Terrin et al., http://www.jcb.org/cgi/content/full/jcb.200605050/DC1

Fig. S1 shows the evaluation of receptor desensitization. HEK293 cells expressing the H30 sensor were subjected to repeated stimulation with either 10 μM PGE_1 or 10 μM isoproterenol in a perfusion chamber. Between each stimulus, the drug was washed away by perfusing with a saline solution (see Materials and methods). Fig. S1 reports the kinetics of the normalized FRET changes recorded as described in Materials and methods and in the aforementioned conditions. It clearly shows that although the β-adrenergic receptors undergo a robust and rapid desensitization (the FRET change is reduced by ∼75% at 12 min after the application of the first stimulus) as expected, the prostaglandin receptor does not show any desensitization at 12 min (second application of PGE_1), and, even after repeated stimulations and at ∼40 min after the first application of the stimulus, the cAMP response is only marginally reduced (by ∼25%).

Fig. S2 shows the evaluation of the dose-response relationship between PKA overexpression and steepness of the cAMP gradient. One of the findings in this study is that PKA has a major role in regulating the steepness of the cAMP gradient via modulation of PDEs activity. If this conclusion is correct, it should be possible to correlate the level of expression of PKA and the consequent activation of PDEs with the steepness of the gradient. In our experimental setup, we define the cAMP gradient by measuring FRET changes in individual compartments on a single cell basis. Therefore, it is impossible to correlate such changes in cAMP with PKA concentrations determined biochemically. To try to correlate the level of PKA expression in a single cell with the steepness of the cAMP gradient measured in the same cell, we took advantage of the GFP-tagged PKA (the PKA-GFP sensor) and estimated the level of overexpression of PKA in the individual cell by measuring the level of fluorescence intensity caused by the expression of PKA-GFP in the same cell. We reexamined the experiments performed on cells expressing GFP-PKA and divided these cells into two groups: a group of dim cells (fluorescence intensity of <500 arbitrary units) overexpressing low levels of PKA-GFP and a group of bright cells (fluorescence intensity of >500 arbitrary units) expressing high levels of PKA-GFP. When we compared the steepness of the cAMP gradient between the subplasma membrane compartment (*mp*PKA) and the bulk cytosol (PKA) in dim versus bright cells, we found that the gradient was steeper in the case of bright cells (expressing higher levels of PKA) as compared with dim cells (expressing low levels of PKA). In fact, as shown in Fig. S2, the cAMP response in the bulk cytosol of dim cells is about half the cAMP response at the plasma membrane ($\Delta R/R_0 = 4.05 \pm 0.9$ [$n = 22$] and $8.2 \pm 1.5\%$ [$n = 34$], respectively; $P = 0.04$), whereas the cAMP response in the bulk cytosol of bright cells is about one third of the cAMP response at the plasma membrane ($\Delta R/R_0 = 2.64 \pm 1.2$ [$n = 11$] and $8.2 \pm 1.5\%$ [$n = 34$], respectively; $P = 0.03$).

Determination of the velocity of FRET change for the cAMP sensors

To compare the speed of FRET change upon cAMP binding to the cAMP sensors, we measured the ΔFRET/Δt changes in the initial linear phase of the FRET response in cells expressing PKA-GFP, *mp*PKA-GFP, H30, *mp*H30, or *nls*H30 and in which 64 μM cAMP

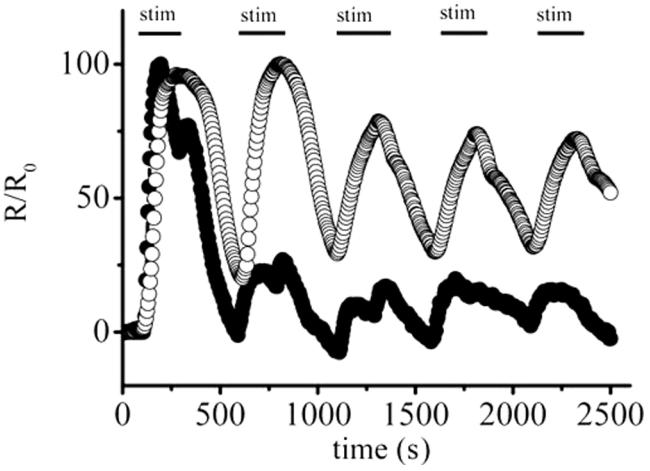

Figure S1. **Evaluation of PGE_1 receptor desensitization.** HEK293 cells expressing the H30 sensor were subjected to repeated stimulation (stim) with either 10 μM PGE_1 (open circles) or 10 μM isoproterenol (closed circles) in a perfusion chamber. Between each stimulus, the drug was washed away by perfusing with a saline solution (see Materials and methods).

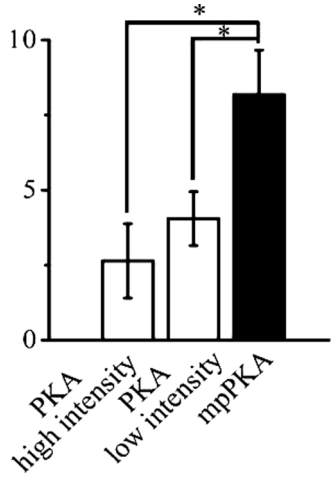

Figure S2. **Evaluation of the dose-response relationship between PKA overexpression and steepness of the cAMP gradient.** FRET change recorded upon PGE_1 stimulation in HEK cells expressing GFP-PKA at a high level (PKA high intensity = fluorescence intensity of >500 arbitrary units), expressing low levels of PKA-GFP (PKA low intensity = fluorescence intensity of <500 arbitrary units), or expressing the membrane-targeted sensor *mp*PKA-GFP. Error bars represent SEM. *, $P < 0.04$.

was injected with a patch pipette (see Materials and methods). We found that the velocity of FRET response for the PKA-GFP sensor was identical to the velocity of FRET response showed by the *mp*PKA-GFP sensor ($\Delta FRET/\Delta t = 0.020 \pm 0.005$ [$n = 10$] and 0.020 ± 0.009 R/R_0s^{-1} [$n = 6$], respectively; $P = 0.8$). On the contrary, we found that the velocity of FRET response for *nls*H30 ($\Delta FRET/\Delta t = 0.027 \pm 0.001$ R/R_0s^{-1}; $n = 6$) was significantly higher ($P = 0.004$) as compared with H30 ($\Delta FRET/\Delta t = 0.011 \pm 0.002$ R/R_0s^{-1}; $n = 10$) and *mp*H30 ($\Delta FRET/\Delta t = 0.0092 \pm 0.002$ R/R_0s^{-1}; $n = 10$; $P = 0.0001$). The difference between H30 and *mp*H30 was not significant ($n = 10$; $P = 0.5$). As a consequence, a direct comparison of the kinetics of FRET changes recorded in the nuclear compartment with the cytosolic or subplasma membrane compartments using the Epac-based sensors is not possible. However, the difference in response kinetics does not affect the value of peak response recorded by the two sensors in the cytosolic and subplasma membrane compartment, as these are steady state measures. On the contrary, the kinetics of FRET changes recorded at the plasma membrane and in the bulk cytosol can be directly compared when both reported by the PKA-based sensors and the Epac-based sensors. It is interesting to note that by applying a high concentration of cAMP, PKA-GFP and H30 show the same velocity of FRET change as previously reported in a comparison between PKA- and Epac-based probes (Nikolaev et al., 2004).

Dynamics interactions between mpRII-CFP and C-YFP upon cAMP binding and release

When cells overexpress *mp*PKA-GFP, both the RII-CFP and the C-YFP subunits appear to be predominantly targeted at the plasma membrane in the presence of basal levels of cAMP. As shown in Video 1, upon the addition of 25 μM forskolin, C-YFP is completely mobilized from the plasma membrane and uniformly diffuses into the cytosol, as expected in the presence of increasing intracellular [cAMP]. Such mobilization of C-YFP is completely reversible upon removal of the stimulus, indicating that *mp*PKA-GFP appropriately responds by dissociation and reassociation of its R and C subunits to changes in [cAMP]. RII-CFP remained localized at the plasma membrane for the entire duration of the experiments (unpublished data).

Reference

Nikolaev, V.O., M. Bunemann, L. Hein, A. Hannawacker, and M.J. Lohse. 2004. Novel single chain cAMP sensors for receptor-induced signal propagation. *J. Biol. Chem.* 279:37215–37218.