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Enzyme-Responsive Procarriers Capable of Transporting Chloride Ions across Lipid and Cellular Membranes

Ye Rin Choi,[†] Bom Lee,[†] Jinhong Park,[‡] Wan Namkung,[‡] and Kyu-Sung Jeong*[,†]

[†]Department of Chemistry, Yonsei University, Seoul 120-749, Korea

[‡]College of Pharmacy, Yonsei International Campus, Incheon 406-840, Korea

Supporting Information

ABSTRACT: Adopting the concept of procarrier for the first time, we demonstrated the controlled transport of chloride ions across lipid and cellular membranes. Procarriers containing highly hydrophilic appendages were initially inactive due to the lack of their partitioning into lipophilic membranes but were activated to transport chloride ions in the presence of specific enzymes that were able to hydrolyze off the appendages to generate an active carrier under specific conditions. Namely, the procarrier with an ester-bond-linked appendage was most activated by an esterase (PLE) at pH = 7.4, whereas the procarrier with a glycosyl-bond-linked appendage was activated only by a glycosylase (AOG) under slightly acidic conditions (pH = 5.5−6). In addition to controlling chloride transport, hydrophilic appendages greatly increase the water solubility of the procarrier, which may improve the deliverability of a hydrophobic active carrier into a plasma membrane.

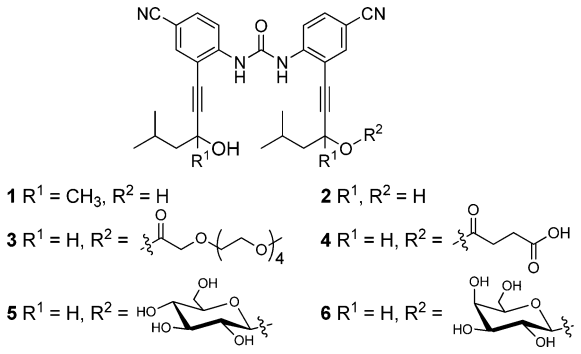
The transport of chloride ions across cellular membranes is facilitated by transmembrane proteins functioning as ion channels or ion transporters, which is precisely controlled and activated by biological stimuli including membrane potential, neurotransmitters, pH and light.[1] For example, the binding of γ-aminobutyric acid (GABA) to the receptor[2] makes the chloride-selective channel open to conduct chloride ions. Halorhodopsins[3] are activated by light to pump chloride ions into the cell against the electrical gradient. In addition, enzyme-catalyzed phosphorylation and dephosphorylation in the hydroxyl residues of serine, threonine and tyrosine are known to reciprocally regulate the activities of Na-K-2Cl (NKCCs) and K-Cl (KCCs) cotransporters. That is, phosphorylation activates NKCCs but inhibits KCCs for chloride uptake in cells whereas dephosphorylation gives rise to the opposite activities of the channels.[4]

A variety of synthetic molecules that can facilitate the transport of chloride ions across lipid and plasma membranes have been prepared in recent years.[5] These synthetic transporters may have therapeutic application in the treatment of channelopathies such as cystic fibrosis and Bartter's syndrome.[6] Moreover, some anion transporters have been proven to induce cell apoptosis and display anticancer activity.[7] A great challenge is to achieve the spatiotemporal control of transport activity to a specific target of interest. To this end, one approach is to design stimuli-responsive molecules that can function only under specific conditions or in the presence of an external stimulus.[8,9] As a stimulus, enzymes are highly useful and relevant in biological systems due to their high selectivity, specificity, and biocompatibility. Consequently, enzyme-catalyzed reactions have been widely implemented in drug delivery,[10] cell imaging[11] and biomolecular sensing,[12] but the enzyme-responsive synthetic anion transporter is not described to date.

Synthetic anion carriers should be highly lipophilic and hydrophobic for efficient partitioning into lipid membranes, but at the same time they should be suitably soluble in water for efficient delivery.[13] It is extremely difficult to attain an optimum balance between two contradicting properties, and therefore many synthetic carriers have shown to give poor transport activities due to their low deliverability. The improvement of deliverability of hydrophobic synthetic carriers is also an important issue in the development of synthetic anion transporters.[13d−f] We envisioned that the deliverability might be enhanced by the incorporation of a hydrophilic appendage to the hydrophobic carriers, which could be in situ removed to generate an active transporter via enzymatic hydrolysis just prior to embedding into cellular membranes.

With these in mind, we herein prepared a series of synthetic anion procarriers **3**−**6** that contained strongly hydrophilic groups such as carboxylates and carbohydrates (Chart 1). These procarriers per se showed negligible activities of chloride transport across lipid and cellular membranes. However, the activities were greatly revived in the presence of specific lipases, esterases, or glycosylases that were able to hydrolyze off the hydrophilic appendages.

Chart 1. Structures of Synthetic Carriers 1 and 2, and Procarriers 3−6



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The design of procarriers **3**–**6** was based on the structure of compound **1** that was previously proven to be an excellent synthetic carrier for chloride ions across a lipid membrane.[14] Given the relative reactivity of the enzymatic hydrolysis of the final procarriers, compound **2** with *sec*-alcohols was used as an active carrier (see Figure S1) instead of compound **1** containing *tert*-alcohols. Four different procarriers, **3**–**6**, were prepared by the introduction of tetraethylene glycol acetate, succinate, glucosyl and galactosyl units, respectively. The syntheses and characterizations are described in the Supporting Information (SI). All the procarriers with these hydrophilic appendages are suitably soluble in aqueous solution.

The chloride transport ability of synthetic molecules was measured by a fluorescence assay in large unilamellar vesicles (LUVs) comprising 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC).[15] The LUVs were loaded with $NaNO_3$ (200 mM) and lucigenin (1 mM) in 10 mM phosphate buffer pH = 7.2, and were suspended in a solution of $NaNO_3$ (200 mM) in 10 mM phosphate buffer pH = 7.2. To this solution were added a procarrier and an enzyme at room temperature. After 30 min,[16] a solution of NaCl (50 mM) was injected to initiate the influx of chloride ions into the LUVs, which was monitored by the gradual decrease of the fluorescence intensity of lucigenin in the LUVs.

The transport behaviors of ester-bond-linked procarriers **3** and **4** were first examined in the presence of commercially available esterase and lipases (Figure 1 and Table S1). Without any

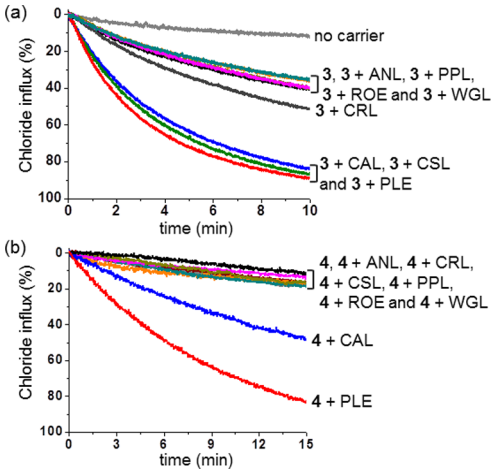

Figure 1. Chloride influx across a POPC membrane facilitated by (a) procarriers **3** (2 μM) and (b) **4** (40 μM) when treated with enzymes (500 μg/mL) at 25 °C for 30 min; *Aspergillus niger* lipase (ANL), *Candida antarctica* lipase (CAL), *Candida rugosa* lipase (CRL), *Candida* sp. lipase (CSL), porcine liver esterase (PLE), porcine pancreas lipase (PPL), *Rhizopus oryzae* esterase (ROE) and wheat germ lipase (WGL).

enzyme, procarrier **3** with a neutral ethylene glycol appendage was weakly active whereas procarrier **4** with a succinate salt was negligible under the given conditions. These observations suggest that hydrophilic appendages, in particular the hydrophilic succinate salt, effectively inhibit partitioning of the procarriers from the aqueous buffer solution into a lipophilic POPC membrane. In the presence of specific enzymes, the procarriers **3** and **4** exhibited much increased activities of transporting chloride ions across a POPC lipid bilayer. For example, procarrier **3** became much more active upon incubation with CAL, CSL and PLE (Figure 1a). However, the transport activity of **3** was not enhanced in the presence of five other enzymes (ANL, PPL, ROE, WGL and CRL). These results imply that the former three enzymes can effectively catalyze the hydrolysis of the ester linkage to generate the active carrier **2** under the given conditions, unlike the later five enzymes. This explanation was confirmed by ^{1}H NMR spectroscopy. When procarrier **3** was incubated with PLE and CSL in a phosphate buffer solution at room temperature, a new set of ^{1}H NMR signals corresponding to an active carrier **2** began to appear within 30 min. After 12 h, each reaction was completed by approximately 90% (see Figures S6 and S8). However, spectral change was not observed in the presence of an inactive enzyme PPL under the same conditions (see Figure S6). Procarrier **4** containing a succinate salt appendage was more resistant and selective to the enzymatic hydrolysis under the same conditions, compared to **3**. Among the eight enzymes we examined, only PLE was effective in generating the transport activity of chloride ions, together with a moderate activation by CAL (Figure 1b).

Next, the transport properties of glycosyl-linked procarriers **5** and **6** were tested with seven commercially available glycosylases (Figure 2 and Table S1). As anticipated, procarriers **5** and **6**

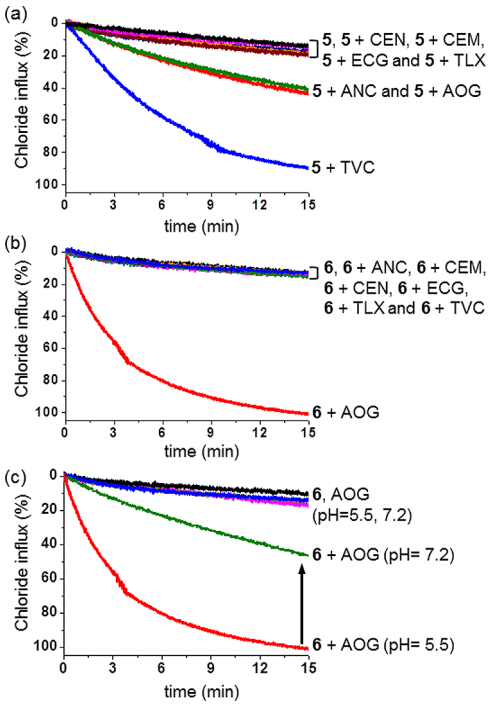

Figure 2. Chloride influx across a POPC membrane facilitated by procarriers (a) **5** (30 μM) after incubation with each enzyme (1 mg/mL) at 37 °C for 3 h and (b) **6** (30 μM) after incubation with enzymes (1 mg/mL) at 25 °C for 30 min; *Aspergillus niger* cellulase (ANC), *Aspergillus oryzae* β-galactosidase (AOG), *Canavalia ensiformis* α-mannosidase (CEM), *Canavalia ensiformis* β-*N*-acetylglucosaminidase (CEN), *Escherichia coli* β-galactosidase (ECG), *Thermomyces lanuginosus* xylanase (TLX) and *Trichoderma viride* cellulase (TVC).

which contain highly hydrophilic glucose and galactose units were completely inactive for the chloride transport across a POPC lipid bilayer. Again, the transport activities of chloride ions were revived upon the incubation of the procarriers **5** and **6** in the presence of specific glycosylases. In the case of procarrier **5** bearing a glucose unit, the enzymatic hydrolysis was extremely slow at 25 °C in a pH = 7.2 phosphate buffer solution and therefore the activation of chloride transport across a POPC membrane was not efficient in the presence of any glycosylase tested here. It was known that the glycosyl bond was more

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susceptible to the enzymatic hydrolysis under acidic conditions.[17] Therefore, the incubation with each enzyme was carried out at 37 °C in a pH = 5.5 phosphate buffer solution for 3 h prior to the transport experiment, separately. As shown in Figure 2a, TVC was most effective among the seven enzymes we examined. It should be noted that only a small portion (<10%) of the added procarrier **5** was hydrolyzed for 3 h under the given conditions in the presence of the most effective enzyme TVC. Under the same conditions, only ~20% of the hydrolysis was completed after 12 h incubation according to ^{1}H NMR spectra (see Figure S9).

To improve the practical applicability, we prepared procarrier **6** with a galactosyl appendage that was more susceptible to the enzymatic hydrolysis under biological conditions. Mild and facile hydrolysis is also desirable to conduct the transport experiment in one pot using POPC vesicles. Otherwise, a separate process of preincubation at an elevated temperature for a prolonged reaction time was required to prevent vesicle decomposition. As demonstrated in Figure 2b, procarrier **6** was activated only in the presence of AOG to generate the active carrier for the transport of chloride ions. The enzymatic hydrolysis of procarrier **6** was much faster than that of procarrier **5** and proceeded smoothly at room temperature; ~20% of **6** was hydrolyzed after 30 min and the reaction was completed after 12 h (25 °C, pH = 5.5, AOG, see Figure S10). It is worthwhile mentioning that hydrolysis at pH = 7.2 was considerably sluggish and consequently the chloride transport was much less effective, as demonstrated in Figure 2c. It has been known that the interstitial or extracellular pH of tumor tissue is more acidic than normal tissue primarily due to the secretion of lactic acid.[18] As mentioned earlier, recent studies[7] demonstrated that the anticancer activities of some synthetic carriers were associated with the facilitated anion transport. Therefore, the concept of the procarrier described in this study could be applicable to the development of a synthetic anion transporter that works selectively in cancer cells, thus eventually exhibiting the anticancer activity.

Finally, we tested if procarriers can be used to facilitate the transport of chloride ions across a plasma membrane using Fischer rat thyroid epithelial (FRT) cells that were stably transfected with a halide sensor YFP-F46L/H148Q/I152L, a mutant EYFP. Procarriers **3** and **6**, which were more susceptible to the enzymatic hydrolysis, were found to be suitable for this study. The chloride influx across the FRT cell membrane was monitored by the fluorescence quenching of the halide sensor YFP-F46L/H148Q/I152L.[19] First, the FRT cells were exposed to procarrier **3** (10 μM) at pH = 7.4 in the absence or presence of three different enzymes (250 μg/mL), ROE, CRL and PLE, each of which showed different degrees of transport activities in the POPC vesicle experiments. As shown in Figure 3a, the relative percentages of YFP quenching were compared. As a reference of the background quenching, the YFP fluorescence in the FRT cells was reduced by ~5% for 1 h under the exeprimental conditions without procarier **3** and any enzyme. In addition, the fluorescence quenching by each enzyme was also negligible. The fluorescence quenching was moderately increased up to ~10% in the presence of procarrier **3** with enzymes ROE and CRL. More drastic quenching (~24%) resulting from chloride influx was observed with PLE (Figure 3a and S11b), implying that an active chloride carrier was generated by the enzymatic hydrolysis. These results are consistent with the trend observed with POPC vesicles.

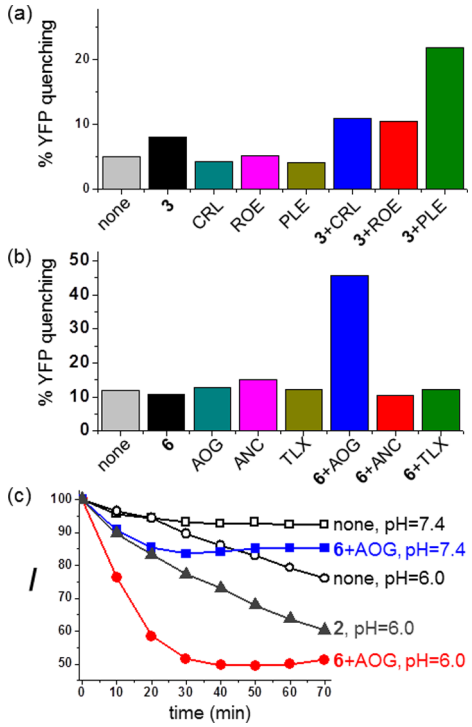

Figure 3. Bar graphs of relative percentages for YFP quenching facilitated by (a) **3** (10 μM, pH = 7.4) for 1 h and (b) **6** (10 μM, pH = 6.0) for 30 min in the absence and presence of each enzyme. (c) Normalized YFP decay ($I = (I_t/I_0) \times 100$) by **2** (20 μM, pH = 6.0) and **6** (10 μM) in the presence of AOG (250 μg/mL) at pH = 6.0 and 7.4 against time (min).

Similarly, chloride influx in the FRT cells was examined using procarrier **6** (10 μM) in the presence of AOG, ANC and TLX (250 μg/mL). At pH = 7.4, chloride influx was negligible but AOG gave rise to a mild influx of chloride ions into the FRT cells (see Figure S11c). At a more acidic pH (pH = 6.0), the chloride influx was much more activated only when in the presence of enzyme AOG. The results are summarized in Figure 3b,c. It is clearly noticeable that AOG is much more efficient under a slightly acidic environment (pH 6 vs pH 7.4). It should be also emphasized that a mixture of procarrier **6** and AOG has a much more pronounced quenching of fluorescence at pH = 6.0, compared to the fluorescence quenching observed with a real active form **2** of the chloride carrier (see Figure 3c). This result is possibly due to the enhanced deliverability of a hydrophobic carrier into the membrane. As mentioned earlier, a hydrophilic appendage in the procarrier greatly increases the solubility in water and can be removed just prior to embedding into a lipophilic membrane, thus possibly enhancing the deliverability of the active carrier.

In conclusion, we demonstrated that the controlled transport of chloride ions across lipid and cellular membranes could be effectively achieved with the concept of procarrier for the first time. Procarriers with hydrophilic appendages were activated to facilitate chloride transport in the presence of specific enzymes that can hydrolyze off the appendages to generate an active carrier. In particular, procarrier **6** with a galactosyl appendage was activated only in the presence of AOG under slightly acidic conditions. Moreover, the chloride influx into the FRT cells was much more efficient in a mixture of procarrier **6** and AOG compared to the case of direct use of the active carrier **2**. This result reflects that the procarrier approach could be used not only

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to achieve selective transport of chloride ions but also to improve the deliverability of a hydrophobic synthetic transport in a specific target.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b10592.

Synthetic procedures with characterization of **2**−**6**, and experimental details and data (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*ksjeong@yonsei.ac.kr

ORCID

Kyu-Sung Jeong: 0000-0003-1023-1796

Notes

The authors declare no competing financial interest.

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