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Supporting Information

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Coumarinylmethyl Esters for Ultrafast Release of High Concentrations of Cyclic Nucleotides upon One- and Two-Photon Photolysis

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Experimental Section

Materials: cAMP, cGMP, 7-Amino-4-methylcoumarin, bromoacetic acid *tert*-butyl ester, SeO_2, $NaBH_4$, CH_3SO_2Cl, phenylacetic acid, 4-dimethylaminopyridine (DMAP), N,N'-dicyclohexylcarbodiimide (DCC), and LiBr were purchased from Sigma-Aldrich (Germany). The free acid of 8-Br-cAMP and the sodium salt of 8-Br-cGMP were purchased from Biolog (Germany). p-Toluensulfonylhydrazide and trifluoroacetic acid (TFA) were obtained from Lancaster (Germany). Pluoronic F-127 and the acetoxymethyl ester of FLUO-4 (FLUO4/AM) were obtained from MoBiTec (Germany), and the phospholipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids (Alabaster, U.S.A.). The sodium salt of 8-Br-cGMP was converted to the acid form as described previously.[1] (7-Diethylamino-coumarin-4-yl)methyl) (DEACM)-caged cAMP was prepared as described.[2] N-(6-Bromo-7-hydroxycoumarin-4-yl)methoxycarbonyl-L-glutamic acid (Bhc-glu) was synthesized according to the published procedure[3] The remaining chemicals were of the highest grade commercially available and used without further purification. TLC plates (silica gel 60 F_{254}) were purchased from E. Merck (Germany) and silica gel for flash chromatography (60 Å, 30–60 µm) from J. T. Baker (The Netherlands). CH_3CN from J. T. Baker (The Netherlands) was HPLC grade. Water was purified with a Milli-Q-Plus system (Millipore, Germany).

Instrumentation: ^1H and ^{31}P NMR spectra were recorded using a Bruker DRX 600 spectrometer at 600 MHz. ^1H chemical shifts are given in parts per million (ppm) relative to tetramethylsilane as an internal standard. ^{31}P NMR chemical shifts are reported in ppm referenced to external 85% H_3PO_4.

Mass spectra were measured by electrospray ionization mass spectrometry in the positive ionization mode using a TSQ 700 (Finnigan MAT) spectrometer. UV/vis spectra were recorded on a UV/vis spectrophotometer Lambda 9 (Perkin-Elmer). Fluorescence spectra were taken on an MPF-2A fluorescence spectrometer (Hitachi/Perkin-Elmer, Japan) combined with a personal computer and data analysis software package (SPECTRACALC).

Analytical reversed-phase HPLC (RP-HPLC) was carried out on a Shimadzu LC-6A system (flow rate: 1 mL min^{-1}) equipped with a UV detector (operating at 254 and 385 nm) and a fluorescence detector (l_{exc} = 385 nm, l_{em} = 495 nm) using a PLRP-S column, 300 Å, 8 µm, 250 × 4.6 mm from Polymer Laboratories (U.K.). Preparative RP-HPLC was run on a Shimadzu LC-8A system (flow rate: 10 mL min^{-1}) with a UV/vis detector (SPD-6AV, l_{exc} = 254 nm) over a Nucleogel RP 100-10 (300 × 25 mm) column from Macherey-Nagel (Germany).

One-photon photolysis of all employed caged compounds in solution was realized by using a high-pressure mercury lamp (HBO 500, Oriel, U.S.A.) with controlled light intensity and metal interference transmission filters (365 nm, Oriel, U.S.A.). For the all experiments, UV and fluorescence quartz cuvettes with a path length of 1 cm and a cross-sectional area of 1 cm^2 were used. During irradiation, the solutions in the cuvettes were moved by a magnetic stirrer.

All synthetic and analytical procedures were performed in darkness or under yellow light provided by sodium vapor lamps. The melting points are uncorrected.

Synthesis

General procedures for the preparation of the BCMACM-caged cyclic nucleotides 3-6. Method A (compounds 3, 4, and 6): A mixture of the free acid of cAMP, cGMP, or 8-Br-cGMP (0.5 mmol) and of **1 (**246 mg of the crude product, containing 0.5 mmol pure **1**) was stirred in 12 mL CH_3CN and 4 mL DMSO at 60 ^oC in the dark for 8 h. An additional quantity of **1** (123 mg of the crude product, containing 0.25 mmol pure **1**) was added, and the mixture was stirred at 60 °C for more 16 h. CH_3CN was evaporated under reduced pressure, and DMSO was removed by repeated extraction with ether/pentane (1:1) and ether. The residue, which contained t he axial and the equatorial isomers of the bis-*tert*-butyl esters of **3**, **4**, or **6**, was dissolved in a small volume of $CHCl_3$/MeOH (1:1 v/v) and separated by flash chromatography on a silica gel column. Elution with $CHCl_3$, MeOH/$CHCl_3$ (1:48 v/v), MeOH/$CHCl_3$ (1:24 v/v), and MeOH/$CHCl_3$ (3:22 v/v) gave fractions containing mixtures of the axial and the equatorial isomer of the bis-*tert*-butyl esters of **3**, **4**, or **6**. The fractions were dried on a rotary evaporator. Lyophilization gave mixtures of the two isomers of the bis-*tert*-butyl ester of **3** (42.9 mg, 12%, axial:equatorial = 42:58), the bis-*tert*-butyl ester of **4** (93.3 mg, 25%, axial:equatorial = 44:56), or the bis-*tert*-butyl ester of **6** (30 mg, 7.3%, axial:equatorial = 38:62) as yellow solids. Axial:equatorial ratios were determined by analytical RP-HPLC at 20 °C using a linear gradient of 30-50% B (bis-*tert*-butyl ester of **3**

and **6**), 30-60% B (bis-*tert*-butyl ester of **4**), or 40-50% B (bis-*tert*-butyl ester of **5**) in 35 min (eluent A, water; eluent B, CH_3CN).

The axial and equatorial isomers were separated from each other by preparative RP-HPLC (using the same linear gradients of B as described for analytical RP-HPLC over 105 min) followed by deprotection of the *tert*-butoxy groups by 20min stirring with TFA/H_2O/CH_2Cl_2 (74:1:25) at room temperature (20 mg of the esters in 4 mL of the solvent). After evaporation, the residue was dissolved in CH_3CN/H_2O and lyophilized to give quantitatively the pure axial or equatorial isomers of **3**, **4**, and **6,** respectively, as yellow solids.

Method B (compounds 3, 5 and 6): A mixture of the tetra *n*-butylammonium salt of cAMP, 8-Br-cAMP or 8-Br-cGMP (0.1 mmol; prepared from the free acids of cAMP or 8-Br-cGMP and the sodium salt of 8-Br-cAMP, respectively, via ion exchange with tetra-*n*-butylammonium hydroxide) and of **2 (**72.4 mg, 0.15 mmol) was stirred in 5 mL CH_3CN at 85 ^oC in the dark for 5 h and worked up as described above (Method A). After flash chromatography, mixtures of the two isomers of the bis-*tert*-butyl esters of **3** (28 mg, 38%, axial:equatorial = 80:20), of **5** (39.5 mg, 49%, axial:equatorial = 75:25), or of **6** (15 mg, 18%, axial:equatorial = 63:37) were obtained as yellow solids. Deprotection of the *tert*-butoxy groups as described in Method A gave the pure axial and equatorial diasteromers of **3**, **5**, and **6**.

Data for axial bis-*tert*-butyl ester of 3: TLC: R_f = 0.77 ($CHCl_3$/MeOH 9:1 v/v); t_R = 24.3 min (analytical HPLC, t_R = retention time), t_R =8 4.5 min (preparative HPLC); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: d = - 5.02; ^{1}H NMR (DMSO-d_6): d = 1.41 (s, 18H), 4.20 (s, 4H), 4.28 (dt, J = 10.3 and 4.7 Hz, 1H), 4.46 (t, J = 9.8 Hz, 1H), 4.67-4,73 (m, 2H), 5.39 (dd, J = 9.7 and 4.9 Hz, 1H), 5.42 (d, J = 6.1 Hz, 2H), 6.07 (s, 1H), 6.32 (s, 1H), 6.38 (d, J = 4.7 Hz, 1H), 6.50 (d, J = 2.3 Hz, 1H), 6.58 (dd, J = 8.9 and 2.3 Hz, 1H), 7.34 (s, 2H), 7.57 (d, J = 8.9 Hz, 1H), 8.15 (s, 1H), 8.34 (s, 1H); ESI MS: 731.1617 $[M+H]^+$.

Data for equatorial bis-*tert*-butyl ester of 3: TLC: R_f = 0.71 ($CHCl_3$/MeOH, 9:1 v/v); t_R = 25.8 min (analytical HPLC), t_R = 88.7 min (preparative HPLC); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: d = - 3.56; ^{1}H NMR (DMSO-d_6): d = 1.42 (s, 18H), 4.20 (s, 4H), 4.49-4.52 (m, 2H), 4.71 (t, J = 4.8 Hz, 1H), 4,74 (dd, J = 12.7 and 3.2 Hz, 1H), 5.34-5.43 (m, 3H), 6.08 (s, 1H), 6.21 (s, 1H), 6.35 (d, J = 4.5 Hz, 1H), 6.49 (d, J = 2.4 Hz, 1H), 6.61 (dd, J = 9.1 and 2.4 Hz, 1H), 7.36 (s, 2H), 7.54 (d, J = 8.9 Hz, 1H), 8.19 (s, 1H), 8.39 (s, 1H); ESI MS: 731.1657 $[M+H]^+$.

Data for axial 3: t_R = 10.6 min (analytical HPLC, 5-60% B in A in 20 min, eluent A, 0.1% TFA/H_2O; eluent B, CH_3CN); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: d = - 5.03; ^1H NMR (DMSO-d_6): d = 4.24 (s, 4H), 4.30 (dt, J = 10.2 and 4.5 Hz, 1H), 4.50 (t, J = 9.9 Hz, 1H), 4.69-4,74 (m, 2H), 5.32 (dd, J = 9.8 and 4.8 Hz, 1H), 5.42 (d, J = 6.0 Hz, 2H), 6.09 (s, 1H), 6.31 (s, 1H), 6.42 (br s, 1H), 6.51 (s, 1H), 6.61 (d, J = 9.0 Hz, 1H), 7.55 (d, J = 9.0 Hz, 1H), 7.82 (br s, 2H), 8.23 (s, 1H), 8.44 (s, 1H); ESI MS: 619.0741 $[M+H]^+$; elemental analysis calcd (%) for $C_{24}H_{23}N_6O_{12}P \times 3H_2O$ (672.50): C 42.86, H 4.35, N 12.50, found: C 43.02, H 4.21, N 11.98.

Data for equatorial 3: t_R = 10.6 min (analytical HPLC, 5-60% B in A in 20 min, eluent A, 0.1% TFA/H_2O; eluent B, CH_3CN); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: d = - 3.56; ^1H NMR (DMSO-d_6): d = 4.23 (s, 4H), 4.49-4.53 (m, 2H), 4.71 (t, J=4.8 Hz, 1H), 4,76 (dd, J = 12.0 and 3.0 Hz, 1H), 5.33-5.38 (m, 3H), 6.08 (s, 1H), 6.20 (s, 1H), 6.37 (d, J = 3.6 Hz, 1H), 6.49 (s, 1H), 6.62 (d, J = 8.4 Hz, 1H), 7.40 (s, 2H), 7.53 (d, J = 9.0 Hz, 1H), 8.21 (s, 1H), 8.40 (s, 1H); ESI MS: 619.0767 $[M+H]^+$; elemental analysis calcd (%) for $C_{24}H_{23}N_6O_{12}P \times 1.5H_2O$ (645.48): C 44.66, H 4.06, N 13.02, found: C 44.74, H 4.18, N 12.28.

Data for axial bis-*tert*-butyl ester of 4: TLC: R_f = 0.39 ($CHCl_3$/MeOH, 9:1 v/v); t_R = 20.5 min (analytical HPLC), t_R = 56.3 min (preparative HPLC); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: d = - 5.03; ^1H NMR (DMSO-d_6): d = 1.41 (s, 18H), 4.20 (s, 4H), 4.22 (m, 1H), 4.54 (t, J = 10.0 Hz, 1H), 4.59 (t, J = 4.9 Hz, 1H), 4.65-4.70 (m, 1H), 4.85 (dd, J = 9.7 and 5.0 Hz, 1H), 5.39-5.41 (m, 2H), 5.84 (s, 1H), 6.31 (s, 1H), 6.35 (d, J = 5.0 Hz, 1H), 6.49 (s, 2H), 6.50 (d, J = 2.4 Hz, 1H), 6.58 (dd, J = 9.0 and 2.4 Hz, 1H), 7.57 (d, J = 9.0 Hz, 1H), 7.94 (s, 1H), 10.67 (s, 1H); ESI MS: 747.1155 $[M+H]^+$.

Data for equatorial bis-*tert*-butyl ester of 4: TLC: R_f = 0.30 ($CHCl_3$/MeOH, 9:1 v/v); t_R = 23.7 min (analytical HPLC), t_R = 63.3 min (preparative HPLC); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: d = - 3.98; ^1H NMR (DMSO-d_6): d = 1.42 (s, 18H), 4.20 (s, 4H), 4.44-4.51 (m, 2H), 4.58 (t, J = 4.6 Hz, 1H), 4.71-4.75 (m, 1H), 5.16 (dd, J = 9.3 and 5.2 Hz, 1H), 5.35-5.37 (m, 2H), 5.85 (s, 1H), 6.21 (s, 1H), 6.27 (d, J = 4.6 Hz, 1H), 6.49 (d, J = 2.4 Hz, 1H), 6.56 (s, 2H), 6.60 (dd, J = 9.1 and 2.4 Hz, 1H), 7.53 (d, J = 9.0 Hz, 1H), 7.93 (s, 1H), 10.69 (s, 1H); ESI MS: 747.1214 $[M+H]^+$.

Data for axial 4: t_R = 10.2 min (analytical HPLC, 5-60% B in A in 20 min, eluent A, 0.1% TFA/H_2O; eluent B, CH_3CN); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: d = - 5.04; ^1H NMR (DMSO-d_6): d = 4.23 (s, 5H), 4.57 (t, J = 10.0 Hz, 1H), 4.60 (d, J = 4.6 Hz, 1H), 4.68

(ddd, *J* = 22.0, 9.0 and 4.5 Hz, 1H), 4,85 (dd, *J* = 9.9 and 4.9 Hz, 1H), 5.37-5.41 (m, 2H), 5.85 (s, 1H), 6.30 (s, 1H), 6.35 (br s, 1H), 6.50 (s, 3H), 6.61 (d, *J* = 8.9 Hz, 1H), 7.55 (d, *J* = 8.9 Hz, 1H), 8.01 (s, 1H), 10.70 (s, 1H); ESI MS: 635.0762 $[M+H]^+$; elemental analysis calcd (%) for $C_{24}H_{23}N_6O_{13}P \times 3H_2O$ (688.50): C 41.87, H 4.25, N 12.21, found: C 41.88, H 4.14, N 11.69.

Data for equatorial 4: t_R = 10.7 min (analytical HPLC, 5-60% B in A in 20 min, eluent A, 0.1% TFA/H_2O; eluent B, CH_3CN); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: d = - 3.99; ^1H NMR (DMSO-d_6): d = 4.23 (s, 4H), 4.42-4.51 (m, 2H), 4.59 (m, 1H), 4.72-4.77 (m, 1H), 5.14-5.18 (m, 1H), 5.34-5.38 (m, 2H), 5.85 (s, 1H), 6.20 (s, 1H), 6.28 (d, *J* =4 .6 Hz, 1H), 6.49 (s, 1H), 6.57 (br s, 2H), 6.62 (d, *J* = 8.2 Hz, 1H), 7.52 (d, *J* = 8.9 Hz, 1H), 7.95 (s, 1H), 10.69 (s, 1H); ESI MS: 635.0742 $[M+H]^+$; elemental analysis calcd (%) for $C_{24}H_{23}N_6O_{13}P \times 2.5H_2O$ (679.49): C 42.42, H 4.15, N 12.37, found: C 42.55, H 4.10, N 12.26.

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Data for axial bis-*tert*-butyl ester of 5: TLC: R_f = 0.95 ($CHCl_3$/MeOH, 9:1 v/v); t_R = 18.7 min (analytical HPLC), t_R = 80.3 min (preparative HPLC); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: d = - 5.12; ^1H NMR (DMSO-d_6): d = 1.41 (s, 18H), 4.19 (s, 4H), 4.30 (dt, *J* = 10.0 and 4.8 Hz, 1H), 4.36 (t, *J* = 10.0 Hz, 1H), 4.68 (ddd, *J* = 22.4, 9.2 and 5.0 Hz, 1H), 4.98 (t, *J* = 4.9 Hz, 1H), 5.43 (d, *J* = 6.0 Hz, 2H), 5.59 (dd, *J* = 9.3 and 5.0 Hz, 1H), 5.90 (s, 1H), 6.29 (s, 1H), 6.41 (d, *J* = 4.7 Hz, 1H), 6.50 (d, *J* = 2.4 Hz, 1H), 6.57 (dd, *J* = 8.9 and 2.4 Hz, 1H), 7.53 (s, 2H), 7.56 (d, *J* = 9.0 Hz, 1H), 8.12 (s, 1H); ESI MS: 809.0428 and 811.0392 $[M+H]^+$.

Data for equatorial bis-*tert*-butyl ester of 5: TLC: R_f = 0.89 ($CHCl_3$/MeOH, 9:1 v/v); t_R = 20.4 min (analytical HPLC), t_R = 85.1 min (preparative HPLC); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: d = - 3.37; ^1H NMR (DMSO-d_6): d = 1.42 (s, 18H), 4.20 (s, 4H), 4.37 (q, *J* = 9.3 Hz, 1H), 4.50 (dt, *J* = 9.8 and 5.6 Hz, 1H), 4.72-4.76 (m, 1H), 5.05 (t, *J* = 4.7 Hz, 1H), 5.37-5.39 (m, 2H), 5.48 (dd, J = 10.3 and 5.2 Hz, 1H), 5.90 (s, 1H), 6.21 (s, 1H), 6.36 (d, *J* = 4.5 Hz, 1H), 6.49 (d, *J* = 2.3 Hz, 1H), 6.60 (dd, *J* = 9.0 and 2.3 Hz, 1H), 7.54 (d, *J* = 9.0 Hz, 1H), 7.55 (s, 2H), 8.20 (s, 1H); ESI MS: 809.0559 and 811.0527 $[M+H]^+$.

Data for axial 5: t_R = 14.2 min (analytical HPLC, 5-60% B in A in 20 min, eluent A, 0.1% TFA/H_2O; eluent B, CH_3CN); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: d = - 5.14; ^1H NMR (DMSO-d_6): d = 4.23 (s, 4H), 4.29-4.32 (m, 1H), 4.39 (t, *J* = 9.2 Hz, 1H), 4.67-4.73 (m, 1H), 4.99 (s, 1H), 5.44 (s, 2H), 5.56-5.58 (m, 1H), 5.90 (s, 1H), 6.28 (s, 1H), 6.43 (br s, 1H),

6.52 (s, 1H), 6.61 (d, J = 9.0 Hz, 1H), 7.54 (d, J = 8.6 Hz, 1H), 7.69 (br s, 2H), 8.14 (s, 1H); ESI MS: 696.9749 and 698.9732 $[M+H]^+$; elemental analysis calcd (%) for $C_{24}H_{22}BrN_6O_{12}P \times 2.5H_2O$ (742.39): C 38.83, H 3.67, N 11.32, found: C 38.79, H 3.68, N 11.16.

Data for equatorial 5: t_R = 14.2 min (analytical HPLC, 5-60% B in A in 20 min, eluent A, 0.1% TFA/H_2O; eluent B, CH_3CN); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: d = - 3.37; ^1H NMR (DMSO-d_6): d = 4.24 (s, 4H), 4.40 (q, J = 9.0 Hz, 1H), 4.49-4.53 (m, 1H), 4.74-4.78 (m, 1H), 5.04 (d, J = 4.1 Hz, 1H), 5.37 (s, 2H), 5.46-5.48 (m, 1H), 5.91 (s, 1H), 6.20 (s, 1H), 6.39 (br s, 1H), 6.50 (s, 1H), 6.63 (d, J = 9.0 Hz, 1H), 7.53 (d, J = 8.9 Hz, 1H), 7.72 (br s, 2H), 8.24 (s, 1H); ESI MS: 696.9729 and 698.9691 $[M+H]^+$; elemental analysis calcd (%) for $C_{24}H_{22}BrN_6O_{12}P \times 3.5H_2O$ (760.41): C 37.91, H 3.84, N 11.05, found: C 37.90, H 3.61, N 10.73.

Data for axial bis-*tert*-butyl ester of 6: TLC: R_f = 0.91 ($CHCl_3$/MeOH, 5:1 v/v); t_R = 24.4 min (analytical HPLC), t_R = 67.7 min (preparative HPLC); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: d = - 5.02; ^1H NMR (DMSO-d_6): d = 1.41 (s, 18H), 4.19 (s, 4H), 4.23 (dt, J=10.1 and 4.8 Hz, 1H), 4.41 (t, J = 10.0 Hz, 1H), 4.68 (ddd, J = 22.2, 9.0 and 4.5 Hz, 1H), 5.01 (m, 1H), 5.10 (dd, J= 9.3 and 5.4 Hz, 1H), 5.43 (dq, J = 17.0 and 6.8 Hz, 2H), 5.75 (s, 1H), 6.28 (s, 1H), 6.35 (d, J = 3.2 Hz, 1H), 6.48 (d, J = 1.7 Hz, 1H), 6.54 (s, 2H), 6.56 (d, J = 9.7 Hz, 1H), 7.57 (d, J = 9.0 Hz, 1H), 10.94 (s, 1H); ESI MS: 825.1644 and 827.1654 $[M+H]^+$.

Data for equatorial bis-*tert*-butyl ester of 6: TLC: R_f = 0.85 ($CHCl_3$/MeOH, 9:1 v/v); t_R = 28.9 min (analytical HPLC), t_R = 76.7 min (preparative HPLC); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: d = - 4.22; ^1H NMR (DMSO-d_6): d = 1.42 (s, 18H), 4.20 (s, 4H), 4.36 (q, J = 10.3 Hz, 1H), 4.48 (m, 1H), 4.73 (m, 1H), 4.86 (m, 1H), 5.35-5.36 (m, 3H), 5.73 (s, 1H), 6.23 (s, 1H), 6.26 (d, J = 4.2 Hz, 1H), 6.49 (d, J = 2.0 Hz, 1H), 6.60 (dd, J = 9.6 and 1.8 Hz, 1H), 6.76 (br s, 2H), 7.54 (d, J = 9.0 Hz, 1H), 10.91 (s, 1H); ESI MS: 825.1682 and 827.1690 $[M+H]^+$.

Data for axial 6: t_R = 14.1 min (analytical HPLC, 5-60% B in A in 20 min, eluent A, 0.1% TFA/H_2O; eluent B, CH_3CN); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: d = - 5.00; ^1H NMR (DMSO-d_6): d = 4.20 (s, 4H), 4.23 (dt, J= 10.3 and 4.8 Hz, 1H), 4.45 (t, J = 10.0 Hz, 1H), 4.68 (ddd, J = 22.0, 9.4 and 4.8 Hz, 1H), 5.02 (t, J = 4.2 Hz, 1H), 5.10 (dd, J = 9.0 and 5.0 Hz, 1H), 5.38-5.46 (m, 2H), 5.75 (s, 1H), 6.27 (s, 1H), 6.35 (d, J = 4.9 Hz, 1H), 6.46 (s, 1H), 6.49 (s, 2H), 6.58 (d, J = 8.7 Hz, 1H), 7.55 (d, J = 8.9 Hz, 1H),

10.82 (s, 1H); ESI MS: 712.9959 and 714.9917 $[M+H]^+$; elemental analysis calcd (%) for $C_{24}H_{22}BrN_6O_{13}P \times 3H_2O$ (767.39): C 37.56, H 3.68, N 10.95, found: C 37.85, H 3.36, N 10.79.

Data for equatorial 6: t_R = 14.7 min (analytical HPLC, 5-60% B in A in 20 min, eluent A, 0.1% TFA/H_2O; eluent B, CH_3CN); ^{31}P NMR (DMSO-d_6) heteronuclear decoupled: d = - 4.24; ^1H NMR (DMSO-d_6): d = 4.21 (s, 4H), 4.37 (q, J = 10.3 Hz, 1H), 4.46-4.50 (m, 1H), 4.73-4.77 (m, 1H), 4.86 (t, J = 4.6 Hz, 1H), 5.33-5.37 (s, 3H), 5.73 (s, 1H), 6.22 (s, 1H), 6.27 (d, J = 4.6 Hz, 1H), 6.47 (s, 1H), 6.61 (d, J = 8.0 Hz, 1H), 6.75 (br s, 2H), 7.53 (d, J = 8.9 Hz, 1H), 10.88 (s, 1H); ESI MS: 713.0015 and 714.9975 $[M+H]^+$; elemental analysis calcd (%) for $C_{24}H_{22}BrN_6O_{13}P \times 2H_2O$ (749.38): C 38.47, H 3.50, N 11.21, found: C 38.63, H 3.30, N 10.91.

7-(*tert*-Butoxycarbonylmethylamino]-4-methylcoumarin (8) and 7-[bis(*tert*-butoxycarbonylmethyl)amino]-4-methylcoumarin (9): 7-Amino-4-methylcoumarin (5.26 g, 30 mmol), bromoacetic acid *tert*-butyl ester (29.56 mL, 200 mmol), diisopropylethylamine (20.54 mL, 120 mmol), and NaI (4.5 g, 30 mmol) in 90 mL CH_3CN were refluxed for 24 h. The mixture was cooled to room temperature, filtered, and the solvent evaporated in vacuo. The residue was dissolved in 250 mL EtOAc, washed with 3×50 mL brine, dried ($MgSO_4$), and concentrated in vacuo to afford a yellow oil. The oil was separated and purified via flash chromatography (SiO_2, 15-45% EtOAc in hexane) to afford **9** (3.86 g, 9.37 mmol, 31%) and **8** (3.2 g, 11.1 mmol, 27%). **8** could be converted to **9** using the same procedures described above with about 25% yield.

Data for 8: *M.p.*: 154.7 °C; TLC: R_f = 0.78 (EtOAc/hexane, 3:2 v/v); ^1H NMR (DMSO-d_6): d = 1.43 (s, 9H) , 2.32 (s, 3H), 3.90 (d, J = 6.3 Hz, 2H), 5.95 (s, 1H), 6.38 (d, J = 2.1 Hz, 1H), 6.63 (dd, J = 8.8 and 2.2 Hz, 1H), 6.89 (t, J = 6.2 Hz, 1H), 7.46 (d, J = 8.7 Hz, 1H); ESI MS: 290.0987 $[M+H]^+$; elemental analysis calcd (%) for $C_{16}H_{19}NO_4$ (289.33): C 66.42, H 6.62, N 4.84, found: C 66.44, H 6.77, N 4.80.

Data for 9: *M.p.*: 150.8 °C; TLC: R_f = 0.83 (EtOAc/hexane, 3:2 v/v); ^1H NMR (DMSO-d_6): d = 1.42 (s, 18H) , 2.34 (s, 3H), 4.18 (s, 4H), 6.03 (s, 1H), 6.42 (d, J = 2.4 Hz, 1H), 6.57 (dd, J = 9.0 and 2.4 Hz, 1H), 7.56 (d, J = 9.0 Hz, 1H); ESI MS: 404.1469 $[M+H]^+$; elemental analysis calcd (%) for $C_{22}H_{29}NO_6$ (403.47): C 65.49, H 7.24, N 3.47, found: C 65.60, H 7.37, N 3.47.

7-[Bis(*tert*-butoxycarbonylmethyl)amino]-4-formylcoumarin (10): 9 (4.03 g, 10 mmol) was dissolved in 50 mL p-xylene by heating, selenium dioxide (2.21 g, 20 mmol) was added, and the mixture was refluxed for 6 h with stirring. The mixture was filtered hot to remove black selenium, and the filtrate was concentrated under reduced pressure. The resulting precipitate gave 3.41 g (8 mmol, 80%) of the aldehyde as orange-red crystals.
M.p.: 156 °C; TLC: R_f = 0.61 (THF/hexane, 1:2 v/v); ^{1}H NMR (DMSO-d_6): d = 1.42 (s, 18H), 4.21 (s, 4H), 6.52 (d, J = 2.6 Hz, 1H), 6.65 (dd, J = 9.2 and 2.6 Hz, 1H), 6.76 (s, 1H), 8.23 (d, J = 9.1 Hz, 1H), 10.08 (s, 1H); ESI MS: 418.1512 $[M+H]^+$; elemental analysis calcd (%) for $C_{22}H_{27}NO_7 \times 0.5\ H_2O$ (426.47): C 61.96, H 6.62, N 3.28, found: C 62.02, H 6.54, N 3.26.

7-[Bis*(tert*-butoxycarbonylmethyl)amino]-4-formylcoumarin tosylhydrazone: A mixture of **10** (2.13 g, 5 mmol) and p-tosylhydrazine (1.024 g, 5.5 mmol) in 20 mL ethanol was stirred at room temperature for 2 h. The resulting precipitate was collected by filtration and washed with ethanol to give 2.5 g (4.2 mmol, 84%) of the desired product as yellow solid.
M.p.: 165-67 °C; TLC: R_f = 0.19 (THF/hexane, 1:2 v/v); ^{1}H NMR (DMSO-d_6): d = 1.42 (s, 18H), 2.37 (s, 3H), 4.20 (s, 4H), 6.27 (s, 1H), 6.43 (d, J = 2.6 Hz, 1H), 6.58 (dd, J = 9.2 and 2.6 Hz, 1H), 7.42 (d, J = 8.1 Hz, 2H), 7.79 (d, J = 8.3 Hz, 2H), 7.96 (s, 1H), 8.07 (d, J = 9.1 Hz, 1H); ESI MS: 586.1740 $[M+H]^+$; elemental analysis calcd (%) for $C_{29}H_{35}N_3O_8S$ (585.67): C 59.47, H 6.02, N 7.17, found: C 59.38, H 5.92, N 7.21.

7-[Bis*(tert*-butoxycarbonylmethyl)amino]-4-(diazomethyl)coumarin (1): Triethylamine (506 mg, 5 mmol) was added to a stirred suspension of the tosylhydrazone of **10** (2.38 g, 4 mmol) in 25 mL methanol. The mixture was stirred at room temperature for 3 h, the solvent removed in vacuo, the resulting resin-like brown solid collected by filtration and washed with methanol/ether to give 2.58 g crude product, containing by HPLC analysis 1.5 g (3.5 mmol) of the diazo compound **2** (yield: 87%). The crude product was used for the preparation of **3**, **4**, and **6** without further purification. A sample of high purity was obtained by preparative HPLC.
TLC: R_f = 0.44 (THF/hexane, 1:2 v/v); ^{1}H NMR (DMSO-d_6): d = 1.42 (s, 18H), 4.18 (s, 4H), 5.56 (s, 1H), 6.40 (d, J = 2.4 Hz, 1H), 6.41 (s, 1H), 6.54 (dd, J = 9.0 and 2.5 Hz, 1H), 7.52 (d, J = 9.1 Hz, 1H); ESI MS: 430.2194 $[M+H]^+$; elemental analysis calcd (%) for $C_{22}H_{27}N_3O_6$ (429.47): C 61.53, H 6.34, N 9.78, found: C 61.10, H 6.39, N 9.59.

7-[Bis*(tert*-butoxycarbonylmethyl)amino]-4-(hydroxymethyl)coumarin (11): $NaBH_4$ (189.2 mg, 5 mmol) was added to **10** (2.13 g, 5 mmol) in 40 mL methanol, and the mixture was stirred at room temperature for 2 h. The reaction mixture was diluted with H_2O (40 mL), acidified (pH 5) with 0.1 N HCl and extracted three times with CH_2Cl_2 (30 mL each). The organic phase was washed with H_2O and brine, dried over $MgSO_4$, and concentrated in vacuo. The crude product was purified by flash chromatography (SiO_2, 30-50% EtOAc in hexane), and 1.6 g (3.7 mmol, 74%) of the hemihydrate of **11** was obtained as yellow solid.
M.p.: 176 °C; TLC: R_f = 0.52 (THF/hexane, 1:1 v/v); ^{1}H NMR (DMSO-d_6): d = 1.42 (s, 18H), 4.18 (s, 4H), 4.68 (d, J = 4.7 Hz, 2H), 5.53 (t, J = 5.5 Hz, 1H), 6.16 (s, 1H), 6.44 (d, J = 2.5 Hz, 1H), 6.54 (dd, J = 9.0 and 2.5 Hz, 1H), 7.48 (d, J = 9.0 Hz, 1H); ESI MS: 420.1692 $[M+H]^+$; elemental analysis calcd (%) for $C_{22}H_{29}NO_7 \times 0.5H_2O$ (428.48): C 61.67, H 7.06, N 3.27, found: C 61.41, H 6.81, N 3.31.

7-[Bis(carboxymethyl)amino]-4-(hydroxymethyl)coumarin (12):
11 (42.8 mg, 0.1mmol) was stirred in a mixture (4 mL) of TFA/H_2O/CH_2Cl_2 (74:1:25) at room temperature for 25 min. The solvents were evaporated, and the residue was coevaporated two times with ether, dissolved in CH_3CN/H_2O and lyophilized to give 32.5 mg (0.1 mmol, 100%) of the pure alcohol **12**.
M.p.: 184-187 °C; ^{1}H NMR (DMSO-d_6): d = 4.21 (s, 4H), 4.68 (s, 2H), 5.53 (br s, 1H), 6.14 (s, 1H), 6.45 (d, J = 2.5 Hz, 1H), 6.56 (dd, J = 9.0 and 2.5 Hz, 1H), 7.47 (d, J = 8.9 Hz, 1H); ESI MS: 308.0554 $[M+H]^+$; elemental analysis calcd (%) for $C_{14}H_{13}NO_7 \times 1H_2O$ (325.28): C 51.70, H 4.65, N 4.31, found: C 51.57, H 4.57, N 4.33.

7-[Bis*(tert*-butoxycarbonylmethyl)amino]-4-(bromomethyl)coumarin (2):
Methanesulfonic acid chloride (0.116 mL, 1.5 mmol) was added to an ice-cooled solution of **11** (427.4 mg, 1 mmol) in anhydrous CH_2Cl_2 (5 mL) containing triethylamine (0.28 mL, 2 mmol). After stirring for 10 min, the reaction mixture was washed two times with ice-cooled 5% aqueous $NaHCO_3$ and water and dried over $MgSO_4$. Evaporation of the solvent gave {7-[bis*(tert*-butoxycarbonylmethyl)amino]coumarin-4-yl}methyl methanesulfonate, which was not isolated but directly converted to **2** by stirring with anhydrous LiBr (347.4 mg, 4 mmol) in 5 mL anhydrous THF for 1 h. After removel of the solvent in vacuo, extraction of the residue with CH_2Cl_2, washing of the CH_2Cl_2 solution with brine, drying over $MgSO_4$, concentration to 1.5 mL, addition of hexane, standing overnight at 6 °C, and filtration of the precipitate, 391 mg (0.81 mmol, 81%) of **2** was obtained as yellow solid.

M.p.: 163-164 °C; TLC: R_f = 0.83 (THF/hexane, 1:2 v/v); ^{1}H NMR (DMSO-d_6): d = 1.42 (s, 18H), 4.20 (s, 4H), 4.79 (s, 2H), 6.35 (s, 1H), 6.46 (d, J = 2.4 Hz, 1H), 6.63 (dd, J = 9.0 and 2.3 Hz, 1H), 7.66 (d, J = 9.0 Hz, 1H); ^{13}C NMR (DMSO-*d*6): 27.91, 28.27, 53.63, 81.30, 98.45, 107.40, 109.31, 110.21, 126.27, 151.67, 151.70, 155.65, 160.62, 168.96; ESI MS: 482.0898 and 484.0858 $[M+H]^+$; elemental analysis calcd (%) for $C_{22}H_{28}BrNO_6$ (482.37): C 54.78, H 5.85, N 2.90, found: C 54.96, H 5.82, N 2.96.

{7-[Bis*(tert*-butoxycarbonylmethyl)amino]coumarin-4-yl}methyl phenylacetate: 11 (42.8 mg, 0.1 mmol) and phenylacetic acid (13.6 mg, 0.1 mmol) were dissolved in EtOAc (1.5 mL) in the presence of DMAP, cooled to 10 °C, and then DCC (25.8 mg, 0.125 mmol) was added under stirring. The mixture was then allowed to warm to RT and was stirred for 2 h. After removal of the solvent, the crude product was purified by preparative RP-HPLC using a linear gradient of 50-95% B in 60 min (eluent A, water; eluent B, CH_3CN) to give 26 mg (0.048 mmol, 48%) of the desired compound as a yellow solid.
TLC: R_f = 0.88 (THF/hexane, 1:1 v/v); preparative HPLC: t_R = 54.3 min; ^{1}H NMR (DMSO-d_6): d = 1.42 (s, 18H), 3.85 (s, 2H), 4.19 (s, 4H), 5.33 (s, 2H), 6.02 (s, 1H), 6.46 (d, J = 2.5 Hz, 1H), 6.54 (dd, J = 9.1 and 2.5 Hz, 1H), 7.26-7.29 (m, 1H), 7.31-7.35 (m, 4H), 7.48 (d, J=9.0 Hz, 1H); ESI MS: 538.1716 $[M+H]^+$.

{7-[Bis(carboxymethyl)amino]coumarin-4-yl}methyl phenylacetate: {7-[Bis*(tert*-butoxycarbonylmethyl)amino]coumarin-4-yl}methyl phenylacetate (53.73 mg, 0.1mmol) was stirred in a mixture (7 mL) of TFA/H_2O/CH_2Cl_2 (74:1:25) at room temperature for 25 min. The solvents were evaporated, and the residue was coevaporated two times with ether, dissolved in AN/H_2O and lyophilized to give 43 mg (0.1 mmol, 100%) of the desired product.
M.p.: 180-182 °C (dec.); t_R =12.5 min (analytical HPLC, 5-95% B in A in 20 min, eluent A, 0.1% TFA/H_2O; eluent B, AN); ^{1}H NMR (DMSO-d_6): d = 3.85 (s, 2H), 4.22 (s, 4H), 5.31 (s, 2H), 6.01 (s, 1H), 6.46 (d, J = 2.0 Hz, 1H), 6.55 (dd, J = 9.1 and 2.3 Hz, 1H), 7.26-7.29 (m, 1H), 7.30-7.35 (m, 4H), 7.46 (d, J = 9.1 Hz, 1H); ESI MS: 426.0517 $[M+H]^+$; elemental analysis calcd (%) for $C_{22}H_{19}NO_8 \times 0.5\ H_2O$ (434.4): C 60.83, H 4.64, N 3.22, found: C 61.17, H 4.14, N 3.29.

Chemical and biological experiments

Solubility: Saturated solutions of the diastereomers of **3**-**6** in CH_3CN/HEPES buffer (10 mM HEPES and 120 mM KCl adjusted to pH 7.2 with 2 N KOH) (5/95) were analyzed by analytical RP-HPLC at room temperature.

Hydrolytic stability: Freshly prepared 25 μM solutions of the axial and the equatorial isomers of **3**-**6** in CH_3CN/HEPES buffer (5/95), pH 7.2, were left in the dark at ambient temperature and monitored over a period of 24 h by analytical RP-HPLC.

Photochemical quantum yields: The differential photochemical quantum yields, j_{chem}, were determined for the diastereomers of **3**-**6** in CH_3CN/HEPES buffer (5/95), pH 7.2, at 365 nm by the relative method as previously described[1] using (6,7-dimethoxycoumarin-4-yl)methyl diethyl phosphate (j_{chem} = 0.08)[2] as a standard. For the kinetic investigations, the irradiated solutions were analyzed by analytical HPLC.

Fluorescence quantum yields: The fluorescence quantum yields, j_f, of the diastereomers of **3**–**6** were determined at 25 °C in CH_3CN/HEPES buffer (5/95), pH 7.2, by the relative method[4] versus quinine sulfate in 0.1 N H_2SO_4 as a standard (j_f = 0.545). At the excitation wavelength used, the absorbance values of the standard and the investigated compound were identical.

Time-resolved fluorescence spectroscopy: Time-resolved fluorescence spectroscopy studies were carried out in CH_3CN/HEPES buffer (5/95), pH 7.2, at about 23 °C. Fluorescence rise and decay curves were recorded in right-angle arrangement. We used an MSC 1600 N_2 laser from LTB (337 nm, pulse width 0.5 ns, maximum pulse energy 0.7 mJ) as excitation source. Details of the equipment and the deconvolution procedure of the experimental decay curves are described elsewhere.[5]

Two-photon uncaging in cuvettes using confocal laser scanning microscopy (LCSM)
Two-photon uncaging was performed by placing a micro-cuvette (30 × 10 × 1 mm), which was filled with 75 μL **6** (axial isomer) or 75 μL Bhc-glu, with its flat side on the table of a confocal laser scanning microscope (LSM510-META NLO, Carl Zeiss, Germany) in the focal plane of the objective lens, and irradiating with light from a Cameleon diode-pumped laser (Coherent, Germany) at 740 nm. For each irradiation time and compound a separate cuvette was used to obtain two-photon uncaged samples at various times. The concentrations of the

remained caged compounds were determined with 20 µL of the samples by RP-HPLC. The laser power exiting the objective lens was 90 mW. The exposed surface was 512 × 512 µm.

Isothermal titration calorimetry (ITC):[6,7] 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) dissolved in $CHCl_3$ at 20 mg mL^{-1} was dried in a rotary evaporator and subsequently under high vacuum overnight. For the preparation of pure lipid vesicles, the dry film was suspended in HEPES buffer (pH 7.2) by vortex mixing for 5 min. Bilayers preloaded with the axial isomer of DEACM-caged cAMP on both leafleats were made in the same manner by suspending the lipid film along with the dry caged cNMP in buffer. Small unilamellar vesicles (SUVs) were obtained by ultrasonication in an ice/water bath for 20 min, followed by spinning for 5 min at 13 krpm in a table-top centrifuge.
ITC was performed on a VP-ITC (MicroCal, Northampton, U.S.A.). In uptake experiments, 10-µL aliquots of 40 mM POPC SUVs were injected into the 1.4-mL sample cell containing **3**, **5**, or DEACM-caged cAMP. For release experiments, 10-µL aliquots of 20 mM POPC SUVs prepared in the presence of 482 µM DEACM-caged cAMP were titrated into pure buffer. All measurements were done at 25 °C after gentle vacuum degassing of the samples. Baseline subtraction and peak integration were accomplished using Origin 5.0 as described by the manufacturer (MicroCal Software, Northampton, U.S.A) The first peak was not taken into account because it is usually affected by sample loss due to leakage effects during the equilibration phase preceding the actual ITC run.
Figure 1 shows the reaction heats for DEACM-caged cAMP obtained in uptake and release experiments performed with unilamellar vesicles composed of POPC.

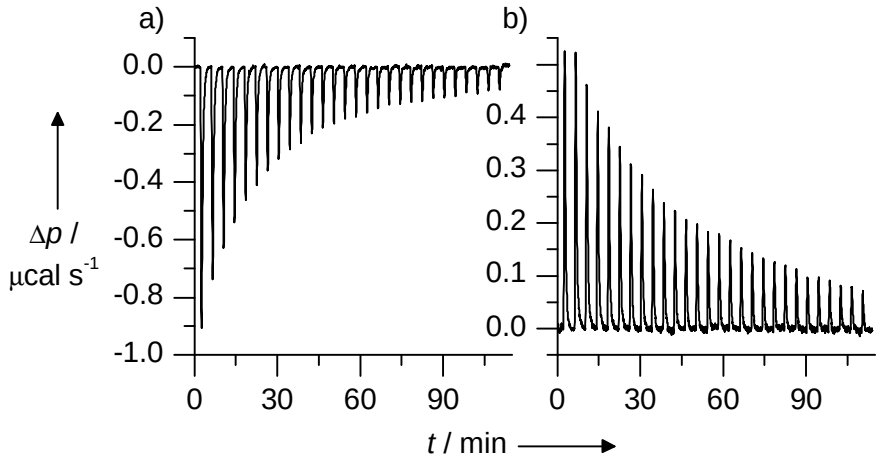

Figure 1. Isothermal titration calorimetry of DEACM-caged cAMP and POPC SUVs. *t* is time, and Δ*p* denotes the differential heat flow, i.e. the compensation power supplied by the

feedback system required to keep the sample cell at the same temperature (25 °C) as the reference cell. a) Uptake experiment. b) Release experiment.

Calorimetric experiments with DEACM-caged cAMP were evaluated in terms of a simple phase partition equilibrium,[7] where the molar ratio of membrane-bound caged compound to accessible lipid,

$$R_b \equiv c_b/(gc_l) \quad (1),$$

is proportional to the concentration of caged compound in the aqueous phase,

$$R_b = Kc_a \quad (2).$$

Here, c_l, c_b, and c_a denote the concentrations of lipid and of caged compound bound to the membrane and in the aqueous phase, respectively. The floating parameters are the adsorption constant, K, (in units of liters per mole), and the lipid accessibility factor, g, which amounts to $g = 1$ for membrane-permeant and to $g = 0.6$ for impermeant compounds, respectively. The change in standard Gibbs free energy was obtained according to

$$\Delta G^o = - RT \ln(Kc_w) \quad (3),$$

where R is the universal gas constant, T the absolute temperature, and c_w the molar concentration of water. In addition, the enthalpy change, ΔH, was determined directly from the ITC experiment, and the change in standard entropy was then calculated from the Gibbs-Helmholtz equation,

$$\Delta S° = (\Delta H° - \Delta G°)/T \quad (4).$$

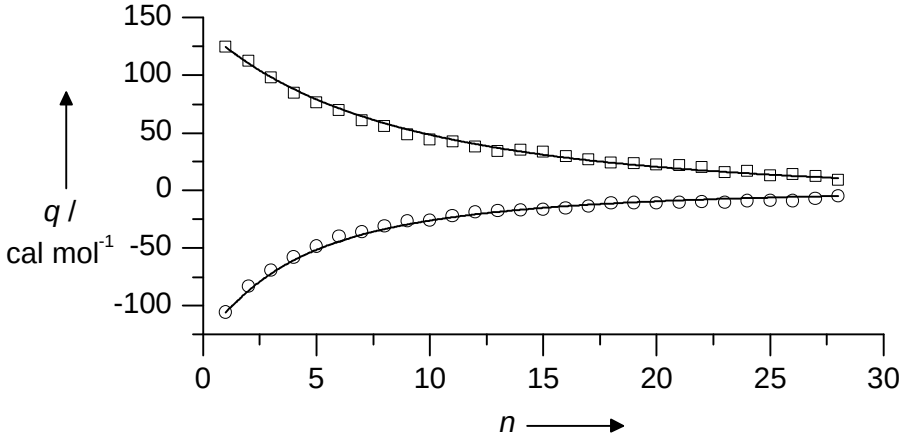

Figure 2. Membrane permeability of small unilamellar POPC vesicles to DEACM-caged cAMP at 25°C. The heats of reaction, q, obtained in an uptake (circles) and a release (squares) experiment are plotted versus the injection number, n. The best fit (solid lines) to both data sets yields the adsorption constant, K = 360 M^{-1}, the changes in Gibbs free energy, ΔG^o = - 5.9 kcal mol^{-1}, in enthalpy, ΔH = -6.6 kcal mol^{-1}, and in entropy, ΔS^o = - 2.6 cal mol^{-1} K^{-1}, and the

lipid accessibility factor, $g = 1$. Thus, DEACM-caged cAMP can equilibrate across the membrane on the time scale of a single ITC injection, i.e., within less than 4 min.

A good fit (Figure 2, solid lines) to both data sets was possible only for the assumption of complete lipid accessibility to the caged nucleotide, indicating that DEACM-caged cAMP is able to rapidly overcome a lipid bilayer. In contrast, the hydrophilic caged cNMPs **3** and **5** do not measurably partition into membranes at pH 7.2, as evidenced by the lack of reaction heats upon titration of 200 μM solutions with lipid vesicles at different temperatures (data not shown).

Optical Ca^{2+} measurements using CLSM: HEK293 cells expressing the α-subunit of the CNG channel from bovine cone photoreceptor cells (CNGA3)[7] were loaded with the Ca^{2+} indicator FLUO-4/AM (4 μM) by preincubation for 30 min in the dark in the presence of 0.05% Pluronic F-127 and 1 mL Dulbecco's modified Eagle's medium (10% fetal bovine serum and 1% penicillin/streptavidin). After washing, the cells were preincubated at 37 °C in the dark for 15 min with **6** (50 μM, axial isomer). The bath solution (pH 7.4) contained (mM) 140 NaCl, 4.6 KCl, 2 $CaCl_2$, 10 HEPES, 10 glucose. For measurements of Ca^{2+}-dependent changes in fluorescence intensity of FLUO-4 and for the photorelease of **6**, we used an LSM510-META UV and an LSM510-META NLO (nonlinear optics) system (both from Carl Zeiss, Germany). The Ca^{2+} indicator was excited with an argon-ion laser (Spectra Physics, Germany), and a cut-off filter (LP 505) was used for the detection of the fluorescence. The caged compounds were photolyzed at 364 nm with an argon-ion laser (Spectra Physics, Germany) and at 745 nm with a Cameleon diode-pumped laser (Coherent, Germany). Irradiation was accomplished in a small cytosolic area (approx. 10% of the total cell area of the confocal image).
As control experiments, measurements were accomplished under frequently UV or IR irradiation in the absence of **6**. No changes in the fluorescence signal of the cells could be detected (data not shown). Blockade of the Ca^{2+} influx through CNG ion channels with 15 mM $MgCl_2$ revealed no changes in the fluorescence intensity of the calcium indicator (data not shown).

Electrophysiological experiments using CNGA1 channels: For patch-clamp experiments, oocytes were obtained surgically from adult females of *Xenopus laevis.* The oocytes were treated for 60-90 min with 1.2 mg/mL collagenase (Type I, Sigma, St. Louis, U.S.A.) and

manually dissected. They were injected with 40-70 nL of a solution containing cRNA specific for bovine CNGA1 channels (accession No. X51604). The oocytes were incubated at 18 °C in Barth medium and were used within 6 days after injection. Currents were recorded in inside-out patches using K^+ solution in the bath and the pipette (in mM: 150 KCl, 1 EGTA, 5 HEPES, pH 7.4 adjusted with KOH). The pipette resistance ranged from 0.6-25 MΩ. Recording was performed with a 200B amplifier (Axon Instruments, U.S.A.). Each patch was first exposed to a solution containing no cyclic nucleotide, and the maximum current was then activated by 700 μM free cGMP.

The experiment chamber was composed of a main compartment and a small photolysis compartment. The solution containing the caged cGMP passed the photolysis compartment (width 0.5 mm, height 1.0 mm) just before entering the main compartment. One wall of the photolysis compartment was formed by the end of a light guide (diameter 1.0 mm). The temperature was 20.3 ± 0.1 ^{o}C. Light flashes were generated by the flash-lamp system JML-C2 (Rapp OptoElectronic, Germany). Photolysis was completed within 150 μs. The tip of the quartz pipette was positioned in the center of the photolysis chamber. The solution flow through the photolysis chamber was adjusted such that the concentration of the liberated cyclic nucleotide was constant for at least 1.5 s.

To determine the concentration of free cGMP liberated by flash photolysis, the ratio of the steady-state current following a flash to the steady-state current at a saturating concentration of free cGMP, I_∞/I_{max}, was inserted in the equation $[cGMP] = EC_{50}\,[(I_{max}/I_\infty)-1]^{(-1/H)}$. EC_{50} and H are the half-maximum concentration and the Hill coefficient, which were determined from the concentration-response relationship for free cGMP to be 46.5 μM and 1.98, respectively. Measurements were controlled and data were collected with the ISO3 soft- and hardware (MFK Niedernhausen, Germany). The sampling rate was 20 kHz (filter 5 kHz). All currents were corrected for capacitive and very small leak components by subtracting the corresponding averaged currents measured in the absence of cGMP.

Electrophysiological experiments using CNGA3 channels: Photorelease of 8-Br-cGMP from **6** in a CNGA3-transfected HEK293 cell [8] was monitored by loading the cell in the whole-cell configuration of the patch-clamp technique with a pipette solution (pH 7.2) that contained (mM) 130 CsCl, 20 TEACl, 2 $MgCl_2$, 10 HEPES, 2 Na_2-ATP, 0.3 Na_2-GTP, 2 EGTA, and 80 μM **6**. The extracellular solution (pH 7.2) contained (mM) 120 NaCl, 3 KCl, 0.3 $CaCl_2$, 50 glucose, 10 HEPES. Cells were voltage-clamped at -70 mV, and the total whole-cell current was measured (EPC-9, HEKA Elektroniks, Germany). 3 min after establishing the

whole-cell configuration, photolysis of **6** was induced by a 380-nm UV flash (UV flash system and monochromator, Till Photonics, Germany). The fluorescence immediately before and after the flash was collected through a dichroic mirror FT 395, detected by a photodiode (Till Photonics, Germany), and acquired simultaneously with the current by the EPC-9.

Measurements of the fluorescence of 12 inside cells: An HEK293 cell was placed on the inverted microscope (Diaphoto 300, Nikon, Japan) with a 40× objective (Fluor 40 / 1.30 oil, Nikon, Japan). 100 μM **12** was loaded into the cell with a patch pipette in the whole-cell configuration. The fluorescence intensity was recorded with a single-cell photon counting system (PhoCal, Life Science Resources, Cambridge, U.K.) after achievement of giga-seal. The recording configuration was monitored by using a patch-clamp amplifier (EPC-7, HEKA, Germany). Excitation light was generated by a 75-W xenon lamp (AMKO, Germany). Solutions (mM): pipette: 140 KCl, 1 EGTA, 10 HEPES; pH 7.2 (KOH); bath: 140 NaCl, 10 EGTA, 10 HEPES; pH 7.4 (NaOH)
Figure 3 shows the increase in the single-cell fluorescence intensity during loading of the cell with **12**.

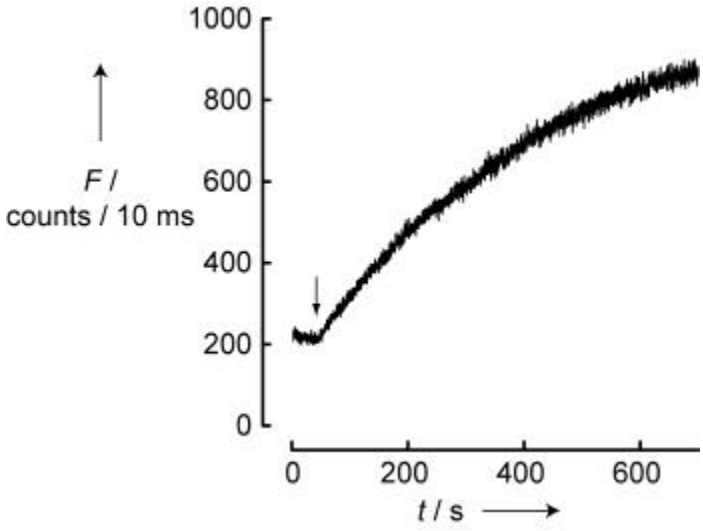

Figure 3. Increase in the single-cell fluorescence intensity during loading of an HEK293 cell with 100 μM **12** in HEPES buffer, pH 7.2 (excitation: 365 nm, emission: >480 nm). The arrow indicates the time point when the whole-cell configuration was formed.

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