Derivation of equation used to calculate cytosolic cAMP concentration.

The absolute maximal FRET response observed *in vitro* (F_{ABS}) and the maximal FRET response observed in the intact cell (F_{MAX}) are described by Eq. 1 and Eq. 2, respectively:

$$\boldsymbol{F_{ABS} = \frac{R_{MAX} - R_{0\ in\ vitro}}{R_{0\ in\ vitro}}} \quad \textbf{(Eq. 1)}$$

$$\boldsymbol{F_{MAX} = \frac{R_{MAX} - R_{0\ cell}}{R_{0\ cell}}} \quad \textbf{(Eq. 2)}$$

where R_{MAX} = maximum CFP/YFP fluorescence ratio; $R_{0\ in\ vitro}$ = minimum CFP/YFP fluorescence ratio *in vitro*; and $R_{0\ cell}$ = minimum CFP/YFP fluorescence ratio in the intact cell.

Based on Eq.1 and Eq. 2, and assuming that R_{MAX} is identical *in vitro* and in the intact cell, we can derive Equation 3:

$$F_{ABS} * R_{0\ in\ vitro} + R_{0\ in\ vitro} = F_{MAX} * R_{0\ cell} + R_{0\ cell} \quad \rightarrow$$

$$\boldsymbol{R_{0\ cell} = \left(\frac{F_{ABS} + 1}{F_{MAX} + 1}\right) * R_{0\ in\ vitro}} \quad \textbf{(Eq. 3)}$$

Furthermore, the FRET response observed under any given set of experimental conditions (F) can be described by Equation 4:

$$F = \frac{R_{cell} - R_{0\ cell}}{R_{0\ cell}} \quad \rightarrow$$

$$\boldsymbol{R_{cell} = R_{0\ cell} * (F + 1)} \quad \textbf{(Eq. 4)}$$

where R_{cell} = the CFP/YFP fluorescence ratio in the intact cell.

Based on Eq. 3 and Eq. 4, we can derive Eq. 5, which describes the *equivalent* FRET response *in vitro* ($F_{eq\ in\ vitro}$):

$$F_{\mathrm{eq}\ in\ vitro} = \frac{R_{cell} - R_{0\ in\ vitro}}{R_{0\ in\ vitro}} = \frac{R_{0\ cell} * (F + 1) - R_{0\ in\ vitro}}{R_{0\ in\ vitro}} \quad \rightarrow$$

$$F_{\mathrm{eq}\ in\ vitro} = \frac{\left[\left(\frac{F_{ABS} + 1}{F_{MAX} + 1}\right) * R_{0\ in\ vitro}\right] * (F + 1) - R_{0\ in\ vitro}}{R_{0\ in\ vitro}} \quad \rightarrow$$

$$\boldsymbol{F_{\text{eq } in\ vitro} = \left(\frac{F_{ABS}+1}{F_{MAX}+1}\right) * (F+1) - 1} \qquad \textbf{(Eq. 5)}$$

The Hill equation describing the *in vitro* FRET response of the probe to different cAMP concentrations is:

$$\boldsymbol{F_{\text{eq } in\ vitro} = \frac{F_{ABS} * [cAMP]^n}{EC_{50}{}^n + [cAMP]^n} = \frac{F_{ABS}}{1 + \left(\frac{EC_{50}}{[cAMP]}\right)^n}} \qquad \textbf{(Eq. 6)}$$

where EC_{50} = the concentration of cAMP that produces half-maximal activation of the probe and n is the Hill coefficient.

Based on Eq. 5 and Eq. 6 we can derive Eq. 7 to calculate the concentration of cAMP that produces a given FRET response in an intact cell (F):

$$[cAMP] = EC_{50} * \left(\frac{F_{\text{eq } in\ vitro}}{F_{ABS} - F_{\text{eq } in\ vitro}}\right)^{1/n} \rightarrow$$

$$[cAMP] = EC_{50} * \left(\frac{(F_{ABS}+1) * (F+1) - F_{MAX} - 1}{F_{ABS} * (F_{MAX}+1) - [(F_{ABS}+1) * (F+1) - F_{MAX} - 1]}\right)^{1/n} \rightarrow$$

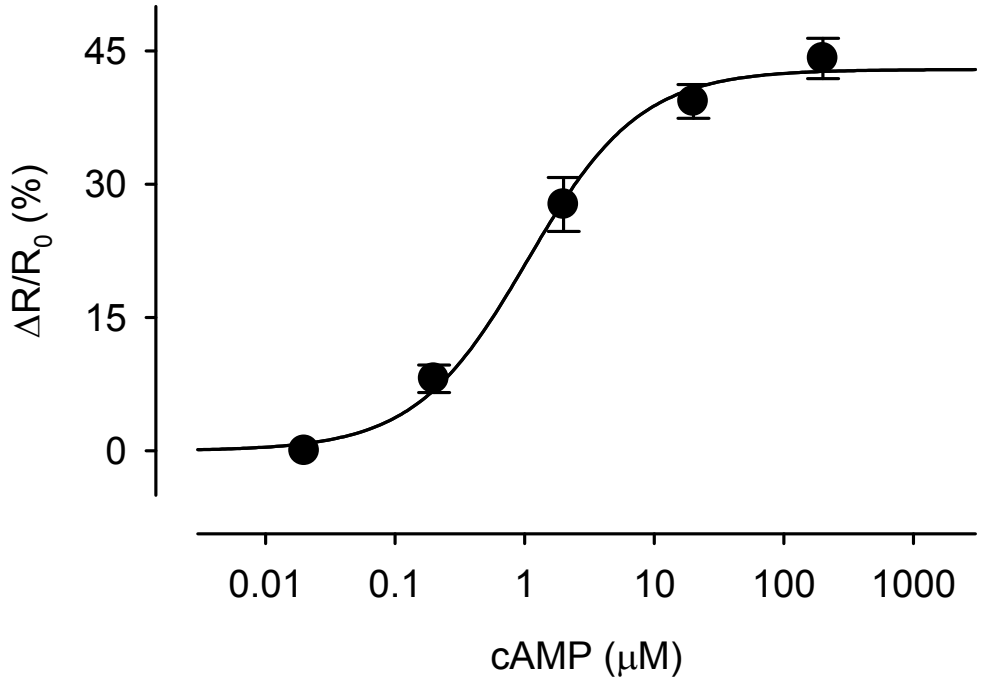
$$\boldsymbol{[cAMP] = EC_{50} * \left(\frac{F_{ABS} * (F+1) + F - F_{MAX}}{F_{ABS} * (F_{MAX} - F) - F + F_{MAX}}\right)^{1/n}} \qquad \textbf{(Eq. 7)}$$

Supplement Table 1: MODEL PARAMETERS			
Parameter	***Value***	***Units***	***Description***
$R_{\beta 1TotalCav}$	0.633	μM	concentration of β_1R in Cav compartment
$R_{\beta 1TotalEcav}$	1.098	μM	concentration of β_1R in Ecav compartment
$R_{\beta 1TotalCyt}$	0.603	μM	concentration of β_1R in Cyt compartment
K_H	0.062	μM	high affinity binding constant between ligand and receptor
K_L	0.567	μM	low affinity binding constant between ligand and receptor
K_C	8.809	μM	affinity binding constant between free receptor and G protein
$R_{M2TotalCav}$	0.506	μM	concentration of M_2R in Cav compartment
$R_{M2TotalEcav}$	0.506	μM	concentration of M_2R in Ecav compartment
$R_{M2TotalCyt}$	0.301	μM	concentration of M_2R in Cyt compartment
K_H	0.16	μM	high affinity binding constant between ligand and receptor
K_L	11	μM	low affinity binding constant between ligand and receptor
K_C	30	μM	affinity binding constant between free receptor and G protein

$G_{sTotalCav}$	10	μM	concentration of G_s protein in Cav compartment
$G_{sTotalEcav}$	10	μM	concentration of G_s protein in Ecav compartment
$G_{sTotalCyt}$	10	μM	concentration of G_s protein in Cyt compartment
$G_{iTotalCav}$	20	μM	concentration of G_i protein in Cav compartment
$G_{iTotalEcav}$	1	μM	concentration of G_i protein in Ecav compartment
$G_{iTotalCyt}$	10	μM	concentration of G_i protein in Cyt compartment
k_{act1Gi}	0.1	s^{-1}	activation rate constant for RG_i complexes
k_{act2Gi}	5	s^{-1}	activation rate constant for LRG_i complexes
k_{act1Gs}	0.1	s^{-1}	activation rate constant for RG_s complexes
k_{act2Gs}	5	s^{-1}	activation rate constant for LRG_s complexes
k_{hydrGi}	0.8	s^{-1}	hydrolization rate constant of $G_{i\alpha\text{-}GTP}$
k_{reasGi}	$1.21*10^3$	$s^{-1}\ \mu M^{-1}$	reassociation rate constant of $G_{i\alpha\text{-}GDP}$ and $G_{\beta\gamma}$
k_{hydrGs}	0.8	s^{-1}	hydrolization rate constant of $G_{s\alpha\text{-}GTP}$

k_{reasGs}	$1.21*10^3$	$s^{-1}\ \mu M^{-1}$	reassociation rate constant of $G_{s\alpha\text{-}GDP}$ and $G_{\beta\gamma}$
$AC_{5/6\text{-}Cav}$	3.379	μM	concentration of Cav $AC_{5/6}$
$AC_{5/6\text{-}Cyt}$	0.126	μM	concentration of Cyt $AC_{5/6}$
ATP	$5 * 10^3$	μM	concentration of ATP (constant)
K_{mATP}	315	μM	$AC_{5/6}$ K_m for ATP
$AF_{5/6}$	500	$\frac{\text{mg purified protein}}{\text{mg membrane protein}}$	amplification factor for $AC_{5/6}$
$MW_{AC5/6}$	130	Kda	molecular weight of $AC_{5/6}$
$AC_{4/7\text{-}Ecav}$	0.200	μM	concentration of Ecav $AC_{4/7}$
$AC_{4/7\text{-}Cyt}$	0.006	μM	concentration of Cyt $AC_{4/7}$
$AF_{4/7}$	130	$\frac{\text{mg purified protein}}{\text{mg membrane protein}}$	amplification factor for $AC_{4/7}$
$MW_{AC4/7}$	130	KDa	molecular weight of $AC_{4/7}$
K_{mPDE2}	50	μM	PDE2 Km for cAMP
K_{mPDE3}	0.08	μM	PDE3 Km for cAMP
K_{mPDE4}	2.2	μM	PDE4 Km for cAMP
k_{PDE2}	20	s^{-1}	rate constant for PDE2
k_{PDE3}	1.25	s^{-1}	rate constant for PDE3
k_{PDE4}	2.5	s^{-1}	rate constant for PDE4

PDE_{2Cav}	4.5	μM	PDE2 concentration in Cav compartment
PDE_{2Ecav}	0.002	μM	PDE2 concentration in Ecav compartment
PDE_{2Cyt}	0.068	μM	PDE2 concentration in Cyt compartment
PDE_{3Cav}	5.6	μM	PDE3 concentration in Cav compartment
PDE_{3Cyt}	0.113	μM	PDE3 concentration in Cyt compartment
PDE_{4Cav}	2.0	μM	PDE4 concentration in Cav compartment
PDE_{4Ecav}	0.01	μM	PDE4 concentration in Ecav compartment
PDE_{4Cyt}	0.027	μM	PDE4 concentration in Cyt compartment
$J_{Cav/Ecav}$	$5 * 10^{-15}$	Liters * s^{-1}	flux rate between Cav and Ecav compartments
$J_{Cav/Cyt}$	$7.5 * 10^{-14}$	Liters * s^{-1}	flux rate between Cav and Cyt compartments
$J_{Ecav/Cyt}$	$0.9 * 10^{-14}$	Liters * s^{-1}	flux rate between Ecav and Cyt compartments



Supplement Figure 1. Concentration dependence of the cAMP-dependent change in Epac2-camps CFP/YFP emission intensity ratio ($\Delta R/R_0$) observed *in vitro (see Methods)*. Parameters of fit to data points: EC_{50}, 1.1 μM, Hill coefficient, 1, maximum FRET response 43%.