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Membrane-active calixarenes: toward 'gating' transmembrane anion transport

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We dedicate this paper to Professor David N. Reinhoudt on the occasion of his 65th birthday

Abstract—This paper describes on-going efforts to develop calixarene amides as transmembrane anion transporters. We report on the transport of Cl^- anions across phospholipid membranes as mediated by some lipophilic calixarenes, all fixed in the *cone* conformation. We present significant findings regarding use of these calixarenes as transmembrane Cl^- transporters: (1) the *cone* conformer **cone-H 2a**, like its 1,3-*alt* and *paco* isomers, transports Cl^- across liposomal membranes; (2) the conformation of the calixarene (**paco-H 1** vs **cone-H 2a**) is important for modulating Cl^- transport rates; (3) the substitution pattern on the calixarene's upper rim is crucial for Cl^- transport function; and (4) at least one of the four arms of the calixarene can be left unmodified without loss of function, enabling development of a pH-sensitive anion transporter (**TAC-OH 3**). This last finding is useful given the interest in gating the activity of synthetic ion transporters with external stimuli.

1. Introduction

As illustrated in Figure 1, we describe the use of lipophilic calixarene amides to transport Cl^- anions across lipid membranes. These studies build on recent work where we showed that the *partial cone* calix[4]arene tetrabutylamide **paco-H 1** mediated transmembrane anion transport.[1] Before describing our new studies we first provide some relevant background about (1) synthetic Cl^- transporters and (2) calixarenes as membrane-active ion transporters.

Structures of transmembrane ion channel proteins have been determined recently, notably for the K^+ and Cl^- channels.[2,3] Understanding relationships between channel structure and function is crucial since maintaining the proper ion balance across cell membranes is essential to life. Knowledge of channel structure is also important because impaired ion transport can cause disease. Thus, cystic fibrosis is caused by a mutation in the CFTR protein that facilitates transmembrane Cl^- transport.[4] These two-fold goals of understanding natural ion transport processes and discovering new therapeutics have driven the development of synthetic transmembrane ion transporters.[5]

Historically, most activity has centered on cation transporters. There has been, however, continuing progress in identifying

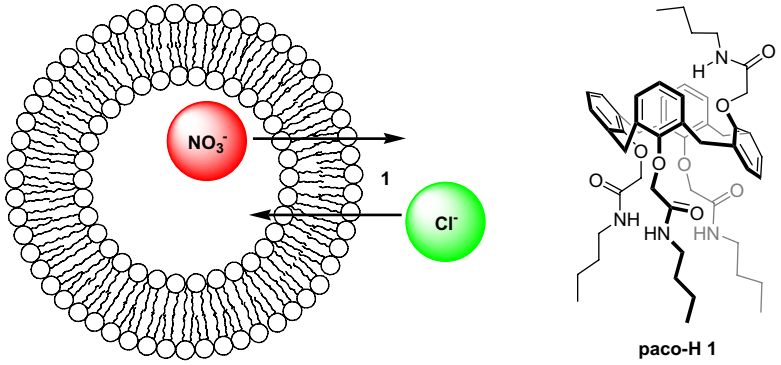

Figure 1. Transmembrane anion transport as mediated by the calixarene **paco-H 1** (see Ref. 1).

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'small molecules' that can transport Cl^- anions across lipid membranes. A recent review by A. P. Davis and Smith provides an excellent discussion of the emerging field of transmembrane Cl^- transport.[6] Synthetic Cl^- transporters include compounds that function as self-assembled channels or as monomolecular carriers. These compounds, which can alter transmembrane ion and pH gradients, may have potential applications as biochemical reagents and/or chemotherapeutic agents. Tomich and colleagues used small peptides derived from the glycine receptor protein to form Cl^- channels in phospholipid bilayers.[7] Gokel and colleagues have reported studies on lipophilic peptides that aggregate to form Cl^- selective ion channels.[8] Regen synthesized a sterol analog whose protonated polyamine side-chain functions as a transmembrane 'anion slide'.[9] Significantly, this analog restored Cl^- transport in cystic fibrosis cells.[10] The groups of A. P. Davis and Smith have also used sterols, coined 'cholapods', to transport Cl^- across liposomal and cell membranes.[11] Gin described an anion channel formed from an amino-cyclodextrin.[12] There is also interest in the prodigiosins, tripyrrole natural products that bind HCl.[13] Recently, the groups of Gale, Smith, Sessler, Thompson, and J. T. Davis have shown that prodigiosin analogs transport Cl^- across lipid membranes, setting the stage for further development of these compounds.[14–17] Recently, we and others have demonstrated that simple isophthalamides can function as efficient transmembrane transporters of Cl^-.[18,19]

Some of our group's contributions in the anion transport field have used the calix[4]arene scaffold.[1,20–22] A review by Cragg and Iqbal nicely describes previous studies that have used calixarene-type macrocycle as transmembrane cation transporters, as channels or as mobile carriers.[23] Kobuke and colleagues,[24] and Gokel and co-workers,[25] have described formation of synthetic cation channels using lipophilic calixarenes. Recently, Carreira et al. discovered a calixarene-amphotericin conjugate that forms K^+ selective channels.[26] Various calixarenes also function as mobile cation carriers. Thus, Beer and colleagues have described resorcin[4]arene-crown analogs that are efficient transmembrane carriers of K^+.[27] Jin has also shown that a calix[4]-arene-crown-5 analog selectively transports K^+ across planar bilayers.[28] Izzo and colleagues have recently described calix[4]arene-cholic acid conjugates that can move both H^+ and K^+ cations across synthetic liposomes.[29]

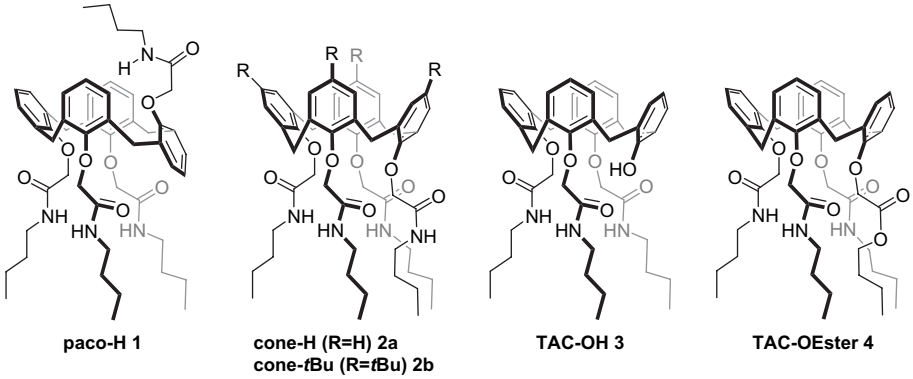
While calixarenes are known to bind anions in solution,[30] their use as membrane-active anion transporters is relatively new.[1,20–22] Our studies on calixarene-nucleoside conjugates,[31,32] designed as ion pair transporters, led us to discover that a tetrabutylamide calix[4]arene fixed in the 1,3-*alternate* conformation forms ion channels that move Cl^- across phospholipid membranes.[20] Presumably, the NH protons on the calixarene's secondary amides hydrogen bonds to Cl^- and help its translocation across the membrane.[33] X-ray structures confirmed that this 1,3-*alt* calixarene self-assembled into channel-like motifs held together by hydrogen bonds to bridging Cl^- anions.[20] Voltage clamp experiments in planar lipid bilayers confirmed that this 1,3-*alt* calixarene tetra-amide formed discrete ion channels. Subsequently, we investigated the structure and Cl^- ion transport activity of a related isomer, the *partial cone* calix[4]arene tetrabutylamide **paco-H 1** shown in Scheme 1.[1] We proposed that Cl^- transport mediated by **paco-H 1** was controlled by both ligand self-association and side-chain conformation.

In this paper, we report on comparative Cl^- transport activity for four calixarene amides all fixed in the *cone* conformation. We present significant findings regarding the use of these calixarenes as transmembrane Cl^- transporters: (1) the *cone* conformer **cone-H 2a** (R=H), like its 1,3-*alt* and *paco* isomers, transports Cl^- across liposomal membranes; (2) the conformation of the calixarene scaffold (**paco-H 1** vs **cone-H 2a**) is important for modulating Cl^- transport rates; (3) the substitution pattern on the calixarene's upper rim is crucial for Cl^- transport function; and (4) at least one of the four arms of the calixarene can be left unmodified without loss of function, enabling the development of a pH-sensitive anion transporter (**TAC-OH 3**). This last finding is timely given the interest in gating the activity of synthetic ion transporters by using external stimuli.[34] There is also an interesting parallel between this 'small molecule' and the ClC chloride transporter protein, as it has been proposed that anion transport by ClC is gated by charge–charge repulsions between Cl^- and negatively-charged glutamate residues within the anion channel.[35]

2. Results and discussion

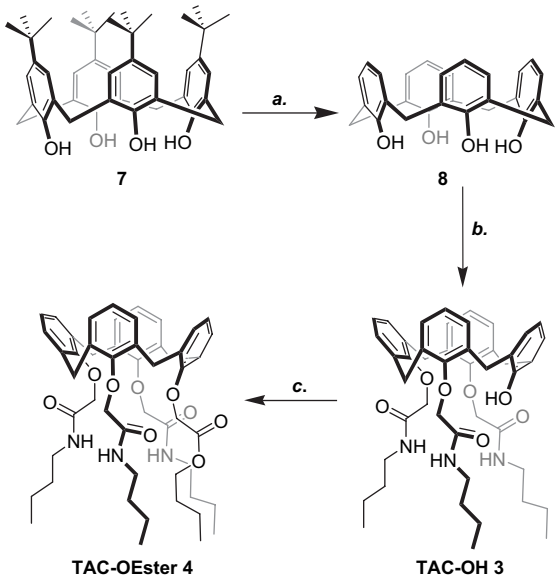
2.1. Rationale for studying calixarenes 1–4

The compounds that we studied are shown in Scheme 1. We previously reported that **paco-H 1** transports Cl^- across liposomal membranes.[1] Herein, we use **paco-H 1** as a standard



Scheme 1. Compounds studied for their transmembrane Cl^- transport activity.

for comparing the Cl^- transport activity of the *cone* calixarenes **2–4**. Compounds **2a** (**cone-H**) and **2b** (**cone-*t*Bu**) are fixed in the *cone* conformation and feature four *n*-butyl secondary amides on their lower rim. They differ in the substitution pattern on their upper rims. In previous work with **paco-H 1** we found that substitution of the calixarene's upper rim with *tert*-butyl groups led to loss of Cl^- transport activity.[1] In this study we set out to determine if this same phenomenon held true for the *cone* conformers **2a** (R=H) and **2b** (R=*t*Bu). The other two analogs, phenol **3** (**TAC-OH**) and ester **4** (**TAC-OEster**), were made so that we could determine the functional effect of either removing or replacing a single *n*-butyl amide on the macrocycle's lower rim. Replacement of a secondary amide with another functional group (as in **4**) without complete loss of activity might provide the opportunity for appending other anion-sensitive groups to the calixarene. This might allow development of new Cl^- sensors and transporters. We also reasoned that deprotonation of the phenolic proton on **TAC-OH 3** might enable formation of a pH-sensitive switch for gating Cl^- transport across lipid membranes.



Scheme 3. Synthesis of tris-*N*-butylamido calixarenes **3** and **4**. (a) $AlCl_3$, PhOH, $PhCH_3$, rt; (b) $BrCH_2CONHBu$, $Ba(OH)_2$, BaO, DMF, 40 °C; and (c) $BrCH_2CO_2Bu$, Cs_2CO_3, DMF, 70 °C.

2.2. Synthesis of calixarenes 2–4

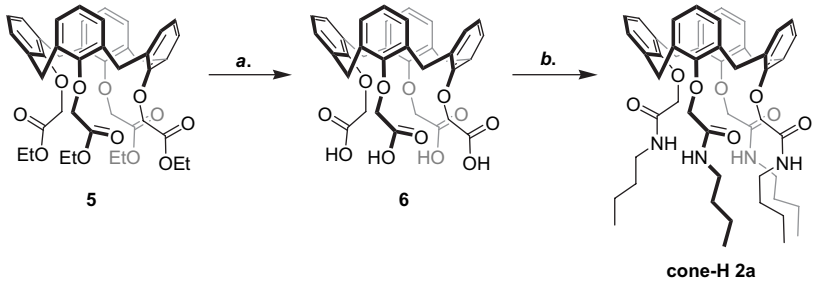
The synthesis of compounds **1–4** was straightforward. Calixarenes **1** and **2b** (R=*t*Bu) were prepared using published methods.[1,36] As shown in Scheme 2, **cone-H 2a** was synthesized from the corresponding tetra-ester **5**[37] via (i) hydrolysis to the tetra-acid **6**; (ii) acid chloride activation; and (iii) amide bond formation.

The tris-*N*-butylamido phenol analog **TAC-OH 3** was prepared from calix[4]arene **8**,[38] by alkylation with 2-bromo-*N*-butylacetamide (Scheme 3). The trisubstituted derivative **TAC-OH 3** was separated from other alkylation products by chromatography. Further alkylation of **TAC-OH 3** with *n*-butyl-2-bromoacetate gave ester **4**.

2.3. Characterization

Compounds **2a**, **2b**, **3**, and **4** were characterized by ^1H, ^{13}C NMR spectroscopy and ESI-MS analysis. The ^1H NMR spectra of **2a**, **2b**, **3**, and **4** indicated that each analog existed as a *cone* conformer in solution, as shown by characteristic AB coupling for bridging $ArCH_2Ar$ protons. Calixarenes that are tetra-substituted at their lower rim and fixed in the *cone* conformation usually display a pair of doublets between δ 3.2 and 4.5 ppm for diastereotopic $ArCH_2Ar$ protons.[39] This was true for **2a** and **2b**. The *cone* conformation for the trisubstituted **TAC-OH 3** was also consistent with the ^1H NMR data, showing two sets of doublets with identical integration between δ 3.2 and 4.5 ppm, corresponding to two AB systems. One AB system consists of doublets at δ 4.27 and 3.35 ppm with J=13.5 Hz. The second AB system for the other four $ArCH_2Ar$ protons in **TAC-OH 3** consists of doublets at δ 4.19 and 3.43 ppm with J=14.4 Hz. The tri-substituted **TAC-OH 3** displays two pairs of doublets because of the asymmetry at its lower rim.[40] The *cone* conformation for **TAC-OEster 4** was confirmed by the presence of a pair of doublets. The AB system for the $ArCH_2Ar$ protons in **4** consists of the doublets at δ 4.44 and 3.26 ppm with J=14.0 Hz.

In addition to spectroscopic analysis, **cone-H 2a** and **TAC-OEster 4** were characterized as *cone* conformers by X-ray crystallography.[41,42] Figure 2 shows the X-ray crystal structures for tetra-amide **cone-H 2a** and tri-amide **TAC-OEster 4**. In the solid-state, both compounds adopt a 'pinched-*cone*' conformation with C_{2v} symmetry.[43] Both compounds also feature two intramolecular hydrogen bonds between two amide NH groups and neighboring carbonyl oxygens. This conformation, if maintained in solution, would leave additional amide NH protons in **cone-H 2a** (two free NH groups) and **TAC-OEster 4** (one free NH group) available for hydrogen bonding to Cl^- anion.



Scheme 2. Synthesis of **cone-H 2a**. (a) KOH aq, MeOH, THF, rt; (b) 1. $SOCl_2$, benzene, reflux, 2. $BuNH_2$, Et_3N, CH_2Cl_2, rt.

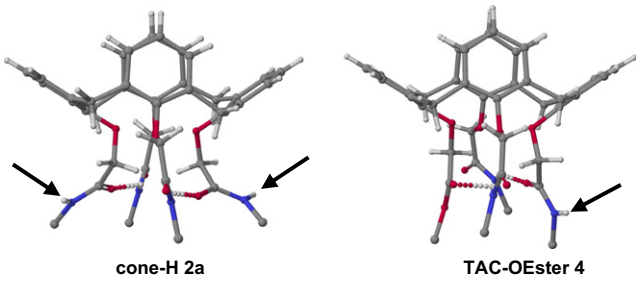


Figure 2. X-ray crystal structures of **cone-H 2a** (left) and **TAC-OEster 4** (right) showing the 'pinched-*cone*' conformations for both compounds. Intramolecular H-bonds between secondary amide NH protons and neighboring carbonyls are indicated by the dotted lines. The butyl side chains are removed for clarity. Arrows point to the 'free' amide NH groups.

2.4. NMR titration study shows Cl^- binding to TAC-OH 3

Compounds **2–4**, which contain either three or four secondary amide NH groups on their lower rim, all bind Cl^- weakly in non-polar organic solvents. Thus, titration of solutions of these calixarenes with tetrabutylammonium chloride (TBACl) in CD_2Cl_2 typically showed a downfield shift of the secondary amide NH protons and, assuming a 1:1 binding stochiometery, gave binding constants on the order of K_a=10–30 M^{-1}. For example, representative NMR titration data for interaction of Cl^- with **TAC-OH 3** are shown in Figure 3. Upon addition of TBACl to a solution of **TAC-OH 3** in CD_2Cl_2 we observed downfield shifts for the two 'outer' amide NH protons ($\Delta\delta$=0.40 ppm, labeled in blue), and for the 'central' amide NH proton ($\Delta\delta$=0.14 ppm, labeled in red). The association constant for binding TBACl by **TAC-OH 3** was determined to be K_a=24.1±5.1 M^{-1} in CD_2Cl_2,[44] which is similar to the value that we previously obtained for binding of Cl^- by the secondary amide NH groups in **paco-H 1** (K_a=28.7±17 M^{-1}).[1]

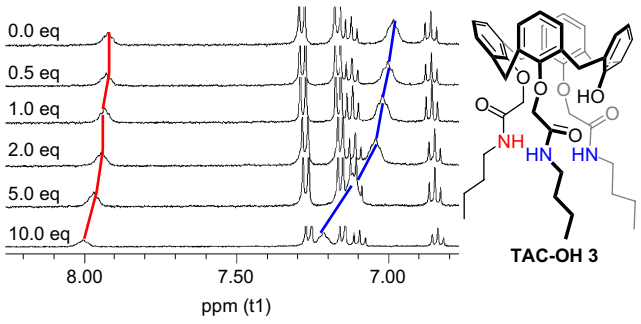


Figure 3. ^{1}H NMR stack plot showing titration of **TAC-OH 3** with TBACl. Blue lines (–) mark the changes in chemical shift for the two terminal NH protons, while the red lines (–) mark the changes in chemical shift for the central NH proton.

2.5. Transmembrane Cl^- transport results

Compounds **1–4** were tested for transmembrane Cl^- transport activity using assays previously reported by our group.[1,22] Liposomes loaded with the Cl^- sensitive dye, lucigenin, were subjected to a Cl^- gradient in the extravesicular solution. Lucigenin is a dye whose fluorescence is quenched upon binding Cl^-.[45] In a typical experiment (in 100 mM $NaNO_3$/10 mM sodium phosphate buffer (pH 6.4)) we incubated calixarenes **1–4** (2:100 ligand/lipid) with egg-yolk phosphatidylcholine (EYPC) liposomes containing lucigenin and 100 mM $NaNO_3$/10 mM sodium phosphate buffer (pH 6.4). We then introduced Cl^- to the extravesicular buffer by adding an aliquot of a NaCl solution in 10 mM sodium phosphate (pH 6.4). The resulting Cl^- concentration gradient across the membrane was relieved by ligand-mediated transport of Cl^- into the liposomes, resulting in the quenching of lucigenin's fluorescence. Intravesicular Cl^- anion concentration was calculated from the dye's fluorescence using the Stern–Volmer constant determined under the assay conditions.

2.5.1. Calixarene conformation attenuates Cl^- transport rate. Figure 4 shows that both **paco-H 1** and **cone-H 2a** transport Cl^- across the EYPC liposomes. It appears that the **paco-H 1** conformer is about twice as efficient as the *cone* isomer **2a** when administered at the same concentration (2:100 ligand to lipid). Despite its lower activity relative to **paco-H 1** the calixarene **cone-H 2a** is still an active transmembrane Cl^- transporter. This result encouraged us to continue our studies on the related *cone* analogs **cone-*t*Bu 2b**, **TAC-OH 3**, and **TAC-OEster 4**, as described below.

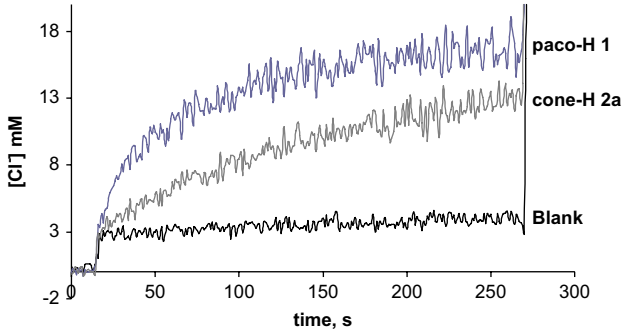


Figure 4. Chloride transport across EYPC liposomes containing lucigenin in a 100 mM $NaNO_3$/10 mM sodium phosphate buffer (pH 6.4). The Cl^- concentration was determined from lucigenin's fluorescence. Compounds **1** and **2a** were added to give a 2 mol % ligand to lipid ratio. At t=15 s, NaCl was added to give an external Cl^- concentration of 24 mM. Lucigenin fluorescence was converted to Cl^- concentration using the Stern–Volmer constant determined under the assay conditions. The traces shown are the average of three trials.

2.5.2. Upper-rim substitution pattern is crucial for Cl^- transport. To investigate the influence of upper-rim substitution on Cl^- transport activity, the substituted analog **cone-*t*Bu 2b** was tested in this lucigenin assay. As shown in Figure 5, **cone-*t*Bu 2b**, unlike its analog **cone-H 2a**, was essentially inactive toward transmembrane Cl^- transport under these standard conditions. This result is consistent with our previous studies wherein we found that substitution of a *paco* calixarene with *t*Bu groups resulted in complete loss of anion transport.[1] Again, the substitution pattern on the upper rim of the calixarene tetra-amide is clearly critical for Cl^- transmembrane transport.

2.5.3. Influence of lower-rim modification on transport: chloride transport by phenol 3 and ester 4. The transmembrane Cl^- transport activity by the trisubstituted amide calixarenes, phenol **3** and ester **4**, was also assessed using the standard lucigenin assay. As shown in Figure 6, the tris-*N*-butylamido **TAC-OH**, **3** transports Cl^- ions across EYPC liposomal membranes at pH 6.4 even more efficiently than

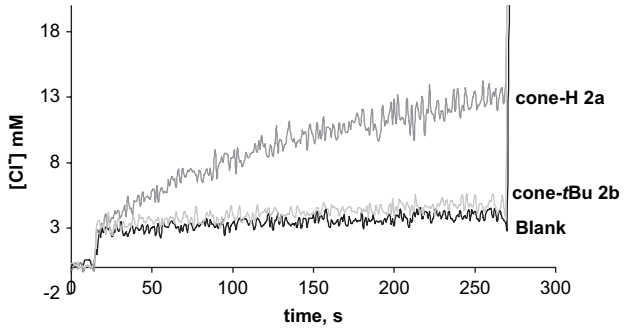


Figure 5. Chloride transport across EYPC liposomes containing lucigenin in a 100 mM $NaNO_3$/10 mM sodium phosphate buffer (pH 6.4). The Cl^- concentration was determined from lucigenin's fluorescence. Compounds **2a** and **2b** were added to give a 2 mol % ligand to lipid ratio. At t=15 s, NaCl was added to give an external Cl^- concentration of 24 mM. Lucigenin fluorescence was converted to Cl^- concentration using the Stern–Volmer constant determined under the assay conditions. The traces shown are the average of three trials.

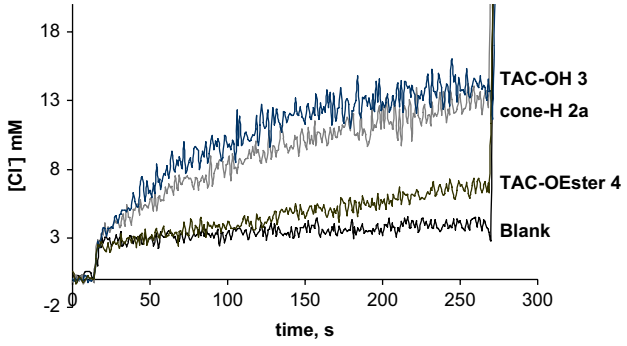


Figure 6. Chloride transport across EYPC liposomes containing lucigenin in a 100 mM $NaNO_3$/10 mM sodium phosphate buffer (pH 6.4). The Cl^- concentration was determined from lucigenin's fluorescence. Compounds **2a**, **3**, and **4** were added to give a 2 mol % ligand to lipid ratio. At t=15 s, NaCl was added to give an external Cl^- concentration of 24 mM. Lucigenin fluorescence was converted to Cl^- concentration using the Stern–Volmer constant determined under the assay conditions. The traces shown are the average of three trials.

does the tetra-amide **cone-H 2a**. This result, indicating that replacement of one of the secondary amide side chains with an acidic OH group could be functionally tolerated, prompted us to study **TAC-OH 3** as a pH-sensitive transporter (see Section 2.5.4). The data in Figure 6 also show that ester **4** is less efficient as a Cl^- transporter than tetra-amide **cone-H 2a**. However, while substitution of one amide side-chain with an ester resulted in reduction in Cl^- flux across the membrane, definite transport activity remained for **TAC-OEster 4** above background. Thus, we are hopeful that substitution on the calixarene *cone's* lower rim with an ester or ether sidechain containing an anion-sensitive reporter may eventually be useful for the development of Cl^- anion sensors. For now, we decided to focus on **TAC-OH 3** as a means for developing a compound whose transmembrane transport activity can be switched on and off by changing pH.

2.5.4. Modulating Cl^- transmembrane transport by pH. We reasoned that the phenolic OH on the unsubstituted arene of **TAC-OH 3** afforded the potential to develop a pH-tunable Cl^- transporter. For example, a trimethylated calix[4]arene with a free phenol has a pK_a=12.5.[46] Deprotonation of the OH group on **TAC-OH 3** should introduce unfavorable electrostatic interactions between the calixarene and Cl^- ions. Such electrostatic repulsions should inhibit anion binding and reduce the efficacy of **TAC-OH 3** as an anion transporter. Second, **TAC-OH 3** has to integrate into the membrane to enable transport. The deprotonated **TAC-OH 3** would undoubtedly have a lower permeability to the lipophilic membrane when it is negatively charged.

On the other hand, transmembrane Cl^- transport by **cone-H 2a** should not be as sensitive to pH since **2a** does not have sufficiently acidic protons to be significantly deprotonated at pH 7–10. The transport function of **TAC-OH 3** and **cone-H 2a** was monitored as a function of extravesicular pH using EYPC liposomes loaded with the Cl^- dye, lucigenin (Fig. 7).

As shown in Figure 7a, the rate of Cl^- transport mediated by **TAC-OH 3** clearly decreased as the extravesicular solution became more basic. In marked contrast, the Cl^- transport rate by **cone-H 2a** was constant over the same pH range

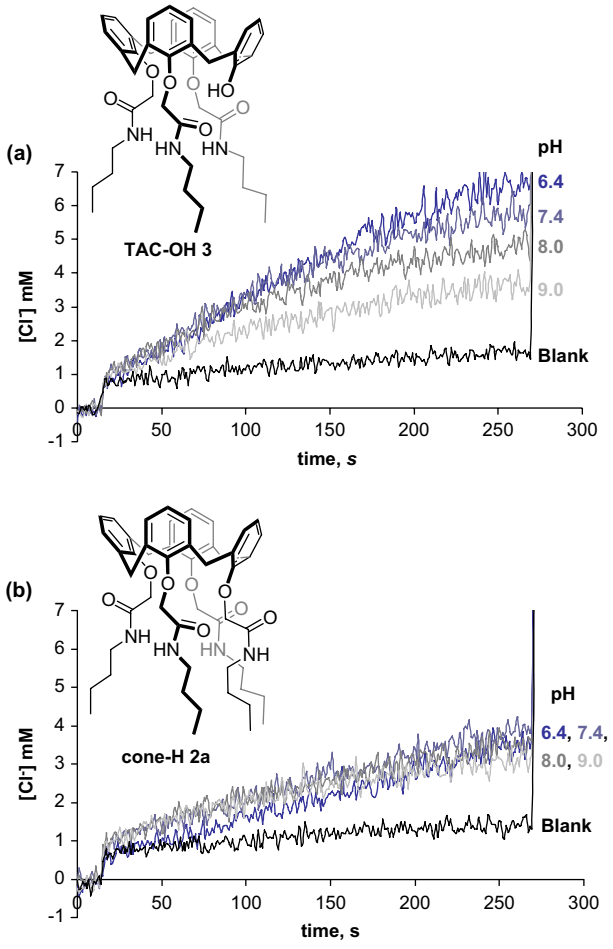


Figure 7. Transmembrane Cl^- transport as a function of pH: (**a**) with **TAC-OH 3** and (**b**) with **cone-H 2a**. Experiments were done using EYPC liposomes with lucigenin (1 mM) in a 100 mM $NaNO_3$/10 mM sodium phosphate buffer at various pHs (6.4, 7.4, 8.0, and 9.0). Compounds **2a** and **3** were added to give a 2 mol % ligand to lipid ratio. At t=15 s, NaCl solution was added to give an external Cl^- concentration of 24 mM. Lucigenin fluorescence was converted to Cl^- concentration using the Stern–Volmer constant determined under the assay conditions. The traces shown are the average of three trials.

between 6.4 and 9 (Fig. 7b). The data are consistent with a negative charge in **TAC-OH 3** acting to inhibit transmembrane transport of the Cl^- anion, by limiting anion binding and/or partitioning of the calixarene into the liposomal membrane. Control experiments with **cone-H 2a** support this conclusion.

3. Summary

In a recent review on the use of calixarenes to effect transmembrane ion transport, Cragg and Iqbal suggested that charge–charge interactions might be used as a strategy for gating transport.[23] The studies reported in this paper, and in another recent paper from our labs,[17] suggest that electrostatic repulsion can be used to turn on and off transmembrane transport of Cl^- anion. This regulatory mechanism, observed with these 'small molecule' transporters, is analogous to the proposal that the self-assembled ClC protein channel makes use of negatively-charged Glu residues to gate Cl^- transport.[35] It may also be possible to modulate anion transport rates by incorporating a positive charge, such as a quaternary ammonium group, into the calixarene framework. Finally, it is possible that a lipophilic compound such as the deprotonated **TAC-OH 3** may be a candidate for transmembrane cation transport. Studies are under way to determine these issues.

4. Experimental section

4.1. General

^{1}H NMR spectra were recorded on a Bruker DRX-400 or a Bruker Avance 400 instrument operating at 400.13 MHz. ^{13}C NMR spectra were recorded on a Bruker DRX-400 instrument operating at 100.52 MHz. Electrospray ionization mass spectra were recorded on a JEOL AccuTOF-CS instrument. Deuterated solvents were purchased from Cambridge Isotope. Chemicals and solvents were purchased from Sigma, Aldrich, Fisher, Fluka or Acros. Lucigenin was purchased from Molecular Probes and EYPC was purchased from Avanti Polar Lipids.

4.1.1. Synthetic procedures. Calixarenes **1**[1] and **2b**[36] were prepared as reported from the corresponding *paco* or *cone* tetra-esters by: (i) hydrolysis to the tetra-acid; (ii) activation to the acid chloride; and (iii) amide bond formation.

4.1.1.1. 25,26,27,28-Tetrakis(2-butylamidomethoxy)-calix[4]arene (cone-H 2a). *Cone* 25,26,27,28-tetrakis-(ethylacetylmethoxy)calix[4]arene **5** (275 mg, 0.36 mmol)[37] and 1 mL of 45% aqueous KOH were stirred in 6 mL of methanol/THF (1:1) at rt for 14 h. The solvent was evaporated under reduced pressure. The resulting solid was dissolved in a minimum amount of water, acidified with 6 N HCl, and the water removed under reduced pressure. The material was dissolved in acetone, solid KCl was removed by filtration and acetone was then evaporated to give the tetra-acid as a white solid. The acid chloride was prepared by stirring a solution of the calixarene tetra-acid **6** (565 mg, 0.85 mmol) in 20 mL of benzene with 5.1 mL of $SOCl_2$. The reaction mixture was heated at reflux for 2.5 h and then concentrated under reduced pressure. The sticky brown solid was dried in vacuo for 1 h and then dissolved in 20 mL of CH_2Cl_2. *N*-Butylamine (1.32 mL, 16 equiv) was added dropwise to the stirring solution at rt. Triethylamine (1.2 mL, 10 equiv) was then added dropwise and the reaction stirred under N_2 for 12 h at rt. The solvent was evaporated and the resulting solid partitioned between $CHCl_3$ and H_2O. The organic layer was dried with sodium sulfate and evaporated. Pure **2a** (332 mg, 44.5% yield from the tetra-ester) was obtained after silica gel chromatography with 3% MeOH–CH_2Cl_2. ^{1}H NMR ($CDCl_3$, 25 °C) δ: 7.34 (br s, 4H, CON*H*CH_2), 6.62–6.59 (m, 12H, Ar*H*), 4.50 (d, J=13.9 Hz, 4H, ArC*H*$_2$Ar), 4.43 (s, 8H, ArOC*H*$_2$CO), 3.34 (q, J=6.6 Hz, 8H, CONHC*H*$_2$), 3.24 (d, J=14.0 Hz, 4H, ArC*H*$_2$Ar), 1.59–1.52 (m, 8H, NHCH$_2$C*H*$_2$), 1.37–1.29 (m, 8H, NH(CH$_2$)$_2$C*H*$_2$), 0.91 (t, J=7.3 Hz, 12H, NH(CH$_2$)$_3$C*H*$_3$). ^{13}C NMR ($CDCl_3$, 25 °C) δ: 170.0, 156.3, 134.7, 129.3, 123.7, 74.5, 39.6, 32.0, 31.4, 20.6, 14.2. Mass calculated for $C_{52}H_{68}N_4O_8$: 876.504. Mass found (+)-ESI-MS: 877.513 (M+H$^+$); 899.477 (M+Na$^+$).

4.1.1.2. 25,26,27,28-Tetrakis(hydroxy)calix[4]arene (8).[38] A slurry of *p-tert*-butylcalix[4]arene **7** (1.03 g, 1.59 mmol) and phenol (0.842 g, 8.94 mmol) in toluene (30 mL) was stirred under N_2 for 0.5 h. Then, $AlCl_3$ (1.29 g, 9.69 mmol) was added and the mixture stirred for 2 days at rt. The reaction was quenched with 0.2 N HCl (35 mL), the solvent was removed in vacuo and MeOH (7 mL) added at which point a precipitate formed. The precipitate was filtered to yield crude **8**, which was purified by recrystallization (2% MeOH–$CHCl_3$) to give **8** (0.337 g, 50%) as transparent crystals. Mp 297–300 °C. ^{1}H NMR ($CDCl_3$, 25 °C) δ: 10.20 (s, 4H, ArO*H*), 7.06 (d, J=7.6 Hz, 8H, Ar*H*), 6.73 (t, J=7.6 Hz, 4H, Ar*H*), 4.27 (s, 4H, ArC*H*$_2$Ar), 3.55 (s, 4H, ArC*H*$_2$Ar). ^{13}C NMR ($CDCl_3$, 25 °C) δ: 149.2, 129.3, 128.8, 122.7, 32.2. Mass calculated $C_{28}H_{24}O_4$: 424.167. Mass found ESI-MS: 425.229 (M+H$^+$); 463.051 (M+K$^+$).

4.1.1.3. 25-Hydroxy-26,27,28-tris(2-butylamidomethoxy)calix[4]arene (TAC-OH 3). A solution of **8** (0.804 g, 1.89 mmol), $Ba(OH)_2$ (1.79 g, 5.67 mmol), and BaO (1.69 g, 11.0 mmol) in DMF (15 mL) was stirred at 40 °C for 1 h under N_2. A solution of 2-bromo-*N*-butylacetamide (1.10 g, 5.67 mmol) in DMF (5 mL) was added dropwise and the mixture stirred for 4 h. The mixture was diluted with CH_2Cl_2 (60 mL), washed with 0.2 N HCl (3×30 mL) and H_2O (3×30 mL), and dried over $MgSO_4$. The solvent was removed in vacuo to give a yellow residue. **TAC-OH 3** (0.376 g, 26%) was separated by silica chromatography (1:1 EtOAc–hexanes) to give translucent flakes. Mp 121–123 °C. ^{1}H NMR ($CDCl_3$, 25 °C) δ: 7.98 (t, J=5.4 Hz, 1H, CON*H*CH_2), 7.27 (d, J=7.8 Hz, 2H, Ar*H*), 7.16 (d, J=7.2 Hz, 2H, Ar*H*), 7.13 (t, J=7.8 Hz, 1H, Ar*H*), 7.01 (t, J=5.8 Hz, 2H, CON*H*CH_2), 6.88 (t, J=7.5 Hz, 1H, Ar*H*), 6.47 (d, J=7.4 Hz, 2H, Ar*H*), 6.44 (td, J=7.5, 1.8 Hz, 2H Ar*H*), 6.35 (dd, J=7.3, 1.8 Hz, 2H Ar*H*), 4.39 (s, 2H, ArOC*H*$_2$CO), 4.36 (d, J=14.9 Hz, 2H, ArOC*H*$_2$CO), 4.27 (d, J=13.6 Hz, 2H, ArC*H*$_2$Ar), 4.19 (d, J=14.5 Hz, 2H, ArC*H*$_2$Ar), 4.15 (d, J=14.8 Hz, 2H, ArOC*H*$_2$CO), 3.75 (s, 1H, ArO*H*), 3.43 (d, J=14.4 Hz, 2H, ArC*H*$_2$Ar), 3.35 (d, J=13.5 Hz, 2H, ArC*H*$_2$Ar), 3.28–3.49 (m, 6H, NHC*H*$_2$CH$_2$), 1.49–1.64 (m, 6H, NHCH$_2$C*H*$_2$), 1.33–1.43 (m, 4H, NH(CH$_2$)$_2$C*H*$_2$), 1.24–1.33 (m, 2H, NH(CH$_2$)$_2$C*H*$_2$), 0.93

(t, J=7.3 Hz, 6H, NH(CH$_2$)$_3$CH_3), 0.75 (t, J=7.3 Hz, 3H, NH(CH$_2$)$_3$CH_3). ^{13}C NMR ($CDCl_3$, 25 °C) δ: 169.1, 168.4, 155.1, 153.6, 152.5, 136.5, 132.9, 132.8, 131.4, 130.8, 129.4, 129.3, 128.7, 125.4, 125.0, 121.2, 75.1, 74.7, 40.2, 39.6, 32.2, 31.6, 31.2, 30.6, 20.8, 20.6, 14.2, 14.0. Mass calculated for $C_{46}H_{57}N_3O_7$: 763.420. Mass found (+)-ESI-MS: 764.477 (M+H$^+$); 786.462 (M+Na$^+$).

4.1.1.4. 25-(2-Butoxycarbonylmethoxy)-26,27,28-tris(2-butylamidomethoxy)calix[4]arene (TAC-OEster 4). A solution of **TAC-OH 3** (0.100 g, 0.13 mmol) and Cs_2CO_3 (0.43 g, 13.1 mmol) in DMF (6 mL) was stirred at 70 °C for 10 min under N_2 gas. A solution of butyl 2-bromoacetate (0.255 g, 1.31 mmol) in DMF (4 mL) was added dropwise and the mixture stirred. After 24 h, the reaction mixture was cooled to rt, and then quenched with 1.0 M HCl (10 mL). The aqueous mixture was extracted with CH_2Cl_2 (3×10 mL) and the resulting organic layer was washed with distilled H_2O (4×15 mL), dried over $MgSO_4$, and the solvent was removed in vacuo. The residue was subsequently purified by silica gel chromatography (1:1 EtOAc–Hexanes) to give compound **4** as a white solid. Yield 45%; ^{1}H NMR ($CDCl_3$, 25 °C) δ: 7.91 (t, 2H, CONHCH$_2$), 6.88–6.77 (m, 7H, ArH, CONHCH$_2$), 6.74 (t, J=7.5 Hz, 2H, ArH), 6.32 (d, J=7.5 Hz, 4H, ArH), 4.77 (s, 2H, ArOCH_2CO$_2$), 4.59 (d, J=14.0 Hz, 2H, ArOCH_2CONH), 4.44 (d, J=14.0 Hz, 4H, ArCH_2Ar), 4.44 (s, 2H ArOCH_2-CONH), 4.31 (d, J=14.6 Hz, 2H, ArOCH_2CONH), 4.13 (t, J=6.8 Hz, 2H, CO$_2$CH_2), 3.45–3.34 (m, 6H, NHCH_2), 3.26 (dd, J=14.0, 4.5 Hz, 4H, ArCH_2Ar), 1.65–1.57 (m, 6H, NHCH$_2$CH_2), 1.51–1.45 (m, 2H, CO$_2$CH$_2$CH_2), 1.42–1.32 (m, 6H, NH(CH$_2$)$_2$CH_2), 1.31–1.24 (m, 2H, CO$_2$(CH$_2$)$_2$CH_2), 0.93 (t, J=7.4 Hz, 6H, NH(CH$_2$)$_3$CH_3), 0.92 (t, J=7.3 Hz, 3H, NH(CH$_2$)$_3$CH_3), 0.90 (t, J=7.7 Hz, 3H, CO$_2$(CH$_2$)$_3$CH_3). ^{13}C NMR ($CDCl_3$, 25 °C) δ: 171.6, 169.6, 169.5, 155.3, 135.5, 135.3, 133.8, 133.5, 129.9, 129.0, 128.7, 123.9, 123.6, 123.5, 78.1, 74.6, 71.9, 65.4, 39.7, 39.5, 39.4, 32.3, 32.1, 31.4, 31.3, 31.0, 30.1, 20.7, 20.5, 19.5, 14.3, 14.2, 14.1. Mass calculated for $C_{52}H_{67}N_3O_9$: 877.488. Mass found (+)-ESI-MS: 878.507 (M+H$^+$); 900.480 (M+Na$^+$); 1014.549 (M+Ba^{2+}).

4.1.2. Preparation of liposomes. Large unilamellar vesicles were prepared using egg-yolk phosphatidylcholine (EYPC) lipid. EYPC (60 mg) was dissolved in 5 mL of chloroform/methanol. The solution was evaporated under reduced pressure to give a thin film that was dried in vacuo overnight. The lipid film was then hydrated with appropriate phosphate buffer solution containing 1 mM lucigenin dye (10 mM sodium phosphate, pH 6.4; 100 mM NaCl) to give a 60 mg/mL solution of lipid. After 10 freeze/thaw cycles, the liposomes were extruded through a 100 nm polycarbonate membrane 21 times at rt using a high-pressure mini-extruder (Avanti). The resulting liposome solution was passed through a Sephadex (G-25) column to remove excess dye (eluant, sodium phosphate buffer, pH 6.4, 100 mM $NaNO_3$). The isolated liposomes were diluted to give a concentration of 25 mM in EYPC, assuming 100% retention of lipid during gel filtration.

4.1.2.1. Chloride transport assay in liposomes. In a typical experiment, 50 μL of the stock EYPC liposomes was diluted into 2 mL of 10 mM sodium phosphate (pH 6.4, 100 mM $NaNO_3$) to give a solution that is 0.5 mM in lipid. Compounds **1–4** were added to give a 2:100 ligand to lipid ratio. To the cuvette containing the EYPC-transporter mixture was added 20 μL of 2.425 M NaCl solution through an injection port, after 15 s, to give an external Cl^- concentration of 24 mM. Intravesicular Cl^- concentration was monitored as a function of lucigenin fluorescence. The fluorescence of lucigenin was monitored at 372 nm and emission at 503 nm for 300 s. The cuvettes were kept at 25 °C during the experiment with a constant water temperature bath. After 270 s, 40 μL of 10% Triton-X detergent was added to lyse the liposomes. Experiments were done in triplicate and all traces reported are the average of the three trials. Lucigenin fluorescence was converted to chloride concentration using the Stern–Volmer constant determined under the assay conditions.[1,22] The Stern–Volmer constant was determined by taking the slope of a plot of f_0/f versus chloride concentration.

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