Gsα stimulation of mammalian adenylate cyclases regulated by their hexahelical membrane anchors

Anubha Seth[1,2], Manuel Finkbeiner[2], Julia Grischin[2] , Joachim E. Schultz[1*]

[1]Pharmazeutisches Institut der Universität Tübingen, Tübingen, Germany;

[2]Max-Planck-Institut für Entwicklungsbiologie, Tübingen, Germany

Corresponding author:
Dr. Joachim E Schultz
Pharmazeutisches Institut der Universität Tübingen,
Auf der Morgenstelle 8, 72076 Tübingen, Germany
Phone: +49 7071-2972475,

Email: joachim.schultz@uni-tuebingen.de

Abstract

Nine mammalian adenylate cyclase (mAC) isoforms are pseudoheterodimers with two dissimilar hexahelical membrane-anchors, isoform-specifically conserved for more than half a billion years. In the past, we have postulated a receptor function for these membrane anchors. Here, we exchanged both membrane anchors of the human AC isoform 2 (hAC2) with the hexahelical membrane domain of the quorum-sensing receptor from *Vibrio*, CqsS, which has a known ligand, *Cholera*-Autoinducer-1 (CAI-1). In the chimera, cyclase activity was stimulated by Gsα, whereas CAI-1 by itself had no effect. Surprisingly, CAI-1 inhibited Gsα stimulation, shifting the concentration-response curve and attenuating the maximal response. Extending these data we report that Gsα stimulation of hAC2 expressed in Sf9 insect cells is inhibited concentration-dependently by human serum whereas human serum albumin does not. The data establish the existence of an allosteric linkage in mACs, in which the membrane anchors, as receptors, can transduce extracellular signals to the catalytic dimer, regulating the extent of mAC stimulation by G-protein-coupled receptor-signaling. A general mechanistic model of AC regulation is presented which is compatible with all known regulatory inputs into mammalian ACs, i.e. stimulation by Gsα as well as modulations by secondary modifications. The data allow to unequivocally designate the membrane anchors of mammalian ACs as orphan receptors, and thus establish a new level of AC regulation in mammals.

This opens the possibility to start a targeted ligand search.

keywords: human adenylate cyclases, isoforms, membrane anchor, receptor, quorum-sensor, cyclic AMP, second messenger

Introduction

The first amino acid sequence of a mammalian adenylate cyclase (mAC) identified two similar catalytic domains (C1 and C2) and two dissimilar hexahelical membrane anchors (TM1 and TM2; (1)). Initially, the membrane domains were proposed to possess a channel or transporter-like function, properties, which were never confirmed. Subsequently, nine independent genes for mAC isoforms with identical domain organizations were identified, indicating substantial subfunctionalization during evolution (mAC isoforms 1 – 9; (2, 3)). Because mACs are the cellular effector proteins for many hormones that signal via G-protein-coupled receptors (GPCRs), their regulation has received broad attention (2, 3). The catalytic center of mACs is formed at the C1/C2 dimer interface. Most biochemical studies have used the startling observation that the separately expressed C1/C2 catalytic domains are regulated by Gsα, i.e. the membrane anchors appear dispensable for catalysis and regulation (4). Why then 2 x 6 transmembrane spans, when 1 or 2 would have been sufficient for membrane-anchoring? The evolutionary conservation of the membrane domains including a cyclase-transducing-element in an isoform-specific manner for more than half a billion years justifies searching for a physiological function beyond membrane-anchoring (5, 6).

Recently, we replaced the 6TM domain of the mycobacterial Rv1625c AC, a monomeric progenitor of the mACs, by the hexahelical quorum-sensing receptor CqsS from *Vibrio harveyi*, generating a CqsS-AC Rv1625c homodimeric chimera (here and in the following, CqsS is used to denote the ligand-binding membrane domain of CqsS, not the full receptor protein (6, 7)). The ligand for CqsS, *Cholera*-Auto-Inducer-1 [CAI-1; (S)-3-hydroxy-tridecan-4-one], stimulated AC activity in this chimera (6, 7). Subsequently, we characterized the family of highly conserved cyclase-transducing-elements (CTEs) which are required for signal transduction (7). CTEs are present, and highly isoform-specifically conserved in all eukaryotic mACs, strongly supporting the hypothesis that the AC membrane domains are receptors for as yet unidentified ligands (5, 7). Here, we asked whether AC regulation by

CqsS is also maintained in a pseudoheterodimeric mAC, in which the TM1 and TM2 domains are replaced by two identical ones, namely by CqsS. Using a chimera between CqsS and the human AC2 (CqsS-hAC2), we report that stimulation by Gsα is preserved. However, whereas the ligand CAI-1 does not by itself affect basal activity, surprisingly, it regulates Gsα stimulation of hAC2. Extending these findings we show that the Gsα stimulation of the hAC2 holoenzyme is similarly inhibited by human serum in a strictly concentration-dependent manner, but not by human albumin. This indicates the presence of as yet unidentified ligands in serum which physiologically act on human ACs and identifies the AC membrane domains as orphan receptors.

Results

CqsS serves as a ligand receptor for hAC2

We generated chimeras in which exclusively TM1 and TM2 of hAC2 were replaced by the quorum-sensing receptor CqsS from *Vibrio harveyi* (Fig. 1). Two questions were obvious: a) is such a chimera expressed in *Escherichia coli* even though so far bacterial expression of mammalian membrane-bound ACs has proven impossible, and b) is regulation by Gsα and the quorum-sensing ligand CAI-1 maintained? The pseudoheterodimeric chimera was expressed as a membrane-bound protein in *E. coli* and had robust basal activity (Fig. 1). AC activity was stimulated by constitutively active Gsα (Q227L, below termed Gsα (8)) demonstrating the formation of a C1/C2 catalytic center. The Gsα concentration-response curve showed a 2.3-fold increase in activity with an EC_{50} of 148 nM Gsα (Fig. 1). By comparison, AC2 expressed in Sf9 insect cells is reported to be stimulated by Gsα about 5 to 12-fold (9, 10). The results support the currently accepted notion that the membrane anchors are not involved in Gsα regulation of mACs (3, 4). The diminished Gsα response and the increased EC_{50} concentration compared to hAC2 may be due to the replacement of the dissimilar TM1 and TM2 domains by two identical CqsS receptors (9-11). The quorum-sensor ligand CAI-1, at concentrations

up to 100 µM, failed to affect basal AC activity. Similarly, the closely related compounds *Legionella*-Auto-Inducer 1 (LAI-1; (S)-3-hydroxy-pentadecan-4-one) and the dihydroxy derivative of CAI-1 (3,4-dihydroxy-tridecane) at up to 100 µM had no effect on basal AC activity (not shown). This posed the following questions: a) are the catalytic domains of mACs at all capable of operating as output domains for transmembrane signals? b) did we fail because of a botched protein design, e.g. an improper helical register in CqsS-hAC2? and, c) which biochemical differences between bacterial and mammalian ACs exist, which might explain the divergent results concerning the CqsS-Rv1625c chimera?

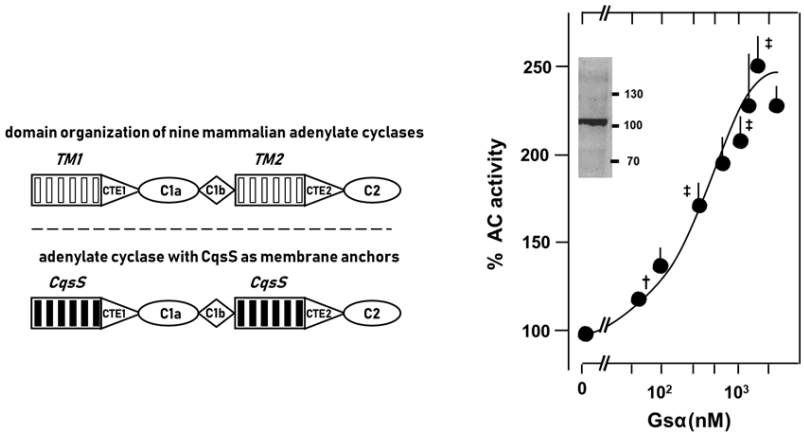

Figure 1. Stimulation of the chimera CqsS-hAC2 by Gsα. **Left:** general domain organization of the nine mammalian membrane-delimited adenylate cyclases and schematic depiction of the chimera between CqsS as membrane anchors and hAC2. The 6TM regions are idealized, CTE1 and CTE2 denote the cyclase-transducing-elements (7), C1a and C2 are the respective catalytic domains, C1b connects C1a to TM2. **Right**: Gsα concentration-response curve. Basal activity (100 %) corresponds to 27.2 ± 2.6 pmol cAMP·mg^{-1}·min^{1}. Half-maximal stimulation was at 148 nM Gsα. Error bars denote S.E.M.'s, n = 2-7. Significances: †: $p < 0.01$; ‡: $p < 0.001$ compared to basal. Insert: A Western blot of the chimera with an anti-His_6-antibody indicated absence of proteolysis. MW standards are indicated on the side.

A crucial difference between bacterial and mammalian ACs are the dissociation constants of the catalytic domains. Bacterial catalytic domains usually have a high 'self'-affinity (Kd $\leq 10^{-7}$ M) and are active when conformationally unconstrained (5-7, 12). The individual C1 and C2 domains of mACs also have a high propensity for self-association, i.e. C1 preferentially associates with C1 and C2 with C2, as documented in the first mAC crystal structure, a C2 homodimer (13). However, homodimers of C1 or C2 are inactive (14). The actual affinity between the C1 and C2 catalytic domains in mACs is rather low (Kd $\geq 10^{-5}$ M (15-18). AC stimulation by Gsα is tantamount to an increase in the apparent affinity of C1 and C2 for each other by approximately two orders of magnitude (15-18). Therefore, a provocative explanation and hypothesis for the lack of a CAI-1 effect would be that CqsS receptor activation causes conformational changes which interfere with Gsα stimulation. This was tested next.

The CAI-1 receptor regulates stimulation of hAC2 by Gsα

In the presence of 5 or 10 µM CAI-1, Gsα activation of CqsS-hAC2 was significantly attenuated (Fig. 2A). Inhibition did not require preincubation and was instantaneous. The effect was ligand-specific as the 3, 4-tridecanediol derivative of CAI-1 or the closely related ligand LAI-1 did not affect Gsα-stimulated activity (see also Fig.3). In presence of 5 or 10 µM CAI-1, the EC_{50} concentrations for Gsα stimulation were increased 1.5-fold and 4-fold, respectively (Fig. 2A). Concomitantly, CAI-1 at 10 µM significantly diminished the Gsα response by 20 % (Fig. 2A). The action of CAI-1 was reversible, as determined by re-assaying membranes which were stimulated, washed and re-isolated (Fig. 2B).The data bolstered the hypothesis that CqsS receptor activation interfered with the actions of Gsα.

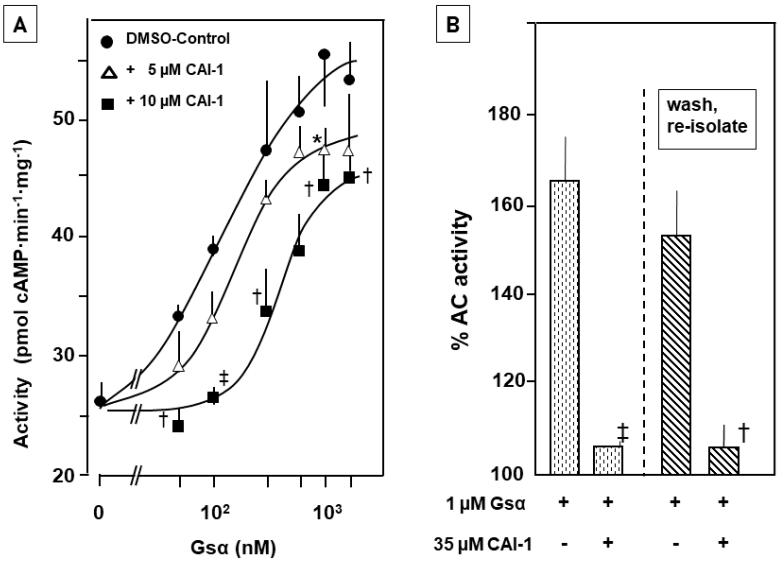

Figure. 2. A) Effect of CAI-1 on the stimulation of the chimera CqsS-hAC2 by Gsα. Filled circles, Gsα alone; open triangles, + 5 µM CAI-1; filled squares, + 10 µM CAI-1. **B) Inhibition of the Gsα response by CAI-1 is reversible**. After stimulation of CqsS-hAC2 for 15 min (left) membranes were washed and re-isolated by ultracentrifugation (total time required about 150 min). Re-stimulation was for 15 min. Basal activity (100%) corresponds to 48.2 ± 9.1 (primary stimulation) and 32.3 ± 5.1 pmol cAMP·mg^{-1}·min^{-1} (washed membranes). 2 % DMSO, serving as ligand solvent, was included in all assays. Error bars denote S.E.M.'s of 5 separate experiments. Significances: *, $p < 0.05$; †, $p < 0.01$ compared to the respective Gsα stimulation without CAI-1 addition. For clarity, not all significances are shown.

Next, a concentration-response curve for CAI-1 in the presence of 1 µM Gsα was carried out. At 35 µM CAI-1, activation of the CqsS-hAC2 chimera by Gsα was almost abrogated (Fig. 3, left). The IC_{50} for CAI-1 inhibition was 5µM. This is about 12-fold higher than that obtained for the stimulatory effect of CAI-1 in the CqsS-Rv1625c AC homodimer (6). This might be due to the fact that we went from a homodimeric CqsS-Rv1625c AC to a linked pseudoheterodimeric CqsS-hAC2 chimera. In controls, the closely related LAI-1 ligand and the dihydroxy derivative of CAI-1 had no effect on Gsα stimulation. To exclude, that CAI-1 might have obstructed the interaction between the catalytic dimer and Gsα, we expressed

hAC2 in Sf9 cells and tested the effect of CAI-1 on Gsα stimulation of hAC2 (Fig. 3, right). Neither CAI-1 nor LAI-1 affected basal or Gsα-stimulated AC activity. This demonstrated unequivocally that a) CAI-1 did not directly affect basal hAC2 activity in the chimera, b) CAI-1 did not interact with Gsα and impair its function, and c) CAI-1 had no effect on the interaction between the catalytic dimer and Gsα. We conclude that the effect of CAI-1 in the chimera was mediated via the CqsS-receptor.

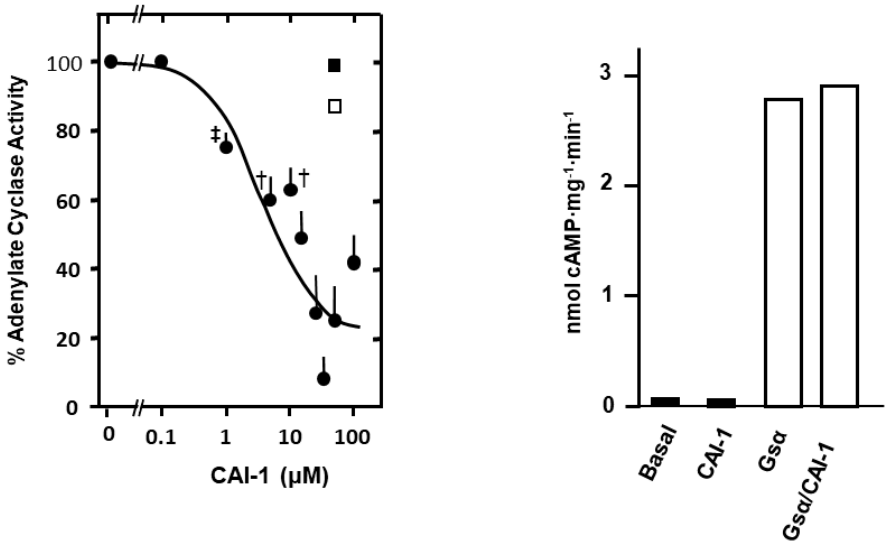

Figure 3. **Left: CAI-1 concentration-response curve for the inhibition of Gsα-stimulated CqsS-hAC2 (1 µM Gsα).** Gsα-stimulated activity above basal (100%) was 39 ± 2.6 pmol cAMP·mg^{-1}·min^{-1} (filled circles); filled square: 50 µM LAI-1; open square, 50 µM 3,4-tridecanediol. The IC_{50} for CAI-1 was 5 µM. Error bars denote S.E.M. of 3-7 separate experiments. Significances: †: $p < 0.01$; ‡: $p < 0.001$. For clarity not all significances are indicated.

Right: CAI-1 has no effect on Gsα stimulation of hAC2. Basal activity was 60 ± 8 pmol cAMP·mg^{-1}·min^{-1}. Concentration of agents: 1 µM Gsα, 50 µM CAI-1. Solvent DMSO was present at 2%. 6-12 assays were carried out with Sf9-expressed hAC2. Inter-assay variability was <10%.

Gsα Stimulation of hAC2 is inhibited by human serum

The above data pose several questions: first, are the experimental results restricted to the bacterial-mammalian chimera CqsS-hAC2? Second, can we demonstrate that mACs are regulated in a similar way? Third, what are potential ligands in mammals and where to expect them? Based on the above data, we are able to experimentally address these questions. Considering that the membrane anchors / receptors are isoform-specifically conserved, prospective ligands are predicted to be similarly primordial (5, 7). Because potential AC ligands should access the mACs from the extracellular solvent space, they are expected to be systemically present in the interconnected extracellular fluid system of the body, such as blood, lymph, and interstitial fluid. Indeed, stimulation of hAC2 with 600 nM Gsα was significantly inhibited by unprocessed human serum (and identically by heat-inactivated human serum or fetal bovine serum [FBS]; data not shown) in a strictly concentration-dependent manner. Additions of human or bovine serum albumin were without an effect on Gsα stimulated hAC2 activity. This suggested that potential receptor ligands for hAC2 are present in serum (Fig. 4, left). However, the following stringent controls are required to unequivocally assign the effect of serum to the AC membrane anchors / receptors: a) exclusion of a salt effect because serum contains about 130 mM NaCl, b) exclusion of an interference of serum components with the dimerization of the C1 and C2 catalytic domains, and c) exclusion of an interference in the interaction between the catalytic dimer and the activating Gsα.

Addition of 10 mM NaCl to the incubations, corresponding to addition of 10% serum in the assays, did not impair hAC2 basal activity or the potency of serum to inhibit Gsα stimulation. Similarly, using dialyzed FBS did not affect serum inhibition of Gsα stimulation excluding an interfering salt effect.

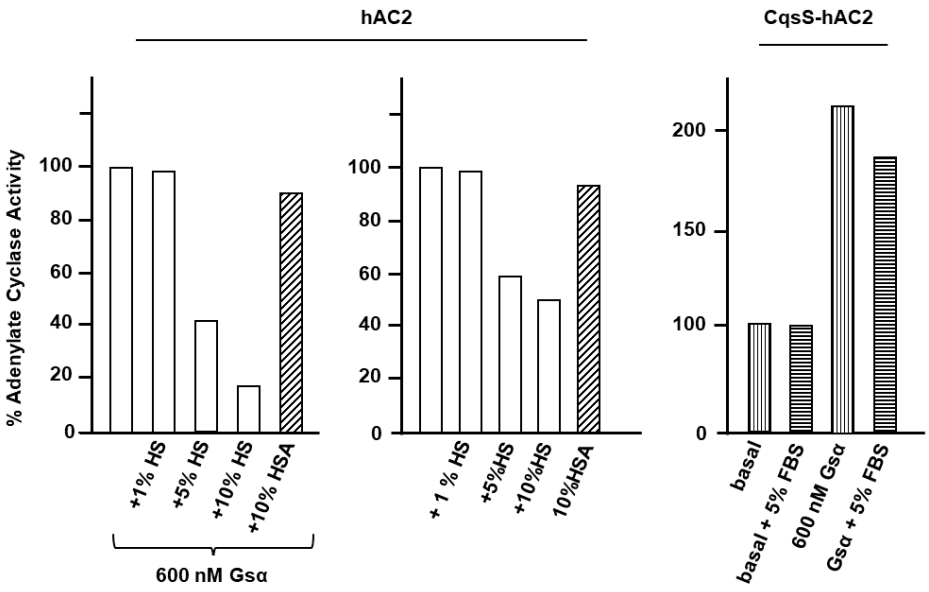

Figure 4. Left: Inhibition of Gsα stimulation (600 nM) of hAC2 (expressed in Sf9 cells) by addition of human serum (HS, 0.1 to 1 µl/10µl assay, i.e. 1 to 10 %; protein content of human serum was determined to 60 µg/µl; open bars); hatched bar: no effect of human serum albumin (HSA) at a comparable protein concentration (450 µg/assay). 100 % Gsα-stimulated activity was 1.6 nmol cAMP/mg•min. **Middle:** Inhibition of basal hAC2 activity by human serum (HS) (open bars); hatched bar: no effect on basal activity was exerted by human serum albumin (450 µg/assay). **Right:** Fetal bovine serum neither inhibited basal hAC2 (12 pmol cAMP/mg•min) nor Gsα-stimulated activity in the CqsS-hAC2 chimera (26 pmol cAMP/mg•min). Please note the different scale of the y-axis at right. All experiments were carried out in triplicates.

Basal activities of all mACs generally are rather low and difficult to determine exactly (19, 20). Therefore, in a control we increased the amount of Sf9 hAC2 membrane protein in the assay 12.5-fold and examined the effect of serum (1 to 10%). Human serum as well as FBS (data not shown) inhibited basal hAC2 activity in a concentration-dependent manner whereas human albumin did not (Fig. 4, middle). This created a critical issue because components of human or fetal bovine serum might either interfere with dimerization of C1 and C2 domains or with activation of the dimer by Gsα. An alternative interpretation would be that serum shifted the equilibrium between different basal states of the cyclase to an inactive state via a specific interaction with a receptor intrinsic to the membrane anchors of mACs. This possibility was explored using the CqsS-hAC2 chimera in which the two membrane anchors

are from a *Vibrio* quorum-sensor whereas all cytosolic domains are from hAC2 (see scheme in Fig. 1). In the CqsS-hAC2 chimera FBS up to 10% did neither interfere with basal hAC2 activity nor with Gsα stimulation, indicating that the action of serum was absolutely contingent on the presence of the membrane anchor of hAC2 (Fig. 4, right). This question was further probed using the class IIIa mycobacterial AC Rv1625c, a progenitor of the membrane-anchored mammalian AC (12). Serum did not inhibit the activity of soluble or membrane-bound Rv1625c AC corroborating the conclusion that unquestionably the serum inhibition of the stimulation of hAC2 by Gsα required the presence of the membrane anchor of hAC2. Taken together the results unequivocally establish the function of the mAC membrane domains as receptors. The data do not establish the chemical identity of ligands present in serum. This is the next challenge in this area.

Discussion

A new model of adenylate cyclase regulation

We observed no direct CqsS-receptor-mediated action of the CqsS ligand CAI-1 as basal hAC2 activity was unaffected; however, CAI-1 did inhibit Gsα stimulation in an unequivocally receptor-mediated process. Extending these observations to hAC2 we demonstrated a likewise effect of serum on Gsα stimulation which was absolutely dependent on the presence of the membrane domain of the hAC2. This functionality was not substituted by the hexahelical quorum-sensor CqsS in the CqsS-hAC2 chimera. Based on these data we propose a new formal concept of regulation of mACs which encompasses all present and former biochemical and structural data (Fig. 5). Three distinct basal states of mACs exist in a dynamic equilibrium, two inactive and one active state, termed A (inactive), B (inactive), and C (active; Fig. 5). States A and B differ in the conformational flexibility of their catalytic domains. In basal state A, the catalytic domains are conformationally constrained and cannot form an active cleft. In state B the catalytic domains are unconstrained, yet because of their

low affinity they only occasionally collapse into a catalytically active dimer (state C). Probably, states A and B are the predominant species. The rather transient state ‘C’ is responsible for the very low basal activity observed in all mACs (3).

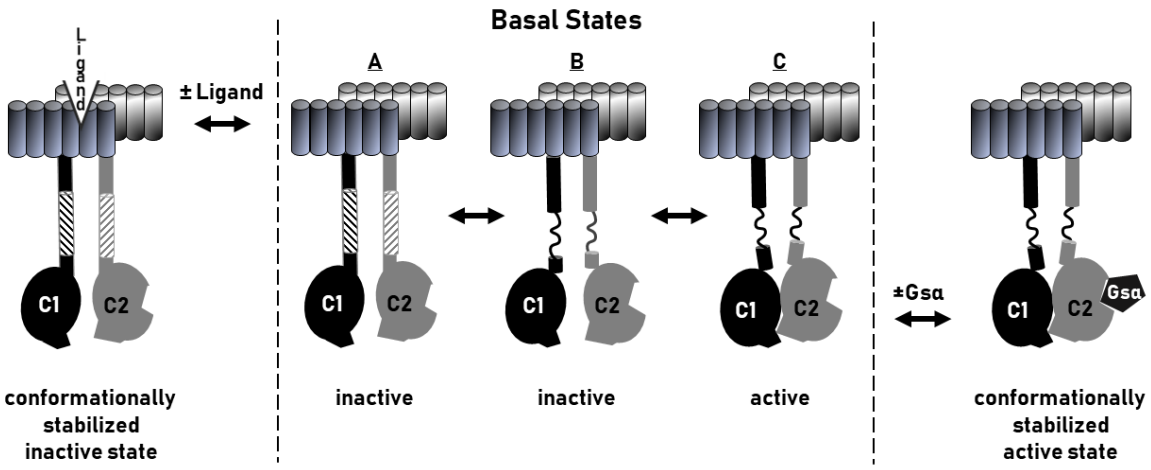

Figure 5. **General scheme of regulation of mammalian adenylate cyclases.** Three basal states are in a thermodynamic equilibrium, two inactive, A and B, and one active state, C. The constant low basal enzyme activity is due to fractional formation of an active dimer as symbolized in C. Contact with Gsα in the cytosol stabilizes the active ‘C’-state (conformationally stabilized active state). Conversely, contact with as yet unknown ligands in the extracellular space stabilizes the inactive state A (conformationally stabilized inactive state; see text for further discussion).

The model proposes that stabilization of the state ‘C’ by the plant diterpene forskolin is responsible for the robust, yet generally limited AC activation observed in mACs with the exception of isoform 9 . Forskolin reversibly increases the apparent affinity of C1 and C2 about tenfold, i.e. it transiently stabilizes the active dimeric state ‘C’ (21, 22). In addition, state ‘C’ is semi-permanently stabilized by Gsα which thus strongly activates mACs and acts synergistically with forskolin, shifting the equilibrium of basal states to a stable active form. The action of Gsα activation is time-limited by hydrolysis of bound GTP. The inactive basal state ‘A’ is characterized by loss of conformational flexibility of the catalytic domains. It is

stabilized by extracellular ligands binding at the cytosolic face of the mACs in a receptor-type interaction. The stabilization of structural rigidity shifts the equilibrium of the three basal states far to the inactive state 'A' (Fig. 5, left) thus attenuating basal AC activity and severely counteracting activation by Gsα (Fig. 4).

The inhibition of the hAC2 by serum most likely unfolds along this model. Indeed, experiments carried out with the intention to exclude the possibility that serum interferes in the formation of the catalytic dimer in the 'C' state resulted in a concentration-dependent inhibition of basal hAC2 activity, fully confirming the existence of the three distinct basal states predicted by the model (Fig. 5). Therefore, we used the closely related mycobacterial AC Rv1625c which probably is a precursor of mACs (12) to demonstrate that serum did not interfere in the formation of an active catalytic dimer (data not shown). The model allows predicting that the presence of forskolin should attenuate the effects of extracellular ligands. This was not tested because we attempted to limit the number of reagents in our assays.

The proposed model establishes the existence of an allosteric linkage in mACs, in which the membrane anchors, as receptors, transduce extracellular signals to the cytosolic catalytic dimer. This way, each mAC isoform can be addressed individually by an extracellular ligand and primed for a physiologically measured GPCR/Gsα response. Such a regulatory network would solve the conundrum of why most often multiple Gsα-stimulated mAC isoforms are expressed in a single cell (23-25). In fact, under particular physiological conditions an AC isoform expressed in a cell may be kept in a completely unresponsive state. The data are in agreement with all other regulatory inputs on mACs reported in the past, such as by Ca^{2+} ions, calmodulin, Gβγ, or secondary modifications such as phosphorylation as well as with the co-localization of mAC isoforms in signalosome-like complexes (3). Further, the recently published complete structure of a bovine AC9 isoform up to a resolution of 3.4 Å contains structural features fully compatible with signal transduction between membrane and catalytic domains (26). In summary, the mAC membrane anchors can now be designated as orphan

receptors and, for the first time, we are in a position to undertake a targeted search for mAC ligands.

Presently, we can only speculate about the chemical nature of the ligands for mACs. Considering the extremely high isoform-specific conservation of mAC membrane anchors including their associated CTEs (>half a billion years; (7)) nine distinct ligands are to be expected. The ligands access the receptor from the extracellular solvent space, i.e. they are present systemically in bodily fluids such as serum. For several reasons the remote alternative hypothesis that components of the membrane itself access the mAC membrane anchors in a ligand-receptor type of interaction such as the adenosine-2 receptor interact with membrane cholesterol (27, 28) is deemed not viable. Expression of hAC2 was in insect Sf9 cells from *Spodoptera frugiperda*, yet insect cells do not share the mammalian AC isoforms (5). The Sf9 insect cells were cultured without any animal-derived components. It appears to overstretch speculation and invoke the presence of nine membrane-integral components in Sf9 membrane as specific ligands (e.g. membrane lipids) for mAC isoforms. The presence of potential ligands for the AC membrane anchors/receptors in systemic fluids such as serum defines an indirect, cytosolic regulatory system regulating all GPCR/Gsα actions connected to mACs. It is tempting to propose that mACs are pivot points between the endocrine and paracrine signaling systems.

Materials and methods

CqsS of *V. harveyi (*acc. # AAT86007) and hAC2 and hAC9 (acc. # Q08462 and NM_001116.3) sequences were used. Position Phe166 in CqsS has been shown to be critical for quorum sensing and was mutated to Leu (F166L) according to Beltz et al.(6, 29). Radiochemicals were from Hartmann Analytic and Perkin Elmer. Enzymes were from either New England Biolabs or Roche Molecular. Other chemicals were from Sigma, Merck and Roth. CAI-1, LAI-1, and 3,4-tridecanediol were synthesized in-house according to(30). The

cAMP assay kit is from Cisbio (Berlin). The constitutively active GsαQ227L point mutant was a gift from Dr. C. Kleuss, Berlin (31). It was expressed in *E. coli* and purified by affinity chromatography via its N-terminal His_6-tag and by size-exclusion chromatography. Human serum albumin (catalog # A3782) was from Sigma-Aldrich (Taufkirchen, Germany), fetal bovine serum was from Gibco, Life Technologies, Darmstadt, Germany (lot number: 1908933).

Plasmid construct

Several CqsS_hAC2-C1_CqsS_hAC2-C2 chimeras were generated using standard molecular biology methods. DNA fragments and vectors were restricted at their 5'-BamHI or EcoRI and 3´-HindIII sites and inserted into $pQE80_L$ (Δ XhoI; Δ NcoI). Gly-Ser and Arg-Ser were introduced to generate required internal restriction sites. All constructs carried N-terminal MRGS-hexa-His-tags to examine potential proteolysis by Western blotting. The fidelity of all constructs was confirmed by double-stranded DNA sequencing. The construct used in this study was: MRGSHis_6-GS–CqsS $(F166L)_{1\text{-}181}$-$hAC2_{221\text{-}603}$-RS-CqsS $(F166L)_{1\text{-}181}$-$hAC2_{836\text{-}1091}$. For virus production, hAC2 was inserted into pLIB. The plasmid was amplified in *E. coli* XL1blue and transformed into *E. coli* EMBacY cells generating the bacmid for Sf9 transfection. Genes for all hACs were bought from GenScript.

Protein expression

CqsS-hAC2 was transformed into *E. coli* BL21 (DE3). Strains were grown overnight in Luria-Bertani broth (LB medium) at 30°C with 100 µg/ml ampicillin. 200 ml LB medium or M9 with glycerol replacing glucose were inoculated with a preculture (to A_{600} of 0.2) and grown at 30-37°C in the presence of antibiotics. At an A_{600} of 0.9 (for M9 medium) / 0.7 (for LB medium), the temperature was lowered to 22°C and expression was initiated by addition of 500 µM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 hrs. Cells were harvested by

centrifugation, washed once with 50 mM Tris/HCl, 1mM EDTA, pH 8, and stored at -80°C. For preparation of cell membranes, cells were suspended in lysis buffer (50 mM Tris/HCl, 0.021 % thioglycerol, 50 mM NaCl, pH 8) containing complete protease inhibitor (in 50ml) cocktail (Roche Molecular) and disintegrated with a French press (1100 psi). After removal of cell debris (4.300 x g, 30min, 4°C) membranes were collected at 100000 x g (1h at 4°C). Membranes were suspended in buffer (40 mM Tris/HCl, 0.016 % thioglycerol, 20 % glycerol, pH8) and assayed for AC activity. Virus-infected Sf9 cells expressing hAC2 were grown in Sf900 III medium which contains no ingredients derived from animal sources, harvested after three days and membranes were isolated, stored at -80 °C and used for testing.

Adenylate cyclase assay

Activity of CqsS-hAC2 chimera AC was determined for 15 min at 30°C- 37°C in 100µl(32). The reactions contained 40 µg membrane protein, 50 mM Tris/HCl pH 8.3, 5 mM $MnCl_2$, 6 mM creatine phosphate and 230 µg creatine kinase, 750 µM [α-^{32}P]-ATP, and 2 mM [2,8-^{3}H]-cAMP to monitor yield during cAMP purification. In all assays, substrate conversion was below 10%. CAI-1 was dissolved in DMSO and 2 µl of DMSO was added with the ligand. Incubations with vehicle alone were carried out as controls. Activity of hACs was determined in a volume of 10 µl using an Assist Plus pipetting robot (Integra Biosciences, Biebertal, Germany) and a cAMP assay kit from Cisbio (Codolet, France) according to the supplier's instructions. cAMP labeled with the dye d2 for binding a Eu^{3+}-cryptate-labeled antibody was used to quantify cAMP by homogenous-time-resolved fluorescence (HTRF).

Western blot analysis

The integrity of the expressed recombinant CqsS-hAC2 was probed by Western blotting. Sample buffer was added to the membrane fractions and applied to SDS-PAGE (10%). For Western blot analysis, proteins were blotted onto PVDF membrane and examined with an RGS-His_4-antibody (Qiagen) and a 1:2500 dilution of the fluorophore-conjugated secondary antibody Cy3 (ECL Plex goat-α-mouse IgG-Cy3, GE Healthcare). Detection was carried out

with the Ettan DIGE Imager (GE Healthcare). Proteolysis of expressed proteins was not observed.

Data analysis and statistical analysis

All incubations were in duplicates (CqsS-hAC2) or triplicates (hACs). Data analysis was performed using GraphPad prism 8.1.2. For each experiment with CqsS-hAC2 membranes of independent expressions in *E. coli* were used. Data are presented as means ± S.E.M. Student's t-test was used to compare corresponding data pairs. For experiments with hACs mean values of 2-4 separate experiments are shown. Errors of reproducibility were within 10%.

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Author contributions: AS, MF and JG designed, carried out and analyzed experiments; JES, concept, analyzed data and wrote manuscript.

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JES Orcid: https://orcid.org/0000-0002-1985-4853

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