Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry

Anion Mediated Activation of Guanidine Rich Small Molecules

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Supporting Information

I. General.

Egg yolk phosphatidylcholine (EYPC) was obtained from Avanti Polar Lipids Inc. 5(6)-carboxyfluorescein (CF) and 1-pyrenebutyric acid (PB) was purchased from Fluka; poly-L-arginine hydrochloride (pR), 1-pyreneacetic acid (PA), and sodium dodecyl sulfate (SDS) were from Sigma-Aldrich. Sodium laurate (SL) and sodium monododecyl phosphate (SDP) were obtained from Tokyo Chemical Industry (TCI), America. All salts and buffers of the best grade were available from Sigma-Aldrich, and used as received. **CPPM1** and **CPPM2** were synthesized and characterized as reported previously.[S1] Stock solution of CPPM was prepared in dimethyl sulfoxide (DMSO, Sigma). Fluorescence measurements were performed on a Jobin Yvon Fluorolog-3 equipped with a stirrer and temperature controller (all measurements were performed at 25 ^{o}C).

II. Preparation of Vesicles

Preparation of EYPC-LUVs⊃CF.[S2] A thin lipid film was prepared by evaporating a solution of 25 mg EYPC in 2 mL $CHCl_3$ on a rotary evaporator (40 °C) and then in vacuum overnight. After hydration (~ 1h) with 1.0 ml buffer A (10 mM Tris, 10 mM NaCl, 50 mM CF, pH 7.5) accompanied by occasional vortex, the resulting suspension was subjected to 5 freeze-thaw cycles (liquid N_2 to freeze and 40 °C water bath to thaw), and 11 times extruded through a polycarbonate membrane (pore size 100 nm). Extra-vesicular components were removed by size exclusion chromatography (Sephadex G-50, Sigma-Aldrich) with buffer B (10 mM Tris, 107 mM NaCl, pH 7.5). The resulting vesicle solution was diluted with buffer B to give CF loaded LUVs stock solution having final lipid concentration of ~ 5.0 mM.

III. Activation Study

Activation Study with Pyrenebutyrate.[S2a] 20 µl of pyrenebutyrate (PB) stock solution (pyrenebutyric acid in H_2O with 1 equivalent of NaOH) and 20 µl EYPC-LUVs⊃CF were added to 1.96 ml gently stirred, thermostated buffer B (10 mM Tris, 107 mM NaCl, pH 7.5) in a disposable plastic cuvette. The time dependent change in fluorescence intensity I_t (λ_{ex} = 492 nm, λ_{em} = 517 nm) was monitored during the addition of 20 µl CPPM or pR stock solution (final concentrations 2.5 µM **CPPM** and 0.25 µM **pR**, respectively) at t = 100 s, and addition of 40 µl 1.2 % (aq.) triton X-100 at the end of every experiment. Fluorescence time courses were normalized and converted into transmembrane activity I_F (equation S1, Figure S1a) and further converted into fractional activity Y using equation S2 (Figure S1b).

$$I_f = (I_t - I_0) / (I_\infty - I_0) \qquad \text{(S1)},$$

where $I_0 = I_t$ before **CPPM** addition and $I_\infty = I_t$ after lysis. I_F at t = 600 s just before lysis was defined as transmembrane activity Y.

$$Y = (Y_t - Y_o) / (Y_\infty - Y_o) \qquad \text{(S2)},$$

where Y_o is Y without activator, and Y_∞ is Y with excess activator. For Hill analysis, Y was plotted against varied pyrenebutyrate (PB) concentration (c_{PB}) at constant transporter and lipid concentration, and fitted to the Hill equation S3 to give effective concentration EC_{50}, Y_{max} and the Hill coefficient n (see Figure S1).

$$Y = Y_o + (Y_{max} - Y_o) / \{1 + c_{PB} / EC_{50})^n\} \qquad \text{(S3)},$$

where Y_o is Y without activator, and Y_{max} is Y with excess activator.

Activation studies with the other activators, pyrene acetate (PA), sodium laurate (SL), sodium dodecyl phosphate (SDP), and sodium dodecyl sulfate (SDS) perfomed similarly as activation study with pyrenebutyrate (PB) and effective activator concentration EC_{50}, Y_{max} and the Hill coefficient n were determined (see Table 1 and Table S1).

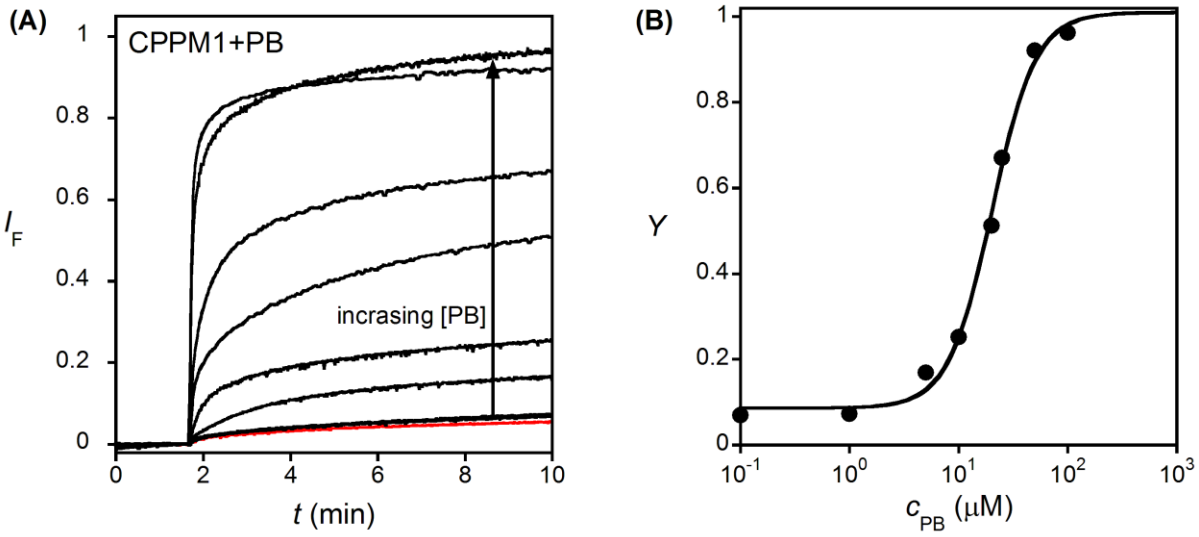

Figure S1. (A) Changes in CF emission (I_F) (λ_{ex} 492 nm, λ_{em} 517 nm) as a function of time (t) during addition of activator [0 (red curve), 0.1, 1, 5, 10, 20, 25, 50, 100 μM PB] at t = 0 s, **CPPM1** (2.5 μM, final concentration) at t = 100 s to EYPC-LUVs⊃CF (50 μM EYPC), calibrated by final analysis (I_F = 1.0, with 40 μL 1.2% aqueous triton X-100). (B) Dose response curves for PB, with **CPPM1** (2.5 μM) against EYPC-LUVs/CF vesicles (50 μM EYPC), with curve fit to Hill equation.

IV. Transporter Efficiency (*E*) Calculation

The effective transporter concentration EC_{50} was denoted as activator concentration needed to reach $Y_{max}/2$ (see Table 1 in the manuscript). Transporter efficiency *E* was calculated [S3] as defined in eqn. S4 (see Table 2 in the manuscript and Table S1).

$$E = Y_{max} \times pEC_{50} / f \qquad \text{(S4)}$$

where Y_{max} is the maximal activity in percent, pEC_{50} the negative logarithm of the effective concentration (EC_{50}) in mM, and $f = 20.6$ as used for classical CPP pR-activator complex.[S3]

Table S1. EC_{50}, Y_{max}, and activator efficiency *E* for the guanidine rich transporters.

CPPM1				
Activators	EC_{50} (μM)	Y_{max} (%)	*n*	*E*
PB	20 ± 1.3	100 ± 4.0	2.2 ± 0.3	8.2
PA	70 ± 2.4	85 ± 1.0	2.2 ± 0.2	4.8
SL	15 ± 2.0	17 ± 2.0	1.3 ± 0.4	1.5
SDP	25 ± 3.1	15± 0.8	2.1 ± 0.5	1.2
SDS	10 ± 1.0	10 ± 0.3	2.3 ± 0.7	1.0
CPPM 2				
Activators	EC_{50} (μM)	Y_{max} (%)	*n*	*E*
PB	24 ± 0.8	100 ± 2.0	3.6 ± 0.5	7.9
PA	88 ± 3.6	96 ± 3.0	3.5 ± 0.4	4.9
SL	76 ± 4.3	17 ± 0.6	5.3 ± 1.2	0.9
SDP	16 ± 0.4	30 ± 0.4	2.4 ± 0.1	2.6
SDS	103 ± 0.02	14 ± 2.0	4.0 ± 0.3	0.7
poly-arginine (**pR**)[a]				
Activators	EC_{50} (μM)	Y_{max} (%)	*n*	*E*
PB	44 ± 2	78 ± 2	2.2 ± 0.2	5.1
PA	86 ± 3	80 ± 3	4.3 ± 0.5	4.1
SL	34 ± 1	10 ± 1	3.8 ± 0.4	0.7
SDP	19 ± 1	61 ± 3	4.2 ± 1.0	5.1
SDS	16 ± 1	27 ± 1	-	2.4

[a]pR activation data incorporated from [S3].

V. Statistical Analysis

The results were analyzed using GraphPad Prism version 5.01. Data are reported as mean ± standard deviation. Three independent experiments were performed and the resulting mean of EC_{50} and Y_{max} values were compared by two-way analysis of variance (ANOVA) followed by Bonferroni's post-test and $P < 0.05$ was considered statistically significant (Figure S2). Although this statistical analyses were performed based on *only* three data points for each EC_{50} or Y_{max} value, all the inferences drawn in the manuscript turned out statistically significant with $P < 0.001$. Statistical analyses were not performed for the E values as equitation S3 includes both EC_{50} and Y_{max}, which makes the overall error (standard deviation) evaluation complicated using two different error levels in a single parameter. Given the statistical significance of both EC_{50} and Y_{max}, the differences in E values are significant.

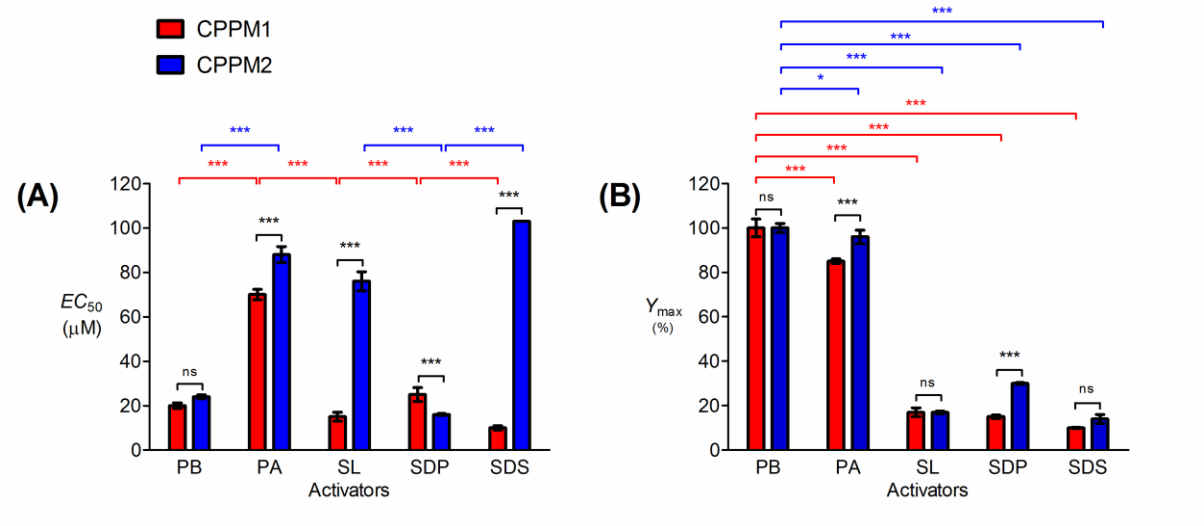

Figure S2. Statistical analyses with (A) EC_{50} and (B) Y_{max} were performed for different activators and CPPMs. Data represents three independent experiments; error bars represent mean ± s.d. (standard deviation). Statistical analyses were performed by two-way analysis of variance (ANOVA) with Bonferroni's post-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ns, non significant. In (B) the P values were shown comparing aromatic activator PB with other aliphatic activators; similar P values were obtained while PA was compared with other aliphatic activators. Statistical analysis of PA with other activators is not shown in the figure (B) to avoid clumsiness.

VI. References

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[S2] (a) A. Hennig, G. J. Gabriel, G. N. Tew, S. Matile, *J. Am. Chem. Soc.* 2008, **130**, 10338-10344; (b) A. Som, A. O. Tezgel, G. J. Gabriel, G. N. Tew, *Angew. Chem., Int. Ed. Engl.* 2011, **50**, 6147-6150.
[S3] M. Nishihara, F. Perret, T. Takeuchi, S. Futaki, A. N. Lazar, A. W. Coleman, N. Sakai, S. Matile, *Org. Biomol. Chem.* 2005, **3**, 1659-1669.