemic insult in the presence of 100 μM TPEN). Similarly, the presence of 100 μM DPESA or TPESA inhibited the transient increase in fluorescence, indicating that DPESA and TPESA chelated synaptically released Zn^{2+} quite rapidly and thereby suppressed influx of Zn^{2+} into the cells. These results suggest that Zn^{2+} is released from the presynaptic vesicles in response to an ischemic insult and is taken up intracellularly into the postsynaptic neurons. Notably, 100 μM DPESA or TPESA was sufficient for Zn^{2+} chelation, whereas 100 μM CaEDTA was not. Thus, our newly designed chelators yielded the biologically significant finding that presynaptic Zn^{2+} can be released in the CA1 region. This confirms the utility of these new chelators as extracellular Zn^{2+} chelators for biological applications.

In conclusion, we have developed new membrane-impermeable Zn^{2+}-selective chelators, DPESA and TPESA, with the advantageous characteristics of high Zn^{2+} selectivity and rapid chelation, that can be applied to cell-biological studies to shed additional light on the function of synaptically released Zn^{2+}.

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Supporting Information Available: Synthesis, experimental details, and characterization of DPESA and TPESA. This material is available free of charge via the Internet at http://pubs.acs.org.

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