Cell swelling enhances GPCR ternary complex formation, underpinning the potentiation of β2 adrenergic receptor-mediated cAMP response

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Abstract

G protein-coupled receptors conformational landscape can be reportedly affected by their local, microscopic interactions within the cell plasma membrane. A pleiotropic stimulus to alter the cortical environment within intact cells, namely osmotic swelling, is employed here to monitor the response in terms of receptor function and downstream signaling. We observe that in osmotically swollen cells the β2-Adrenergic receptor, a prototypical GPCR, favors an active conformation, resulting in cAMP transient responses to adrenergic stimulation that have increased amplitude. The results are validated in primary cell types such as adult cardiomyocytes, a relevant model where swelling occurs upon ischemia-reperfusion injury. Our results suggest that receptors function is finely modulated by their biophysical context, and specifically that osmotic swelling acts as a potentiator of downstream signaling, not only for the β2-Adrenergic receptor, but also for other receptors, hinting at a more general regulatory mechanism.

Introduction

Further to the development of the ternary complex model, our canonical understanding of G protein-coupled receptors' (GPCRs) function has progressively evolved from that of two-state switches, to rheostats exploring a rich conformational landscape, that in turns entails a broad repertoire of intracellular interactions[1]. A prototypical example are molecules from the extracellular space acting as allosteric modulators of receptor function: they favor a specific set of conformations -further stabilized upon ligand binding- that is different from those conformations that would be observed in the absence of the modulator[2]. An emerging concept is that changes of the biophysical context around the receptor can 'allosterically' modulate its function. Mechanistically this involves the action of accessory proteins[3], local lipid composition[4], interaction with intracellular scaffolds[5], and -as highlighted even more recently- local curvature[6]. These interactions can modulate each of the canonical signaling steps (ligand binding, ternary complex formation with cognate G protein, β-arrestin recruitment, trafficking), endowing these receptors with a broad modulatory repertoire able to nuance specific signals in a dynamic and flexible way in space and time.

Some of these interactions may be receptor-specific and associated with specific modulatory proteins, such as Receptor Associated Modifying Proteins (RAMPs)[3]. Other interactions may be more diffuse, such as the cell-wide interaction of GPCRs containing a PDZ binding sequence with the cortical actin, or, as it was proposed more recently, the segregation of

selected GPCRs to areas of higher or shallower curvature within the cell membrane[7]. Suggestively, we observed recently µm-sized receptor-specific segregation to a subcellular compartment, such as the T-tubular network of adult Cardiomyocytes, characterized by high local curvature[8].

While the hypothesis that subcellular receptors localization may indeed modulate -to a sizable extent- their behavior[9] is being increasingly tested, nonetheless the molecular mechanisms underpinning such effects are harder to unravel. These may be ultimately ascribed to heterogeneities in lipid composition of the plasma membrane[10], in the local density of the cortical actin network and the variability of its interactions with the membrane[11]. Moreover, if modulation of GPCR signaling indeed occurs across multiple length-scales at the plasma membrane, from nanometer to micrometer scale, its microscopic identification challenges current technologies[12].

We reasoned that osmotic swelling, a process that occurs in several physiological settings may offer the type of pleiotropic biophysical modulation that we are seeking to test this hypothesis. Cell swelling is associated to a complex rearrangement of the cell membrane environment, ranging from alteration of local geometry, F-actin remodulation and ultimately, increases in cell surface largely originating from flattening of the membrane, not unlike ironing out wrinkles in a cloth. We decided to focus on β-Adrenergic Receptors (β-ARs), both because of their prototypical nature (i.e. they couple to the stimulatory G protein and elicit an increase in cAMP upon Gαs interaction with the Adenylate Cyclase) but also because they display a reportedly rich conformational landscape[13].

Our key observation is that swollen cells yield sizably higher cAMP concentrations in response to β-AR than non-swollen cells. To pinpoint at what step this occurs, we set to explore the whole signaling cascade of the β2-AR. Each step of the signaling cascade was investigated with a specific fluorescent spectroscopy method, ranging from TIRF to fluorescence polarization and FRET biosensing, that allowed us working in intact cells. We could determine that this effect does not arise from alterations in Adenylate Cyclase activity (i.e. the enzyme responsible for converting ATP in cAMP), nor by alterations of receptors trafficking, but rather by a distinctive increase in receptor-G protein coupling, mirrored by an increased affinity of the agonist isoproterenol for the receptor.

These findings demonstrate a novel way of modulating GPCR conformation by biophysical factors, while also showing a previously unappreciated effect of cell swelling on 2nd messenger production, which is potentially transferable to other GPCRs and transmembrane receptors, and thus relevant in a broad range of physiological settings.

Results

In order to size the effect of osmotic swelling on the downstream signalling cascade of the β-ARs we first employed cAMP FRET fluorescence biosensors. The layout of our readout is schematically depicted in **Figure 1a**. If the experiment is conducted at the level of a single cell under a microscope, this means that a false-color image reflecting the degree of FRET between the donor and acceptor of the sensor will change upon agonist addition (100 pM Isoproterenol (Iso) in our case). The relative change between the swollen and control state of representative HEK293 cell is displayed in **Figure 1b**, together with the separate donor and acceptor channels. It is clear that upon addition of the swelling medium (containing the same concentration of Iso as the isotonic medium) to a cell expressing endogenous levels of receptors, the cAMP response is sizably increased. To achieve statistical significance, we then moved to a high-throughput configuration where the signal from tens of thousands of cells was observed in 96-well plates using a fluorescent plate reader. A representative trajectory of intracellular cAMP concentration (proportional to the change in normalized FRET ratio) upon Iso addition in control vs swollen cells is displayed in **Figure 1c**. Trajectories from swollen cells are displayed in blue throughout the manuscript, as opposed to cells maintained in isotonic medium (control) which are displayed in red. A strong peak in cAMP concentration is observed in the swollen cells a few minutes upon non-saturating Iso addition, which then declines to a steady state comparable to the concentration observed in the controls (**Supplementary Figure 1a**). Saturating cAMP levels are then obtained upon Fsk/IBMX stimulation for normalization purposes. When repeated for increasing concentrations of the agonist, ranging from pM up to 100 nM concentration, it was thus possible to build a concentration response curve, as displayed in **Figure 1d**. We observe a statistically significant difference in the log(EC50), which drops from -9.3 ± 0.1 to -9.84 ± 0.09 for the average curves. To rule out any effect of osmotic swelling on the readout of our fluorescence biosensor, a concentration response curve was calculated in the two conditions using the cell permeable cAMP moiety 8-Br-cAMP, displaying overlapping curves for swollen and control cells (**Supplementary Figure 1b**). Individual log(EC50) values from separate experiments are displayed for the two conditions in **Figure1e**, which in turn allows to appreciate the fact that the observed difference applies only to the transient cAMP response and not to the steady state concentration. Interestingly, similar results can be obtained when looking at cells stimulated with other β-Adrenergic agonists, such as Salbutamol, Salmeterol and Terbutaline (**Supplementary Figure 1 c-e**). When challenging other Gs coupled receptors such as the Histamine H1 receptor or the MC4R receptor **(Supplementary Figure 1 f-g)** a similar outcome in terms of cAMP response can be observed upon osmotic swelling. Moreover, a similar behavior is reported for the μ Opioid Receptor upon stimulation with the synthetic agonist Damgo (**Supplementary Figure 1 i-j)**.

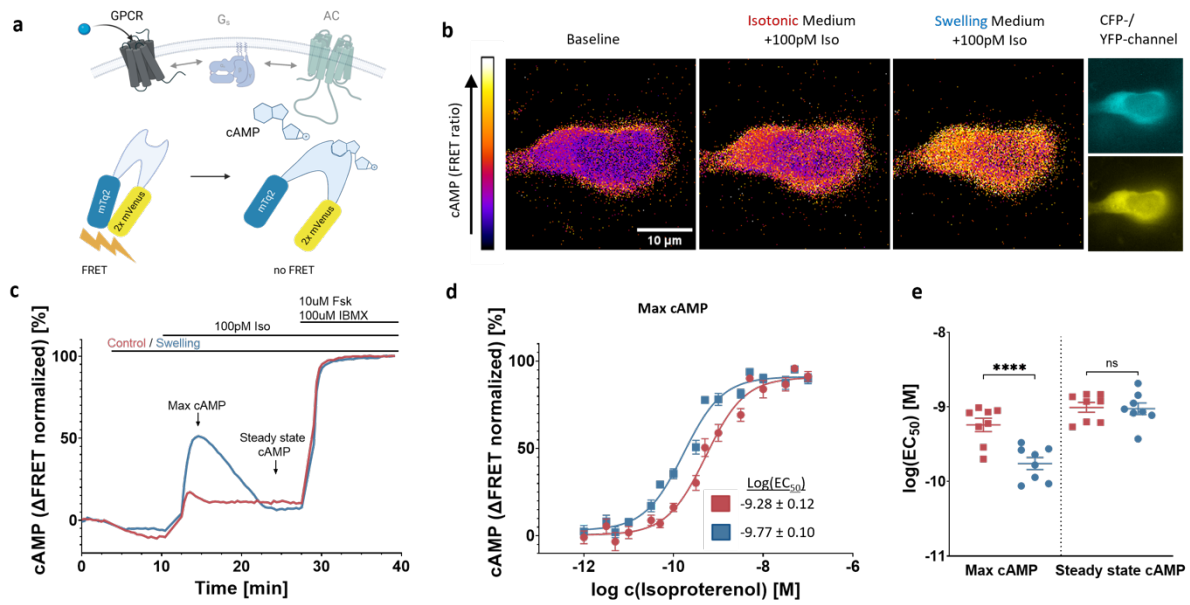

Figure 1 Cell swelling increases β-adrenergic-mediated cAMP production in HEK293 cells.

a Schematics of GPCR-mediated cAMP production and its detection by a FRET biosensor; **b** Representative images of a HEK293 cell stably expressing cytosolic Epac1^{H187s} [14] showing acceptor/donor ratio in false colour; the cells are exposed to 100 pM isoproterenol first in isotonic (300 mOsm) and subsequently in swelling medium (200 mOsm); **c** representative curve showing kinetics of acceptor/donor ratio measured in HEK293T cells stably expressing Epac1^{H187s} in a microplate reader (normalized to baseline and 10µM Forskolin + 100µM IBMX); **d** averaged concentration response curves representing maximal cAMP concentrations, as indicated in (**c**) (mean ± SEM; n = 6 independent experiments); **e** $\log EC_{50}$ values from individual experiments comparing swelling and control condition for maximal and steady state cAMP concentrations (statistical analysis performed by a paired two-tailed t-test).

We then addressed the question if this remarkable behavior was observed also in primary cells, that are known to be subject to osmotic swelling in physiological or pathophysiological conditions, namely adult cardiomyocytes (CM). CMs undergo swelling during the reperfusion phase following ischemic shock[15]. Murine adult ventricular CMs were isolated and seeded on multiwell imaging plates and imaged on the same day, after labeling using a cell membrane dye (**Materials and Methods)**. Upon exposure to the swelling media the CMs display a visible increase volume as illustrated in **Figure 2a**. The increase was quantified as the relative change in cell area, as detected in 2D confocal sections (**Figure 2b, Supplementary Figure 2a,b and Supplementary Movie 1).** Following the same approach used to generate the data in **Figure 1b**, we employed CMs isolated from a transgenic mouse expressing the EPAC1-cAMPs cAMP sensor[16,17] and imaged them under a microscope (**Supplementary Movie 2**). The relative changes in cAMP concentration upon agonist addition were quantified for each cell, and representative traces for a control vs a swollen CMs are displayed in **Figure 2c**. Upon osmotic swelling, the cAMP response to non-saturating concentrations of Iso is clearly enhanced, consistent with the observations reported in **Figure 1** for HEK293 cells.

Notably, in the swollen CMs there is a significant increase of cAMP relative to control conditions also upon application of saturating concentrations of Iso. The data for 10 nM and 10 µM iso additions are summarised in **Figure 2d**, clearly illustrating that cAMP signalling is

strongly enhanced in swollen adult CMs for Iso stimulations around the reported EC50, while being equal at saturating concentration. Albeit it was not possible to generate a full concentration response curve based on single cell images, these data point to an effect consistent with the one clearly displayed in **Figure 1d**. Overall, these results point to the potential physiological relevance of this mechanism.

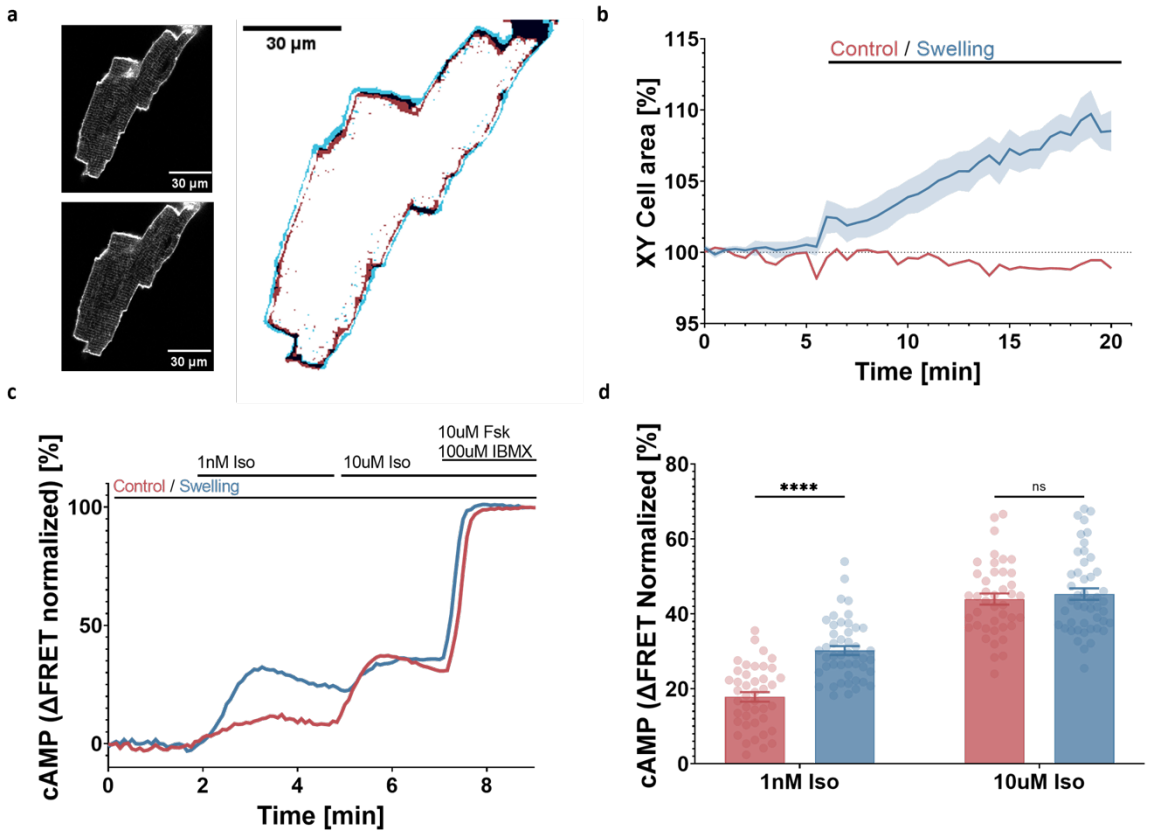

Figure 2 Cell swelling increases β-adrenergic-mediated cAMP production in adult mouse ventricular cardiomyocytes (CM)

a Confocal images of CM under isotonic conditions (upper inset) and after 20 minutes of exposure to swelling medium (lower inset), with an overlay of cell edges under both conditions (main); **b** average curve showing the area of a confocal CM slice (mean ± SEM; n = 7 cells from 3 independent experiments); **c** representative curve showing kinetics of acceptor/donor ratio measured in CM stably expressing Epac1-Camps under an epifluorescence microscope (normalized to baseline and 10µM Forskolin + 100µM IBMX); **d** max cAMP concentrations reached upon stimulation with 1nM and 10µM isoproterenol respectively, normalized to 10µM Forskolin + 100µM IBMX (mean ± SEM; n = 42 (control) and 47 (swelling) cells from 3 independent experiments; statistical analysis performed by an unpaired two-tailed t-test).

We thus set out to identify the molecular determinants of the observed changes in cAMP response in swollen cells. Since swelling is a pleiotropic modulator of cell homeostasis, we systematically investigated all steps of the signalling cascade, from PDE-mediated degradation of cAMP up to G protein coupling. We have graphically summarised these steps in **Figure 3a**, that acts as a legend to the other panels of **Figure 3**. We first set to rule out that impaired receptor trafficking, namely desensitization or downregulation supported by clathrin-mediated endocytosis was at the origin of the observed effect[18]. We treated cells with Dyngo4a, a well characterised dynamin inhibitor (see **Materials and Methods**). **Figure 3b**

illustrates that the transient cAMP production increase reported in **Figure 1c** is still present in HEK293 cells treated with 10 μM Dyngo4a (the full concentration response curve is displayed in **Supplementary Figure 3a**). We then investigated the potential role of altered phosphodiesterase activity[19], as altered degradation rates (in this case reduced) may account for the observed increase in cAMP concentration transients in swollen cells. The addition of the pan-PDE inhibitor IBMX at the saturating concentration of 100 μM did not abolish the observed relative increase in transient cAMP concentration in the swollen cells, as also illustrated in **Supplementary Figure 3b**. Similarly, the localization of the sensor did not seem to affect the outcome, since a membrane bound cAMP sensor[20] also confirmed the observed effect (**Supplementary Figure 3c**). We thus moved upstream along the signalling cascade: the next target is the enzyme responsible for cAMP synthesis, namely the Adenylate Cyclase (AC). AC is canonically activated by the GTP-bound Gαs subunit of the heterotrimeric G protein. However, the diterpene Forskolin and Gαs have a synergistic/cooperative action in activating ACs. In cells possessing endogenous Gαs, direct stimulation of AC by Forskolin leads to obvious cAMP concentration increases, and the differential effect elicited by cell osmotic swelling persists (**Figure 3d and Supplementary Figure 3d**). However, interestingly, in cell lines carrying a Gs knockout[21], we observed that we lose any effect of osmotic swelling upon the amplitude or kinetics of the cAMP concentration increase (**Figure 3e and Supplementary Figure 3e**). The effect is however recovered upon heterologous transfection of Gs in the knockout lines (**Figure 3f and Supplementary Figure 3f**), thus pinning on the alpha subunit of the heterotrimeric G protein a key role in modulating the signalling cascade's response to osmotic swelling. The overall results are summarised as log(EC50) values shifts in **Figure 3f**.

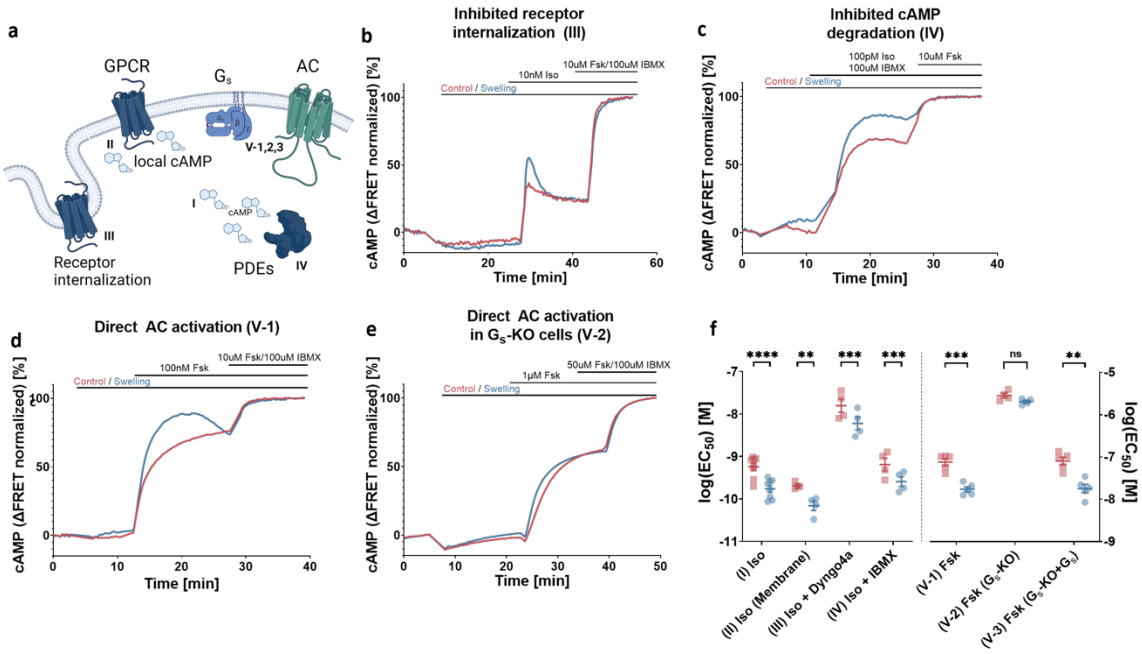

Figure 3 Impact of cell swelling on the molecular steps of the β-adrenergic signalling cascade a Schematics of key steps of the signalling cascade that may be affected by swelling; **b** representative curve of intracellular cAMP concentration under inhibition of receptor internalization activity by 10 μM Dyngo4a; **c** representative curve of intracellular cAMP concentration under inhibition of PDE activity by 100 μM IBMX; **d** representative curve of intracellular cAMP concentration upon direct stimulation of ACs by 100nM Forskolin; **e** representative curve of intracellular cAMP concentration upon direct stimulation of ACs by 1μM Forskolin in a Gs knockout cell line transiently transfected with Epac1-S^{H187s}; **f** $logEC_{50}$

values from individual experiments comparing swelling and control condition for maximal cAMP concentrations under following conditions: (I) cytosolic cAMP upon Iso stimulation, (II) local cAMP at the plasma membrane upon Iso stimulation measured with an Epac-Camps1-Caax sensor, (III) cytosolic cAMP measured upon Iso stimulation after treatment with 30μM Dyngo4a for 30 min, (IV) cytosolic cAMP measured upon Iso stimulation with simultaneous addition of 100μM IBMX, (V-1,2,3) cytosolic cAMP measured upon Fsk stimulation in wt Gs phenotype (V-1), Gs-KO cells (V-2) and Gs-KO cells transiently transfected with Gsα (V-3) (statistical analysis performed by a paired two-tailed t-test).

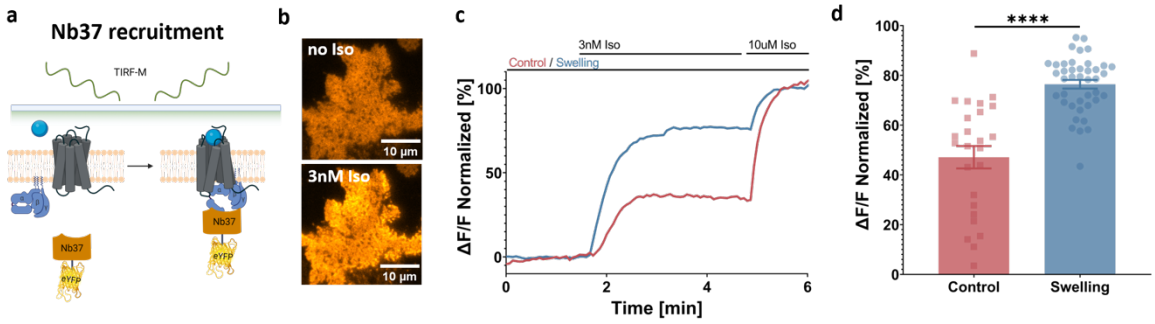

Figure 4 Swelling leads to an increased effector recruitment

a Gs activation by the β2-AR is monitored by recruitment of the fluorescently tagged nanobody 37 (Nb37) at the plasma membrane of the cell together with overexpression of Gs; **b** representative TIRF-M images of Nb37-eYFP expressed in HEK293-AD cells before and after application of 3 nM isoproterenol; **c** representative curve showing relative increase of membrane fluorescence collected as first 3nM, and then at saturating 10μM concentration of Isoproterenol is added to the cell; **d** relative fluorescence increase measured upon Nb37-eYFP recruitment upon 3nM isoproterenol stimulation in swollen vs non-swollen cells (mean ± SEM; n = 25 (control) and 41 (swelling) cells from 3 independent experiments; statistical analysis performed by an unpaired two-tailed t-test).

Based on these observations we decided to investigate further the role of Gs in mediating this remarkable response of drug-stimulated cAMP transients to cell osmotic swelling. In order to do so we first asked the question if Gs recruitment to the receptor is enhanced in swollen cells. This question was addressed exploiting fluorescently tagged Nb37[22], reported to bind the active β2-Gs ternary complex, as schematically illustrated in **Figure 4a**. After co-transfecting HEK293 cells with β2-AR, the heterotrimeric G protein and Nb37-eYFP (as discussed in **Materials and Methods**) we imaged their basolateral membrane using Total Internal Reflection Fluorescence[23], in order to reject as much as possible, the fluorescence signal from the cytosolic Nb37-eYFP and to enhance the membrane signal. This approach allowed us to monitor the membrane recruitment of Nb37-eYFP upon increasing concentrations of Iso (**Figure 4b, Supplementary movie 4.1**), and in turn confirm that in swollen cells the recruitment is enhanced (**Figure 4c**). Overall, the relative recruitment of Nb37 is 25% higher in swollen cells than in the control (**Figure 4d**). This suggests that in swollen cells there is a sizably higher proportion of active ternary complexes, and that osmotic swelling is thus a modulator of the receptor active conformation.

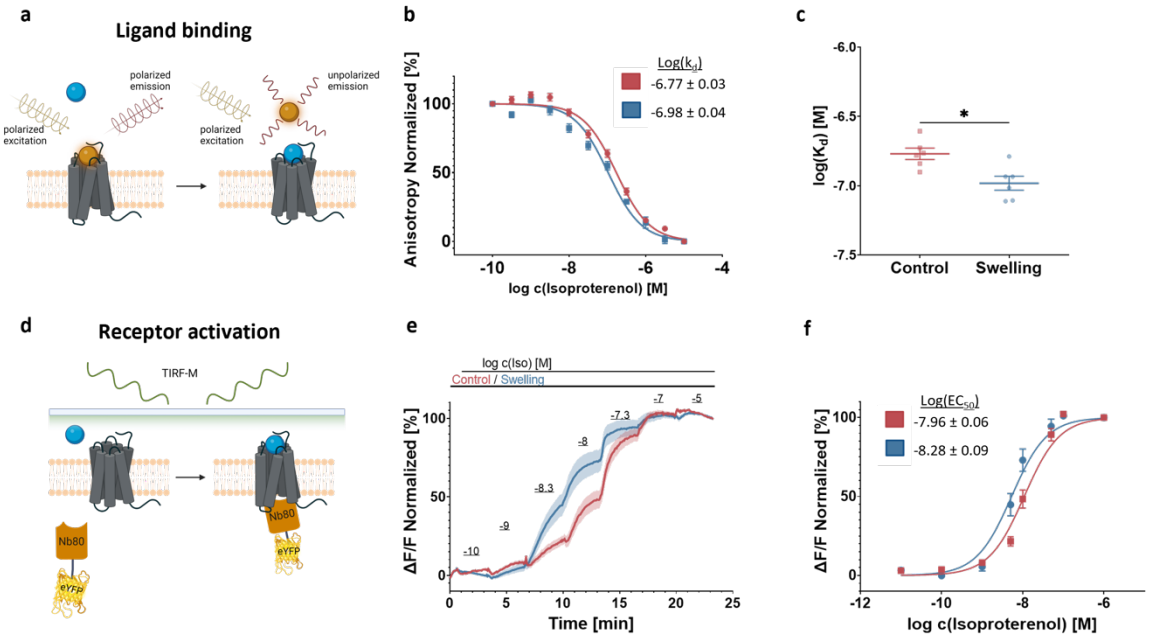

Figure 5 Swelling favors the active receptor conformation

a Ligand binding affinity is determined by employing a fluorescently labelled ligand and measuring the degree of fluorescence anisotropy; **b** average curve of increasing concentrations of isoproterenol displacing 2 nM of fluorescently labelled JE1319 from the β2-AR in swollen and non-swollen cells with the corresponding Kd values **c** scatter plot comparing Kd values obtained from single binding experiments (for **b** and **c** - mean ± SEM; n = 9 plates from 5 independent experiments; statistical analysis by a paired two-tailed t-test) **d** conformational activation of the β2-AR is monitored by recruitment at the plasma membrane of the cell by employing the fluorescently tagged nanobody 80 (Nb80); **e** averaged time-sequence of the relative increase of membrane fluorescence collected as increasing concentration of Isoproterenol are added to the biosensor; **f** the resulting concentration response curves for cells in isotonic and swelling media and associated Log(EC_{50}) values (for **e** and **f** - mean ± SEM; n = 46 (control) and 45 (swelling) cells from 5 independent experiments).

If osmotic swelling indeed favors the formation of a ternary ligand-receptor-Gs complex, we can reasonably expect this to be reflected in a direct modulation of receptor conformation[24]. In order to test for this possibility, we conducted a set of assays aimed at monitoring ligand binding and G protein recruitment at the receptor in intact cells. We employed a recently reported fluorescent ligand against the β-ARs, based on the inverse agonist carazolol[8], to conduct a set of fluorescence anisotropy binding assays[25], as graphically illustrated in **Figure 5a**. As the fluorescence anisotropy increases proportionally to the fraction of bound ligand, it is possible to incubate cells overexpressing β2-AR as well as Gs and Nb37 with a fixed concentration of fluorescent ligand, and then measure changes in fluorescence anisotropy as the fluorescent ligand is progressively displaced by increasing concentrations of the non-fluorescent Iso (**Figure 5b** and **Supplementary Figure 4**). When comparing swollen and control cells, this leads to a small but significant shift in the ligand Kd, from Log(Kd)=-6.77 ± 0.03 to -6.98 ± 0.04 (**Figure 5c**). On the intracellular side the active receptor conformation can be probed by using fluorescently tagged Nb80 (Nb80-eYFP)[26], as schematically described in **Figure 5d**. By employing TIRF to measure nanobody recruitment, we can observe that there is a significant enhancement in Nb80 recruitment in swollen cells (**Figure 5e**), as also reflected by the full concentration response curve's (Log(EC50)) shifts from –7.96 ± 0.06 to –8.28 ±

0.09 (**Figure 5g**). Altogether these data confirm that osmotic swelling modulates the β2-AR conformational landscape, favouring an active conformation that in turn leads to an enhanced downstream cAMP cascade.

Discussion

The canonical GPCR signaling cascade, from the ternary receptor complex model down to the more recent structural determination of receptor conformational states, relies on the assumption that the receptor-G protein signalling complex is forming in relatively aseptic, unperturbed conditions. Cartoons displaying GPCR signalling in textbooks highlight the receptor on the membrane, possibly the ligand, and then the downstream interaction partner, either the heterotrimeric G protein or, increasingly so, β-Arrestins. On the other hand, GPCRs are exposed to hundreds, if not thousands of intracellular interactions with other proteins, some already well documented. While the reductionist approach leading to the 'two-states' switch model has been instrumental in our understanding of the function of this important family of receptors, this in turn has allowed to reach a point where it is now possible to begin investigating the modulatory effects of other, less described, interactions.

In particular, the intracellular domain of plasma membrane GPCRs is exposed to the cell cortical environment and its distinct organization[27]. In this work we decided to investigate a more general mechanism that can fundamentally alter the cortical environment and at the same time has a role in several physiological processes, ranging from cardiac ischemia reperfusion[15] to renal function[28], namely osmotic swelling. In our hands, osmotic swelling became a tool to induce pleiotropic changes of the cell-wide membrane environment, to in turn affect receptor function.

It shall be noted that we explore receptor function in the few minutes upon the onset of osmotic swelling, thus restricting the cohort of potential effects impacting the signalling cascade to rapid responses, arising from a local reorganization of the membrane and cortex (**Supplementary figure 5**), while ruling out more lasting effects such as alterations to gene expression patterns[29]. Two prominent effects that can be immediately associated to osmotic swelling are a remodelling of the cortical actin cytoskeleton and a flattening of plasma-membrane ruffles. The role of cortical actin in connection to adrenergic receptor signaling, in particular the β2-AR, is well documented.

The latter effect happens both at a global as well as at a local scale, i.e. the overall curvature radius of the membrane decreases as the volume increases, while at the same time, small-scale ruffles on the plasma membrane are 'flattened out'[30]. In this context, we can speculate that rather than significant changes to membrane tension, or overall curvature, specific GPCRs may face 'eviction' from regions of higher curvature, such as villi, filopodia or other invaginations of the cell membrane, to flatter regions of the plasma membrane, albeit n the future, we shall clearly need methods to elicit such processes at a controllable and more precise spatial-temporal scale.

Within this scenario, we observe a striking increase in cAMP response in cells undergoing osmotic swelling, and we set to dissect all steps of the β2-AR signalling pathway in intact cells, to pinpoint where swelling is having an impact on the signalling cascade. To do this, we employed, developed and implemented a wide set of fluorescence spectroscopy techniques that allowed monitoring the activity of each of the signalling partners supporting cAMP production and its regulation. Fluorescence cAMP biosensors allowed us to report on the effect (**Figure 1)** in HEK293 cells as well as in the more physiological setting of adult cardiomyocytes (**Figure 2**). The sensor was then combined with specific drug combinations

and knockout cell lines to rule out the role of trafficking (**Figure 3b**), cAMP degradation (**Figure 3c**) or an altered activity of the Adenylate Cyclase (**Figure 3d,e**). As a matter of fact, Fsk-induced AC activity appears unchanged in absence of the Gs, suggesting that a key player in modulating the signalling cascade of the β-ARs is indeed Gs. To further appreciate the role of Gs in modulating receptor signalling we employed a fluorescent reporter for the active conformation of Gαs, namely Nb37, originally developed to stabilize the structure of the active β2-AR in complex with Gαs[31]. By combining the fluorescently tagged Nb37 to TIRF imaging we were able to show not only nanobody recruitment to the ternary complex, but also to correlate the intensity (i.e. the number of ternary complexes present at the membrane), to the strength of the agonist stimulation. With this assay, we can then demonstrate that in swollen cells an equal concentration of agonist leads to an increased number of active receptor-Gαs complexes (**Figure 4a-c**). This is an important result, since it points to improved receptor to G protein coupling as the driving mechanism behind the observed increase in cAMP upon osmotic swelling. According to the ternary complex model[32], a high affinity state characterized by increased receptor-G protein coupling also correlates to increased affinity of the receptor towards its agonist, since the intracellular G protein coupling stabilizes the active receptor conformation. We thus investigated the possibility that osmotic swelling stabilizes the active receptor conformation, first by overexpressing the Gs heterotrimer and Nb37 (**Figure 4b**) and observing using TIRF the recruitment of a fluorescently labeled analogue, then by monitoring ligand binding in intact cells using a fluorescence anisotropy-based displacement assay (**Figure 5a**) and finally by a proxy for G protein coupling, namely the Nb80, originally reported to stabilize the active receptor conformation[26] (**Figure 5d**) also using a TIRF assay.

Upon ligand displacement experiments, the measured k_d displays a small but statistically significant change in swollen cells (**Figure 5b-c**), with increased affinity towards isoproterenol observed for the receptors in swollen cells. We shall note here that a 0.5-1 log-unit shift in EC50 for cAMP production (as summarized in **Figure 1e**), can arise from a minute k_d change, given the large amplification of the signaling cascade[33]. Moreover, the enhancement in Nb80 recruitment upon swelling displayed in **Figure 5e-f** is striking, corroborating the notion that receptors active conformation is indeed strongly favoured by osmotic swelling.

Overall, these results point to a clear modulation of receptor activity upon cell swelling, that we ascribe to cell swelling favouring the active conformation of the β2-AR. This observation would agree with a recent report suggesting that osmotic swelling leads to increased hydration of the intracellular side of the receptor, ‘forcing open’ the G protein binding pocket and thus favouring coupling[34]. Other explanations, such as an enhanced 'collision coupling’ of the receptor to the G protein[35], are possible, but less supported by data. While we observed a slight increase in receptor diffusion coefficients, we did not measure any increase in G protein (Gαs) diffusion rate in swollen cells as opposed to control. Since osmotic swelling has rather broad effects on the cell cortical environment and plasma membrane, such as actin remodelling, altered lipid composition, as well as changes in both local (flattening of ruffles and invaginations) and low (overall increase in volume) geometrical curvature[29], we believe it is difficult to pinpoint its effect as one specific molecular mechanism. Nonetheless, our observation with other Gs-coupled receptors as well as with a Gi coupled receptor such as the μOR, point to the generality of this regulatory mechanism. Moreover given the physiological relevance of this process in several contexts, ranging from cardiac ischemia-reperfusion to swelling of adipocytes[36], we believe that these are novel and timely observations that further our understanding of receptors’ molecular pharmacology and its role in pathophysiological conditions.

Materials and Methods

Cell culture. HEK293 and HEK293 derived cells (H187 stable cell line, HEK293-AD, Gs-KO cell line) were cultured in DMEM medium containing 4.5 g/l glucose (Gibco) supplemented with 10% fetal calf serum (FSC, Biochrome), 2mM l-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin, at 37 °C, 5% CO_2. To split cells, growth medium was removed by aspiration and cells were washed once with 10 ml of PBS (Sigma), followed by trypsinization for 2 min in 1.5 ml of trypsin 0.05%/EDTA 0.02% (PAN Biotech) solution and resuspended in the desired amount of DMEM medium.

Transfection. For the single cell microscopy experiments the cells were transfected directly in the imaging dishes 24 hours prior to conducting the experiments, using JetPrime transfection reagent (Polyplus) according to manufacturer's protocol. For the plate reader experiments the cells were transfected on 10cm plates 48 hours before the experiment using a reduced amount of JetPrime transfection reagent and total DNA (5ul and 2.5ug respectively) and transferred to black 96-well plates (Brandt) 24 hours before the experiment.

Swelling medium. Throughout all experiments two types of imaging buffers (control – MES300 and swelling – MES200) were used. A basic buffer with a low osmolarity – ES150 – was produced by solving 60 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES in distilled water and resulting in a solution of 150 mOsm. It was than supplemented with 150 mM Mannitol (Sigma) to produce MES300 and reach 300 mOsm. MES300 was then used for washing and incubation steps as described in specific experiments. To achieve hypotonic conditions of 200 mOsm during the experiments, 2/3 of the total volume of MES300 was removed from the wells and replaced with the same volume of ES150. In control condition 2/3 of MES300 were also removed and replaced with fresh MES300 to avoid ascribing any effects to mechanical artifacts.

Cardiomyocyte isolation. Cardiomyocyte isolation involved obtaining cardiac myocytes from adult ventricles of CAG-Epac1-camps mice aged 8 to 12 weeks[9], regardless of their gender. The process employed enzymatic collagen digestion and retrograde perfusion through the aorta using a Langendorff perfusion apparatus, as previously described[8]. In brief, the hearts were swiftly removed from the mice after cervical dislocation and connected to a custom-built perfusion system. Initially, the hearts were perfused with perfusion buffer for 4 minutes at a rate of 3 ml/min, followed by an 8-minute perfusion with myocyte digestion buffer containing 5 mg of liberase dispase high DH enzyme per digestion. Subsequently, the heart was detached from the perfusion system, and the ventricles were dissected into small pieces using scalpels. After sedimentation, removal of the supernatant, and resuspension the cells were filtered through a nylon mesh cell strainer (100-µm pore size, Falcon) to eliminate any remaining tissue fragments. The cells were then gradually exposed to physiological Ca^{2+} concentrations (~1 mM), resuspended in myocyte plating medium, and seeded on freshly coated Matrigel-coated µ-slides for each experiment. More detailed information about the buffers and materials used can be found in the methods section of Bathe-Peters et al[8].

Cell area measurements with confocal microscopy. To measure the effect of hypotonic treatment on cell area murine cardiomyocytes were seeded in 8-well Ibidi® µ-slides with a density of 1,000 cells per well and labeled with Cell Mask™ Deep Red according to manufacturer's protocol. XY and XZ movies of cells were then aquired on a confocal laser scanning microscope, Leica SP8, with a white-light laser at the wavelength of 633 nm and laser power of 5%. All measurements were conducted with an HC PLAP CS2 40×1.3 numerical aperture (NA) oil immersion objective (Leica). Movies were aquired at 30 seconds per frame with a hybrid detector in the range of 643 to 693 nm. Medium change to induce

swelling was performed as described in previous sections. ImageJ was used to perform thresholding and object detection on the cells and the extracted area was plotted with Prism v. 9.5.1.

FRET microscopy. HEK293 cells stably expressing Epac-S^{H187} sensor were seeded in 8-well Ibidi® µ-slides with a density of 25,000 cells per well. Cells were washed twice in MES300 and imaged at room temperature. An inverted microscope (DMi8, Leica Mircosystems), equipped with an x63 HC PL APO, 1.40-0.60 numerical aperture (NA) objective (Leica Microsystems), dichroic beamsplitter T505lpxr (Visitron Systems), xenon lamp coupled with a continuously tunable Visichrome high-speed polychromator (Visitron Systems) and a metal-oxide-semiconductor camera (Prime95B, Teledyne Photometrics) with a dual image splitter (OptoSplit II, Cairn Research), was used. Excitation wavelength of 445 nm was used and fluorescence emission was simultaneously recorded at 470/24 nm and 535/30 nm. The movies were obtained at 5 seconds per frame for the number of frames needed for the cAMP concentrations to equilibrate. ImageJ was used to extract fluorescence intensity values from single cells, which were corrected for background and used to calculate FRET/CFP ratio.

Plate reader cAMP FRET measurements. HEK293 cells stably expressing Epac-S^{H187} sensor were seeded in black 96-well plates (Brand) with a density of 50,000 cells per well. 24 h later cells were washed twice with 90 µl MES300 per well. After 5 min incubation at 37°C, baseline measurement was conducted in a Neo2 plate reader (Biotek) using 420 nm excitation and 485 nm / 540 nm emission filters. As the second step, 60 µl MES300 was removed from each well and replaced with 60 µl of either MES300 or ES150 for the “control” and “swelling” conditions respectively and the plate was measured for 15 minutes. Afterwards the desired dilution series with increasing concentrations of ligand was added (10 µl per well of a 10x concentration in MES300) and the plate was measured again for 15 minutes. At last, a mix of forskolin/IBMX in MES300 was added to an end concentration of 10 µM forskolin and 100 µM IBMX and measured for 10 more minutes. The change in acceptor/donor ratio was normalized to 0% baseline and 100% forskolin/IBMX and plotted in Prism v.9.5.1. For the concentration response curves “Dose-response stimulation fit (three parameters)” was applied.

TIRF microscopy. HEK293AD cells were seeded in 8-well Ibidi® µ-slides with a density of 25,000 cells per well and transfected with JetPrime® transfection according to manufacturer’s protocol. Following cDNA ratios were used: for Nb80 recruitment – 9x Snap-ß2AR and 1x Nb80-eYFP; for Nb37 recruitment – 1x Snap-ß2AR, 1x Gs(tricistronic) and 3x Nb37-eYFP; Transfected cells were labeled with 1 µM SNAP-Surface 674 dye (NEB) for 30 min followed by washing two times for 10 min with 300 µl MES300 per well. After labeling, cells were subsequently taken for imaging to an Attofluor Cell Chamber (Fisher Scientific, GmbH) in MES300. A TIRF illuminated Eclipse Ti2 microscope (Nikon), equipped with a x100, 1.49 NA automated correction collar objective and 405-, 488-, 561-, 647-nm laser diodes coupled via an automated N-Storm module and four iXon Ultra 897 EMCCD cameras (Andor), was used. Objective and cell chamber were kept at 37°C during imaging. The automated objective collar was on and hardware autofocus was activated. Movies were acquired at 4 s per frame for 400 frames. After a baseline measurement of 50 frames increasing concentrations of isoproterenol were added to the imaged well in 50 frame steps. Before each isoproterenol addition 30 µl of solution was removed from the well and 30 µl of 10x isoproterenol in either MES300 or MES200 was applied. ImageJ was used to extract fluorescence intensity values, which were corrected for background and normalized to 0% baseline and 100% 10 µm isoproterenol stimulation. In case of F-actin content measurment HEK293AD cells were transfected with Lifeact-eGFP and imaged as described above at 30 seconds per frame.

Plate reader ligand binding assays. HEK293T cells were seeded in 10cm culture plates, transfected with 1x Snap-ß2AR, 1x Nb37-eYFP and 3x Gs(tricistronic)-mTq2 after 24h and

reseeded in black 96-well plates with a density of 50,000 cells per well another 24h later. The plate was incubated with 100ul MES300 containing 2nM JE1319. As the second step, 60 µl MES300 was removed from each well and replaced with 60 µl of either MES300 or ES150 (both containing 2nM JE1319) for the "control" and "swelling" conditions respectively and the plate was incubated for further 15 minutes. Afterwards the desired dilution series with increasing concentrations of isoproterenol was added (10 µl per well of a 10x concentration in MES300) and the plate was incubated for 90 minutes and measured in a Neo2 plate reader (Biotek) using DualFP polarization filter and an 620 nm/680 nm excitation/emission filter. A G-factor of 0.12 was measured for the instrument. Fluorescence anisotropy was calculated as $r = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$ and plotted in Prism v.9.5.1. For the concentration response curves "Dose-response inhibition fit was applied with min and max being constrained to 0% and 100% respectively.

Plate reader Gi-FRET sensor assay. HEK293T cells were seeded in 10-cm cell culture dishes at a density of 4×10^6. Cells were co-transfected with 500 ng of SNAP-µOR and 2,000 ng of G_i2 FRET sensor 24 h after seeding, as described in previous sections; 24 h after transfection, cells were trypsinized and transferred into black 96-well plates (Brand), at a density of 50,000 cells per well; 16 h to 24 h later, cells were washed with MES300, and then 90 µL of MES300 was added to each well. After 10-min incubation at 37 °C, measurement was performed at 37 °C using a Synergy Neo2 Plate Reader (Biotek) using a CFP/YFP FRET filter set. After basal FRET measurement, medium was changed as described above and after 15 minutes of further measurement 10 µl of ligand solution were applied to each well. The change in acceptor/donor ratio was normalized to 0% baseline and plotted in Prism v.9.5.1. For the concentration response curves "Dose-response stimulation fit (three parameters)" was applied.

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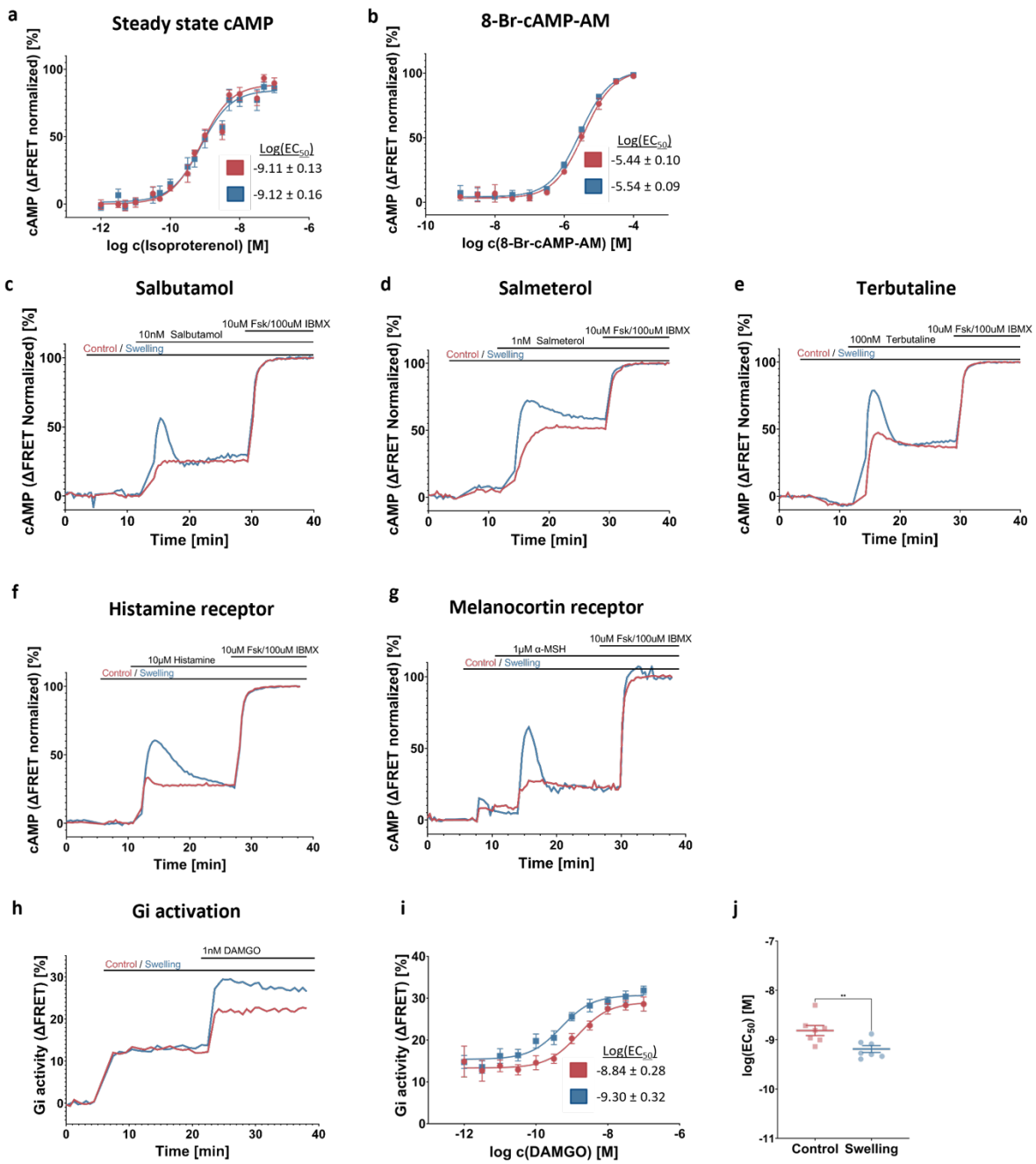
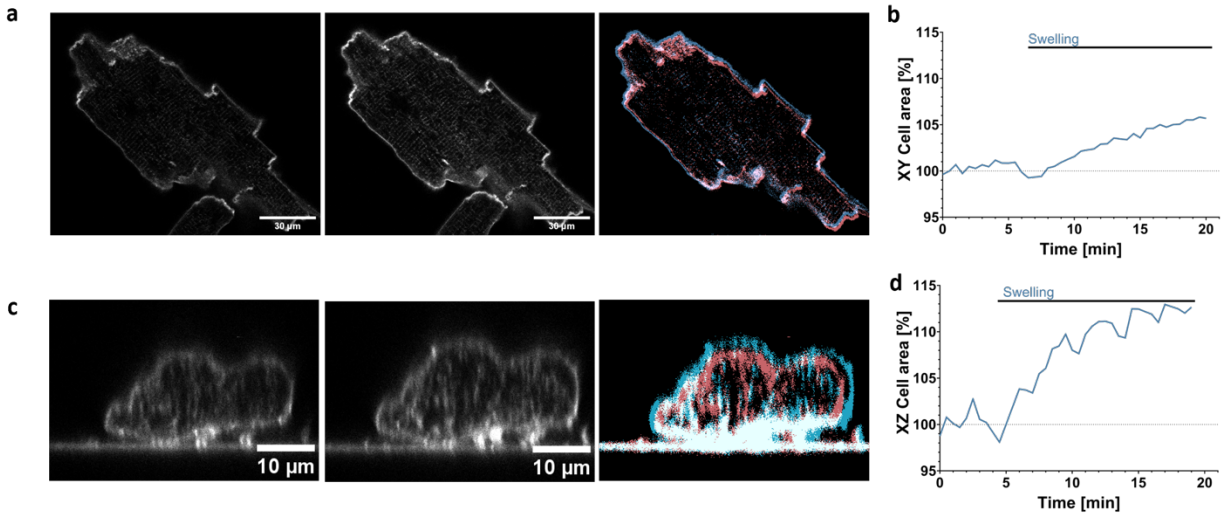
Author contributions

A.S. and P.A. designed research; A.S. performed research; A.I. contributed new reagents/analytic tools; A.S. and P.A. analyzed data; M.B.-P. supported the preparation of primary cardiomyocytes; P.A and M.J.L. supervised the project and acquired funding; P.A. and A.S. wrote the paper with input from all authors.



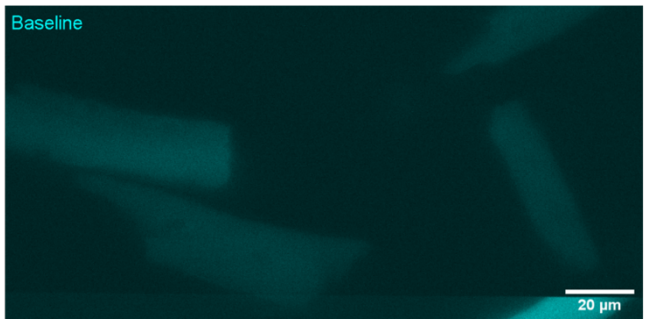
Supp. Fig. 1: Effect of swelling on receptor signaling is generalizable to other ß-AR agonists, to other Gs-coupled GPCRs and to the Gi pathway a averaged concentration response curves representing steady state cAMP concentrations, as indicated in (**Fig1. c**) (mean ± SEM; n = 6 independent experiments); **b** averaged concentration response curves of H187 stable cell line being stimulated by increasing concentrations of 8-Br-O-Me-cAMP (mean ± SEM; n = 3 independent experiments); **c-e** representative curves showing kinetics cAMP concentrations as a response to different ß-adrenergic agonists (normalized to baseline and 10μM Forskolin + 100μM IBMX); **f,g** representative curves showing kinetics cAMP concentrations as a response to histamine (**f**) and α-MSH (**g**) (normalized to baseline and 10μM Forskolin + 100μM IBMX); **h-j** representative curve (**h**), averaged concentration response curve (**i**) and $\log EC_{50}$ values from individual experiments (**j**) comparing a Gi2 FRET-sensor response to DAMGO in control and swollen cells, transfected with μOR.

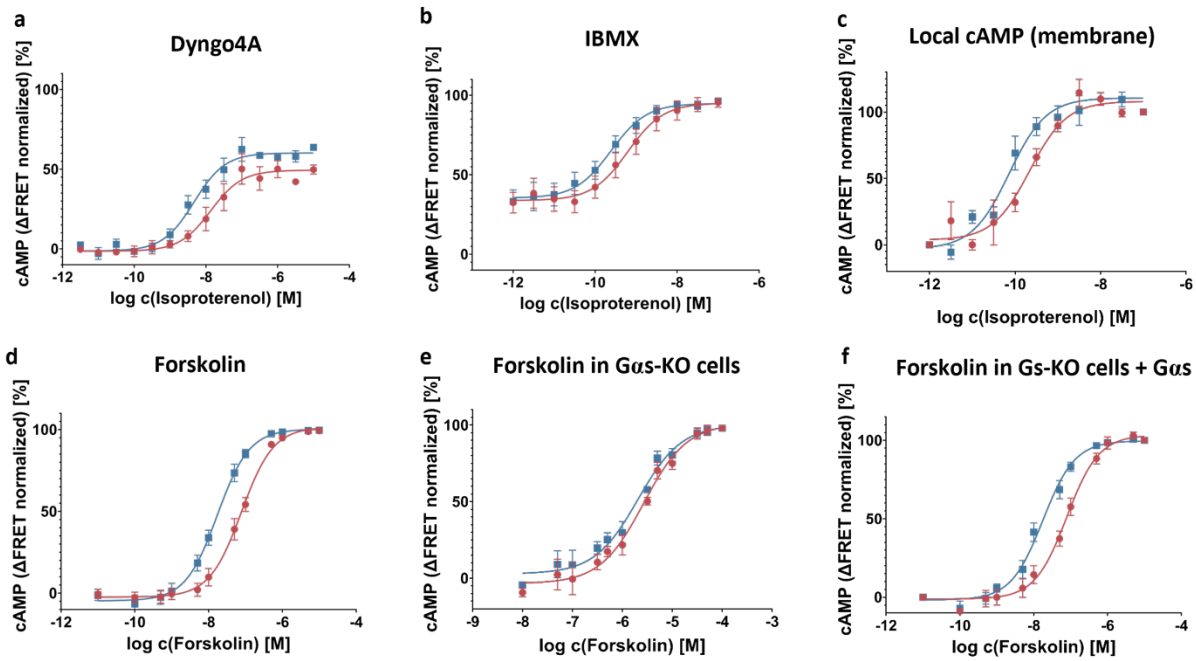


Supp. Fig. 2: Characterization of cardiomyocyte swelling

a-d Confocal images of CM under isotonic conditions (left) and after 20 minutes of exposure to swelling medium (middle), with an overlay of cell edges under both conditions (right) and the quantification of cell area imaged in XY plane (**a,b**) and XZ plane (**c,d**);

Supplementary movie 1 (cAMP in CM upon Iso and Fsk/IBMX stimulation)

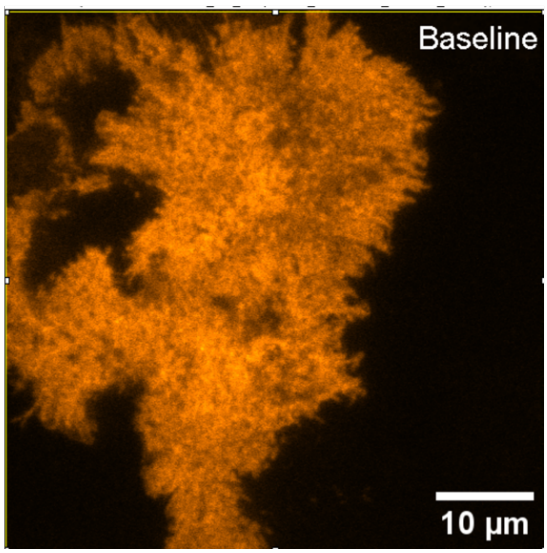


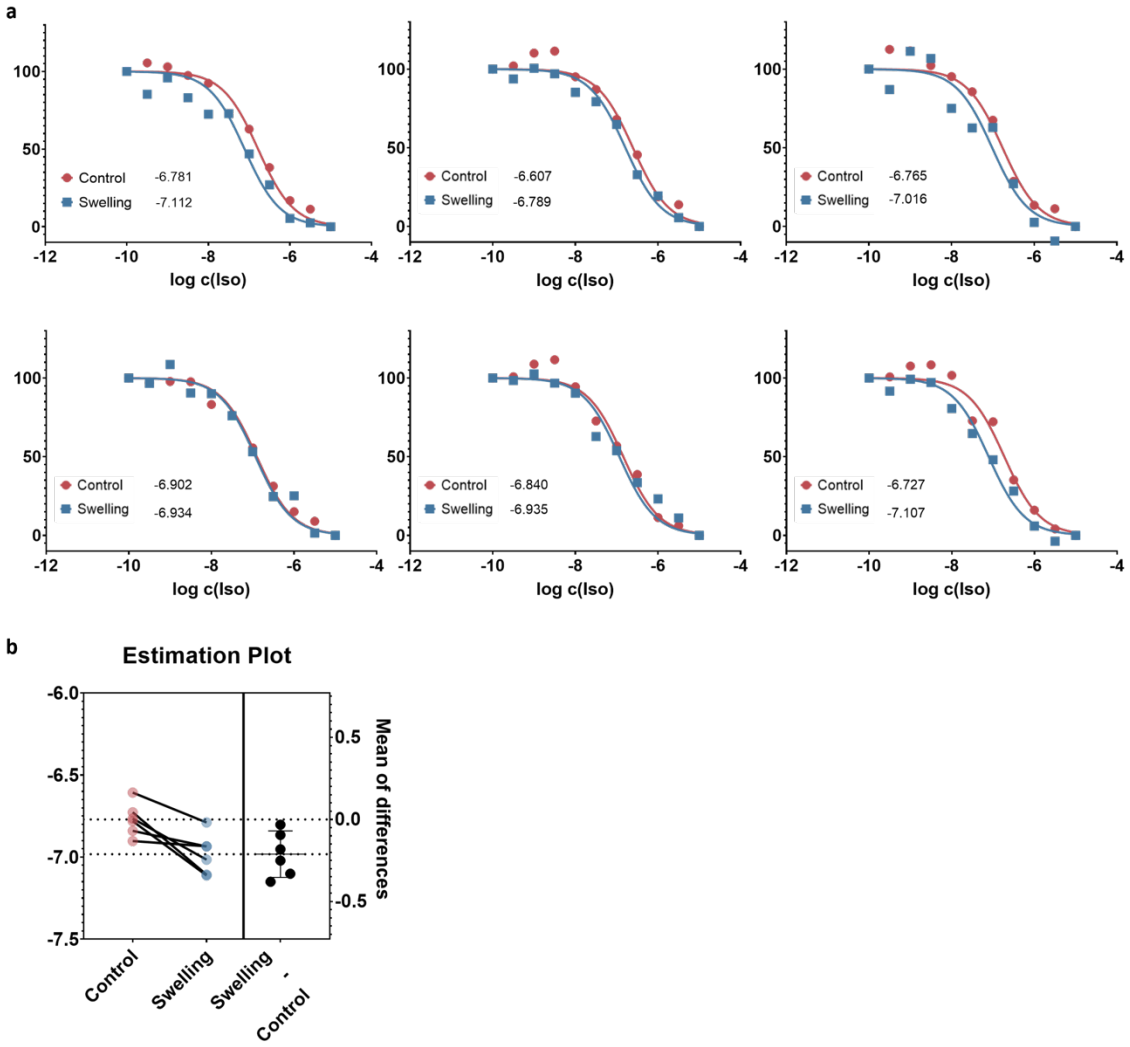


Supp. Fig. 3: Impact of cell swelling on isoprenaline potency under selective regulation of the β-adrenergic signalling cascade

a-f averaged concentration response curve of intracellular cAMP concentrations under following conditions: (**a**) cytosolic cAMP measured upon Iso stimulation after treatment with 30µM Dyngo4a for 30 min; (**b**) cytosolic cAMP measured upon Iso stimulation with simultaneous addition of 100µM IBMX; (**c**) local cAMP at the plasma membrane upon Iso stimulation measured with an Epac-Camps1-Caax sensor; (**d,e,f**) cytosolic cAMP measured upon Fsk stimulation in wt Gs phenotype (**d**), Gs-KO cells (**e**) and Gs-KO cells transiently transfected with Gsα (**f**)

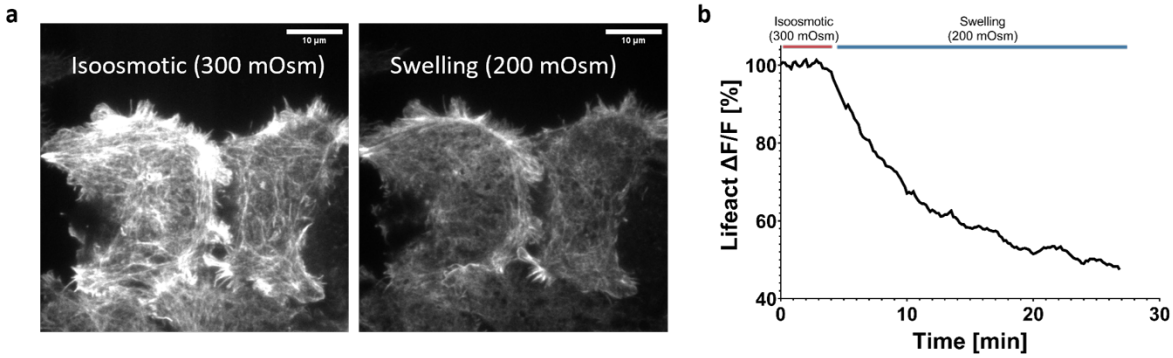
Supplementary movie 2 (Nb37-eYFP recruitment)





Supp. Fig. 4: Fluorescence anisotropy measurements show an increase of isoproterenol binding affinity in swollen cells

a single displacement curves from individual experiments, averaged in **Fig. 5 (b) b** pairwise comparison of log (Kd) values represented in **Fig. 5c,** comparing swollen to control cells, and the average of the difference overlay.



Supp. Fig. 5: F-actin labeling shows a decreasein cortical actin polymerization upon induction of cell swelling

a representative TIRF-M images of HEK293 cells transfected with Lifeact-eGFP and visualizing F-actin in basal conditions (left) and after 20 minutes of incubation in swelling medium (right) **b** quantification of Lifeact-eGFP intensity over the course of time for one representative cell

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