Distinct glycerophospholipids potentiate Gsα-activated adenylyl cyclase activity

Anubha Seth[1*†], Marius Landau[2*], Andrej Shevchenko[3], Sofia Traikov[3], Anita Schultz[2], Sherif Elsabbagh[2], Joachim E. Schultz[2]

[1]Max-Planck-Institut für Biologie, Tübingen, Germany

[2]Pharmazeutisches Institut der Universität Tübingen, Tübingen, Germany

[3]Max-Planck-Institut für molekulare Zellbiologie und Genetik, Dresden, Germany

*both contributed equally

†: present address:
Dept. of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520-8066, USA

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 adenylyl cyclases (AC) are pseudoheterodimers with two hexahelical membrane domains which are isoform-specifically conserved. Previously we proposed that these membrane domains are orphan receptors (10.7554/eLife.13098; 10.1016/j.cellsig.2020.109538). The identity of the ligands is unknown. Lipids extracted from fetal bovine serum at pH 1 inhibited mAC activities. Guided by a lipidomic analysis we tested glycerophospholipids as ligands. Contrary to the effect of the lipid extract we surprisingly discovered that 1-stearoyl-2-docosahexaenoyl-phosphatidic acid (SDPA) potentiated Gsα-activated activity of human AC isoform 3 seven-fold. The specificity of fatty acyl esters at position 1 and 2 was rather stringent. 1-Stearoyl-2-docosahexaenoyl-phosphatidylserine and 1-stearoyl-2-docosahexaenoyl-phosphatidylethanolamine significantly potentiated several Gsα-activated mAC isoforms to different extents. SDPA does not interact with forskolin activation of AC isoform 3. SDPA enhanced Gsα-activated AC activities in membranes from mouse brain cortex. The action of SDPA was reversible. SDPA did not affect isoproterenol-stimulated cAMP generation in HEK293 cells indicating a role as an intracellular effector. In summary, we discovered a new dimension of intracellular AC regulation by chemically characterized glycerophospholipids.

Keywords: adenylyl cyclase / cyclic AMP / membrane anchor / glycerophospholipids / phosphatidic acid

Abbreviations used: mAC, membrane-delimited adenylyl cyclase; GPL, glycerophospholipid; SDPA, 1-stearoyl-2-docosahexaenoyl-phosphatidic acid

Introduction

cAMP is a universal regulator of numerous cellular processes (Dessauer, Watts et al., 2017, Schultz & Natarajan, 2013, Sinha & Sprang, 2006, Sunahara & Taussig, 2002). Its biosynthesis is via adenylyl cyclases. This report deals with the nine mammalian, membrane- integrated pseudoheterodimeric ACs (mACs; reviewed in (Bassler, Schultz et al., 2018, Dessauer et al., 2017, Ostrom, LaVigne et al., 2022, Schultz & Natarajan, 2013). Currently, a direct regulation of mACs does not exist. The generally accepted mAC regulation is indirect, i.e. it sequentially involves *(i)* the extracellular activation of G-protein-coupled receptors, *(ii)* intracellular release of the Gsα subunit from a trimeric G-protein and, *(iii),* as a last step mAC activation by the free α-subunit (Dessauer et al., 2017, Sadana & Dessauer, 2009). In contrast, we recently have assigned a direct regulatory role mediated by the membrane domains of the 9 mAC isoforms as receptors (Beltz, Bassler et al., 2016, Seth, Finkbeiner et al., 2020). In this proposal the mAC receptors are comprised of the two hexahelical domains each connected to a cytosolic catalytic domain, C1 and C2, via highly conserved cyclase-transducing-elements (Dessauer et al., 2017, Seth et al., 2020, Ziegler, Bassler et al., 2017). The proposal for a receptor function is based on *(i)* the evolutionary conservation of the membrane anchors in an isoform-specific manner for more than 0.5 billion years (Bassler et al., 2018), *(ii)* on highly conserved cyclase-transducing-elements (Ziegler et al., 2017), and *(iii)* catalytic domains conserved from cyanobacteria to mammals (Bassler et al., 2018, Kanacher, Schultz et al., 2002, Linder & Schultz, 2003, Seth et al., 2020). Recently we reported ligand-mediated inhibition of a Gsα-activated mAC2 in a chimera in which the AC membrane domains were replaced by the hexahelical quorum-sensing receptor CqsS from *Vibrio sp.* which has a known ligand, cholera-auto-inducer-1 (Beltz et al., 2016, Ng, Wei et al., 2010, Seth et al., 2020).

In an initial approach to identify ligands for the mAC receptors we used fetal bovine serum (FBS) which had been shown to contain inhibitory components (Seth et al., 2020). Eliminating peptides or proteins as possible ligands we fractioned lipids by extraction with chloroform/methanol at different pH values (Bligh & Dyer, 1959). Expecting to isolate inhibitory components we report the most surprising discovery that 1-stearoyl-2-docosahexaenoyl-phosphatidic acid (SDPA) potentiated Gsα-activated mAC3 activity up to 7-fold. The actions of SDPA resemble, to a limited extent, those of the plant diterpene forskolin (Dessauer et al., 2017). The data establish a new layer of direct mAC regulation and emphasize the importance of glycerophospholipids (GPLs) in regulation of intracellular cAMP generation.

Results

Lipids as possible mAC effectors

We extracted lipids from FBS with chloroform/methanol at pH 1, pH 6, and pH 14 (Bligh & Dyer, 1959). After evaporation of solvent the solid residue was dissolved in DMSO and tested against human mAC isoforms 1, 3, 5, 6, and 7 in membrane preparations from HEK293 cells transfected with the respected ACs. The pH 1 extract inhibited Gsα-activated mAC activities to slightly different extents. The pH 6 and pH 14 extracts were not inhibitory (table 1).

Table 1.

Lipid Extraction of FBS with Chloroform / Methanol

remaining Gsα activated AC activity (%)

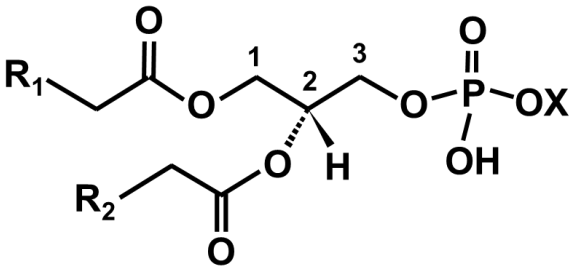
	pH 14	pH 6	pH 1
hAC1	99	91	25
hAC2	175	167	41
hAC5	96	112	44
hAC6	83	87	36
hAC7	140	122	51

2mL FBS were extracted with chloroform/methanol (1:2) according to (Bligh & Dyer, 1959). The organic phase was evaporated and the residue was dissolved in 35 µl DMSO. Adenylyl cyclases were activated by 600 nM Gsα and 33 nl of the DMSO extract were added. Basal activities were: 0.13, 0.02, 0.04, 0.16, and 0.2 nmol cAMP·mg^{-1}·min^{-1}, respectively. 600 nM Gsα-activated activities were 0.49, 0.36, 2.22, 0.71, and 0.25 nmol cAMP·mg^{-1}·min^{-1}. Values are the mean of two separate experiments.

We then carried out a lipidomic analysis with the pH 1 and the pH 6 fractions (Matyash, Liebisch et al., 2008, Vvedenskaya, Rose et al., 2021). Based on our previous data we expected ligands which inhibit Gsα-activated mAC activities (Seth et al., 2020). Therefore we concentrated on lipids prevalent in the pH 1 fraction. Apart from several constituents from different lipid classes the major constituents in the acidic fraction were phosphatidic acids, phosphatidylcholine, phosphatidylethanolamine and phosphatidylserines (see Appendix Fig. 1 and 2). Next we examined the effect of commercially available bulk lipids on Gsα-activated mACs (Appendix

Table 3). Egg phosphatidic acids significantly stimulated, whereas other bulk lipids did not show unequivocal effects.

The lipidomic analysis showed that the unsaturated fatty acids C20:4, arachidonic acid, and C22:6, docosahexaenoic acid, are prominent acyl substituents in phosphatidic acids (Appendix Fig. 2). These acyl residues are only minor components in the tested egg phosphatidic acids. Therefore, we assayed commercially available synthetic GPLs containing polyunsaturated fatty acids as acyl esters. The general structure of glycerophospholipids is shown below (see Appendix Table 1 for a complete list of lipids examined in this study).



Basic structure of glycerophospholipids: $R_1$and R_2 are fatty acyl residues esterified at glycerol positions 1 and 2; X can be a proton H^+ as in phosphatidic acid, choline (phosphatidylcholine), serine (phosphatidylserine), glycerol (phosphatidylglycerol), or ethanolamine (phosphatidylethanolamine).

The assays used membranes containing human mAC isoforms expressed in HEK293 cells. The mACs were activated by 600 nM of a constitutively active Gsα (Q227L, here termed Gsα) because we expected to characterize an inhibitory input (Graziano, Freissmuth et al., 1991, Seth et al., 2020). Most surprisingly, we discovered that 1-stearoyl-2-docosahexaenoyl-phosphatidic acid (SDPA) potentiated mAC3 up to 7-fold above the 16-fold activation already exerted by 600 nM Gsα alone (Fig. 1). The EC_{50} of SDPA was 0.9 µM. In the absence of Gsα 10 µM SDPA

alone had no significant effect (Fig. 1). The effect of SDPA was as far as the synergism between Gsα-activated mAC3 is concerned reminiscent of the cooperativity between forskolin and Gsα activated mACs (Dessauer et al., 2017).

Does the action of SDPA require a membrane-anchored AC holoenzyme or is the activity of a Gsα-activated C1/C2 catalytic dimer potentiated as well? We produced a soluble active AC construct connecting the catalytic C1 domain of mAC1 and the C2 domain of mAC2 by a flexible linker (Tang & Gilman, 1995). The construct was expressed in *E. coli* and purified via its His_6-tag. It was activated 12-fold by Gsα (from 12 to 150 pmol cAMP•mg^{-1}•min^{-1}). SDPA up to 10 µM did not affect basal activity and barely enhanced Gsα-activated activity of the chimera. We conclude that the SDPA action requires membrane anchoring of mACs.

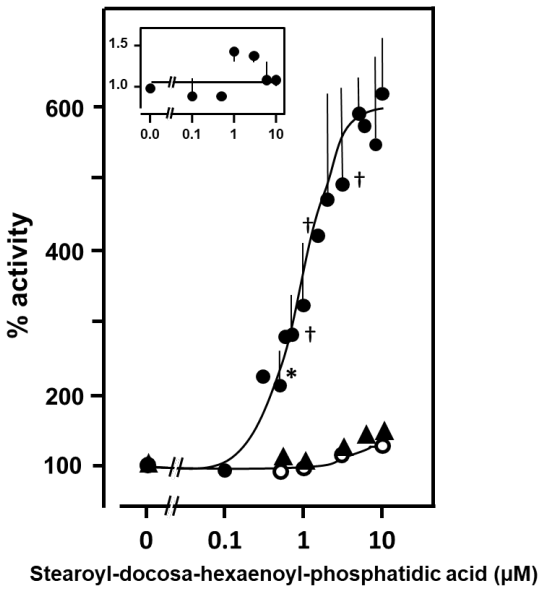


Figure 1. 1-Stearoyl-2-docosahexaenoyl-phosphatidic acid concentration-dependently potentiates mAC3 activated by 600 nM Gsα (filled circles). 100 % Gsα-activated mAC3 activity corresponded to 707 ± 187 pmoles cAMP•mg^{-1}•min^{-1}. Basal mAC3 activity is not significantly affected by SDPA (open circles; 100 % basal activity corresponds to 34 pmoles cAMP•mg^{-1}•min^{-1}). Triangles: Effect of SDPA on the C1-C2 soluble AC construct activated by 600 nM Gsα (Basal activity was 12 pmol cAMP•mg^{-1}•min^{-1}, 600 nM

Gsα activated activity was 150 pmol cAMP•mg^{-1}•min^{-1} corresponding to 100 %. Insert: Activity of the mycobacterial AC Rv1625c is unaffected by SDPA (activity was 23 nmoles cAMP•mg^{-1}•min^{-1}). Data were normalized to respective 100 % activities. Significances: *: $p<0.05$; **: $p<0.01$ compared to 100% activity. For clarity, not all significances are marked. N =2-6; error bars denote S.E.M.'s.

Does SDPA affect the activity of a Gsα-insensitive membrane-bound bacterial AC? We used the mycobacterial AC Rv1625c, a monomeric progenitor of mammalian mACs, which has a hexahelical membrane domain and is active as a dimer (Guo, Seebacher et al., 2001). The activity of the Rv1625c holoenzyme was unaffected by SDPA (Fig. 1 insert). The particular intrinsic properties of the mammalian membrane domains in conjunction with Gsα-activation may be required to confer SDPA sensitivity.

Which kinetic parameters are affected by SDPA? For mAC3, the enzymatic reaction rates ± SDPA were linear with respect to protein concentration and time up to 30 min. The Km for substrate ATP (0.1 mM) was unaffected. The most striking effect of SDPA was the increase in Vmax (from 4 to 8 nmol cAMP•mg^{-1}•min^{-1}). Concentration-response curves for Gsα in the presence of different SDPA concentrations showed that the affinity of mAC3 for Gsα was significantly increased (Fig. 2). Most likely, Gsα and SDPA act at distinct sites of the protein and potentiation by SDPA is due to concerted structural interactions, reminiscent of the cooperativity between Gsα and forskolin (Dessauer et al., 2017).

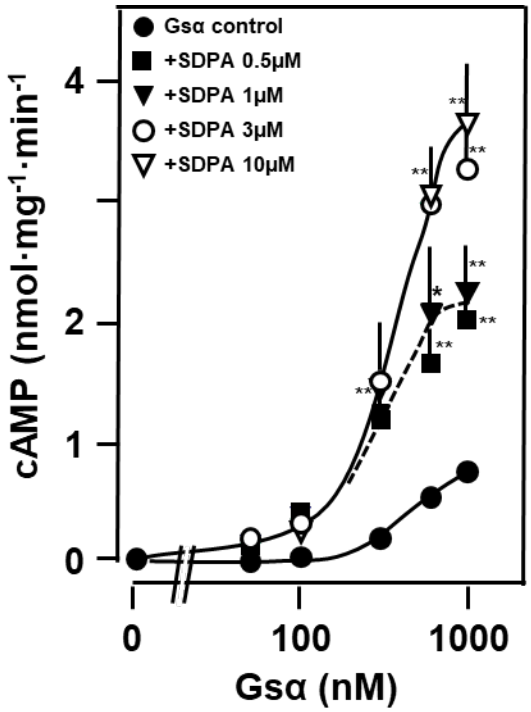


Figure 2. SDPA increases the affinity of mAC3 for Gsα. The EC_{50} concentration for Gsα in the absence of SDPA was 518 nM and in the presence it was 336 ± 29 nM ($p<0.02$; n=5-6; basal mAC3 activity was 27 ± 21 pmoles cAMP•mg^{-1}•min^{-1}; 1000 nM Gsα increased mAC3 activity to 791± 128 pmoles cAMP•mg^{-1}•min^{-1}). Significances: *: $p<0.05$; **: $p<0.01$ compared to corresponding activities without SDPA. N =2-6; error bars denote S.E.M.'s. Often, error bars did not exceed the symbol size.

Specificity of 1- and 2-acyl substituents in phosphatidic acid

Phosphatidic acid is the simplest GPL consisting of a glycerol backbone to which two fatty acids and phosphoric acid are esterified. It potentially carries two negative charges. Generally, at positions 1 and 2 of glycerol a variety of fatty acyl residues haves been identified. We examined the biochemical specificity of the fatty acyl-groups in phosphatidic acid (Fig. 3).

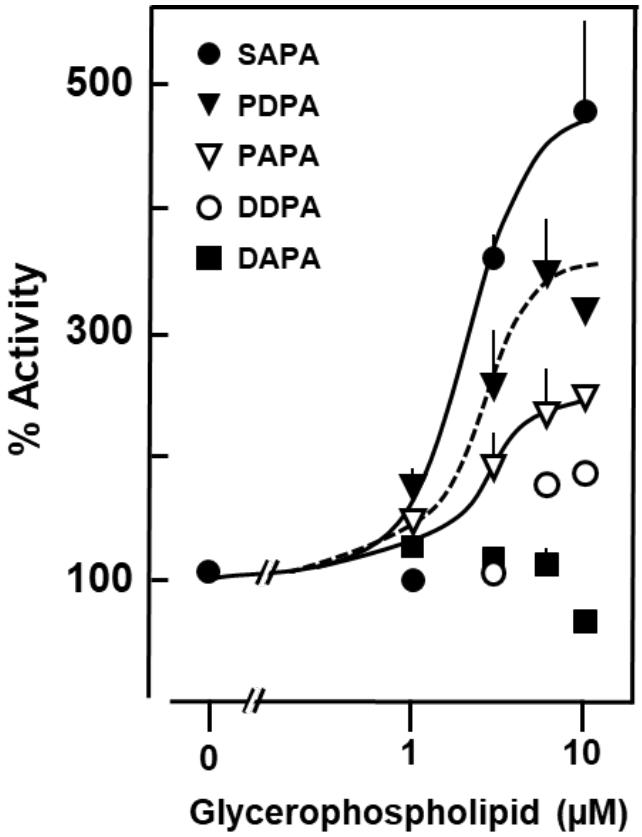


Figure 3. Specificity of fatty acyl esters in phosphatidic acids for potentiation of Gsα-activated mAC3. 600 nM Gsα-activated activity (100%) was 446 pmol cAMP•mg^{-1}•min^{-1} (basal mAC3 activity was 15.2 pmol cAMP•mg^{-1}•min^{-1}). Abbreviations: SAPA, 1-stearoyl-2-arachidonoyl-phosphatidic acid; PDPA, 1-palmitoyl-2-docosahexaenoyl-phosphatidic acid; PAPA, 1-palmitoyl-2-arachidonoyl-phosphatidic acid; DDPA, di-docosahexaenoyl-phosphatidic acid; DAPA, di-arachidonoyl-phosphatidic acid. The EC_{50} concentrations were 4.8, 1.3, and 1.4 µM, for SAPA, PDPA, and DDPA, respectively (differences not significant; n=3). Error bars denote S.E.M..

10 µM 1-Stearoyl-2-arachidonoyl-phosphatidic acid (SAPA) potentiated Gsα-activated mAC3 about 5-fold (EC_{50} = 4.8 µM; Fig. 3). Exchanging the stearic acid at position 1 by a palmitic acid, i.e. 1-palmitoyl-2-docosahexaenoyl-phosphatidic acid (PDPA) reduced biochemical activity by about 50 % compared to SDPA (EC_{50} = 1.3 µM). Strikingly, the corresponding 1-palmitoyl-2-arachidonoyl-phosphatidic acid (PAPA) lost about 70% of activity compared to SDPA (Fig. 3),

highlighting the structural contribution of the 1-fatty acyl residue to biochemical activity. The importance of the substituent at position 1 was further emphasized when assaying 1, 2-di-docosahexaenoyl-phosphatidic acid (DDPA). The potency was reduced by 80 % compared to SDPA (compare respective curves in Fig. 1 and 3). The EC_{50} for DDPA was 1.4 µM. Even more drastic was the absence of an effect using 1, 2-di-arachidonoyl-phosphatidic acid (DAPA; Fig. 3). Expectedly then, 1-stearoyl-2-linoleoyl-phosphatidic acid was inactive (not shown). The data show a remarkable positional specificity for the 1- and 2-acyl substituents of the glycerol backbone and indicate a specific and concerted interaction between the fatty acyl esters. The specificity of fatty acyl-substitution also strongly indicated that SDPA is not acting in its property as a general membrane GPL because other phosphatidic acids should be equally suitable as membrane lipids. Further, the peculiar biochemical properties of SDPA in its relation with AC isoforms suggest that the negative charges of phosphatidic acid are not sufficient to determine specificity, but that the lipid substitutions on position 1- as well as 2- probably are equally important.

Head group specificity of glycerophospholipids

The next question is whether 1-stearoyl-2-docosahexaenoyl-GPLs with different head groups might affect Gsα-activated mAC3. First, we replaced the phosphate head group in SDPA by phosphoserine generating SDPS. This greatly reduced potentiation of Gsα-activated mAC3 activity (2.8-fold potentiation; Fig. 4). A concentration-response curve of SDPS with mAC3 showed that the EC_{50} concentration was similar to that of SDPA (1.2 vs 0.9 µM; n=6-9; n.s.), but its efficacy is significantly lower indicating that identical binding sites are be involved.

We further used 1-stearoyl-2-docosahexaenoyl-ethanolamine (SDPE), 1-stearoyl-2-docosahexaenoyl-phosphatidylglycerol (SDPG) and 1-stearoyl-2-docosahexaenoyl-

phosphatidylcholine (SDPC; Fig. 4). In this order, efficacy to enhance the Gsα-activated mAC3 declined, with SDPC having no significant effect (Fig. 4). The surprising specificity of the 1- and 2-fatty acyl-substituents of the glycerol backbone was emphasized once again when we used 1-stearoyl-2-arachidonoyl-phosphatidyl-ethanolamine (SAPE) and 1-stearoyl-2-arachidonoyl-phosphatidylcholine. In both instances biochemical activity was lost (not shown). Consequently, we did not further probe GPLs with differing fatty acyl combinations at the glycerol 1- and 2-positions because, as demonstrated, changes in acyl substitutions resulted in considerable reduction or loss of biological activity (see Fig 3).

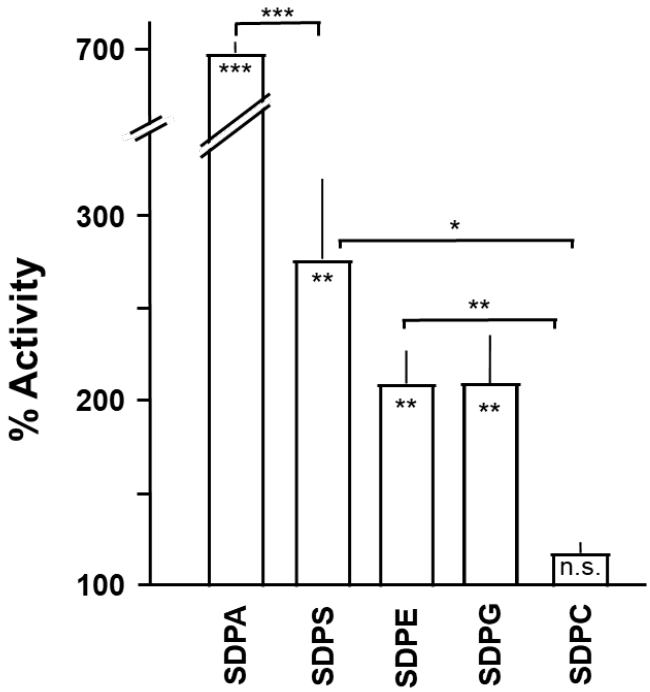


Figure 4. Head group specificity of glycerophospholipids enhancing Gsα-activated mAC3 activity. Basal and Gsα-activated mAC3 activities were 0.03 and 0.56 nmol cAMP•mg^{-1}•min^{-1}. Concetration of lipids was 10 µM. Error bars denote S.E.M., *: p< 0.05; **: p< 0.01; ***: p<0.001; n = 10.

Effect of glycerophospholipids on Gsα-activated adenylyl cyclase isoforms

So far, we examined only the mAC3 isoform. Does SDPA equally potentiate the Gsα-activated activities of the other mAC isoforms? More generally, do GPLs display an mAC isoform specificity in the regulation of intracellular cAMP biosynthesis? We expressed the nine human mAC isoforms in HEK293 and, first, tested how SDPA affected the Gsα-activated activities (Fig. 5).

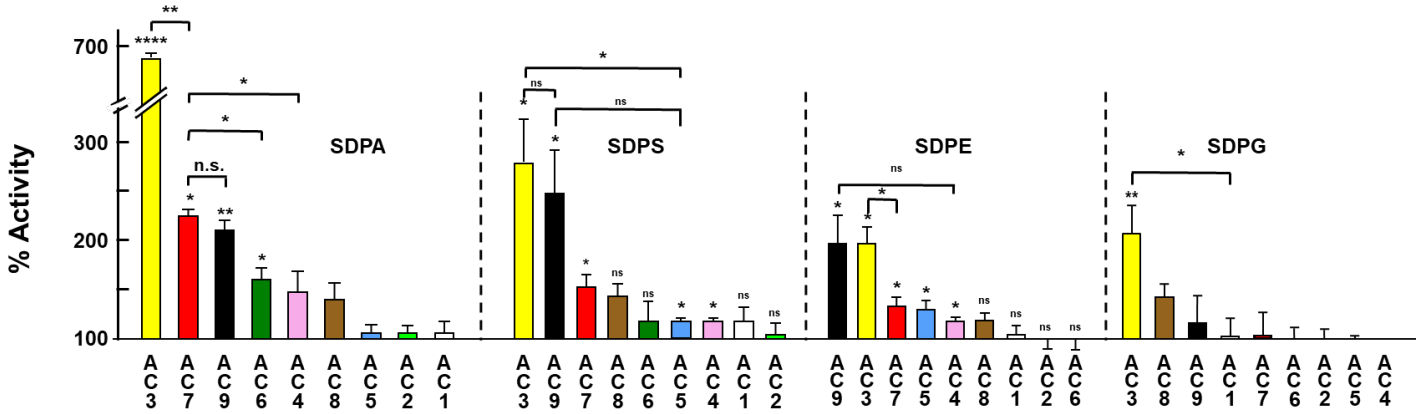


Figure 5. Effect of 10 µM of glycerophospholipids on various mAC isoforms activated by 600 nM Gsα. Basal activities and Gsα-activated activities are listed in supplementary Table 2). Error bars denote S.E.M., n = 7-12. *: $p< 0.05$; **: $p< 0.01$; ***: $p<0.001$ compared to Gsα-activated activity.

Under identical experimental conditions 10 µM SDPA significantly potentiated mAC7 (2.4-fold), mAC9 (2.1-fold) and mAC6 activities (1.5-fold). Concentration-response curves were carried for mACs 1, 2, 6, 7 and 9 (Appendix Figure 3). The EC_{50} concentrations of SDPA for mAC6, 7 and 9 were 0.7 µM, i.e. not significantly different from that for mAC3 indicating equal binding affinities. The other Gsα-activated mAC isoforms were not significantly affected (Fig. 5 and Appendix Figure 3). In summary, the data demonstrated that the mAC isoform specificity of SDPA was not absolutely stringent. The data then pose the question whether other GPLs may exert similar effects on mAC activities or display a different panel of isoform specificity. This was investigated using another four stearoyl-2-docosahexaenoyl-GPLs (Fig. 5). 10 µM SDPS potentiated mAC3 and mAC9. Smaller, yet significant effects were measured with mACs 7, 8, 5, and 6 (Fig. 5). 10 µM SDPE significantly potentiated Gsα-activated mAC isoforms 9, 3, 7, 5, and 4 (in this order). 10 µM SDPG significantly enhanced only mAC3 activity (Fig. 5). Compared to the seven-fold effect of SDPA on mAC3 these effects were small, yet, in mammalian biology such enhancements in mAC activity may well have profound physiological consequences. Up to 20 µM SDPC which is a major constituent of the outer leaflet of membranes had no effect on any mAC isoform (not shown). Taken together, the data then demonstrate the capacity of chemically defined GPLs to enhance or potentiate the activation of Gsα-activated mACs. We can virtually exclude coincidental and unspecific effects of the amphiphilic phospholipids because mAC isoforms were affected differentially. The results strongly indicate that a defined conformational space must exist at mACs which allows specific interactions with GPLs. Presently, the molecular details of the binding mode remain unknown.

Relationship between SDPA and forskolin

SDPA failed to activate basal mAC3 activity and only potentiates Gsα-activated mAC3 activity (Fig. 1). The plant diterpene forskolin stimulates basal as well as Gsα-activated mAC activities

(Dessauer et al., 2017, Tesmer, Sunahara et al., 1997, Zhang, Liu et al., 1997), i.e. the effects of SDPA and forskolin are only partly similar. Forskolin stimulates mACs expressed in Sf9 cells to rather different extents and with discrepant potencies, e.g. the EC_{50} concentrations for AC1 (0.7 µM) and AC2 (8.7 µM) differ more than 12-fold (Pinto, Papa et al., 2008). We established forskolin concentration-response curves for all mAC isoforms expressed in HEK293 cells under identical experimental conditions using Mg^{2+} as divalent cation, a study which is lacking so far (Appendix figure 4).

Stimulations at 1 mM forskolin were between 3-fold for mAC1 and remarkable 42-fold for mAC3. The EC_{50} concentrations for stimulation of basal mAC activities ranged from 2 to 512 µM forskolin (mAC7; Appendix Figure 4). We also observed forskolin activation of mAC9 although the current consensus regarding this isoform is that it is forskolin insensitive. The latter conclusion is based on experiments with mAC9 expressed in insect Sf9 cells using Mn^{2+} as a cation (Dessauer et al., 2017). Another report described forskolin activation of mAC9 when expressed in HEK293 (Premont, Matsuoka et al., 1996), in line with our data (Appendix Figure 4). We examined potential interactions between forskolin and SDPA using mAC3 activated by 600 nM Gsα. Up to 10 µM SDPA did not significantly affect forskolin stimulation. We reason that the absence of interactions or cooperativity between forskolin and SDPA indicates that both agents affect mAC regions which exclude mutual cooperative interactions. Nevertheless, considering the structural dissimilarity of forskolin and SDPA and the obvious lack of a molecular fit an identical binding site for both lipophilic agents is rather unlikely. On the other hand, both agents do interact with distantly-binding Gsα in a cooperative manner.

SDPA enhances Gsα-stimulated cAMP formation in mouse brain cortical membranes

Above we tested GPLs with individual mAC isoforms. At this point the question is whether SDPA would potentiate mAC activity in membranes isolated from mammalian organs. Several mAC isoforms are expressed in any given tissue and cell, yet the ratios of isoform expression differ. In mouse brain cortex all mAC isoforms with the exception of mAC4 are expressed (Ludwig & Seuwen, 2002, Sanabra & Mengod, 2011). Depending on the expression ratios we might expect at least a moderate potentiation of the Gsα-activated AC activity by SDPA. In mouse cortical membranes the basal AC activity of 0.3 nmoles $cAMP \cdot mg^{-1} \cdot min^{-1}$ was unaffected by 10 µM SDPA (Fig. 6A). 600 nM Gsα stimulated AC activity 20-fold (7.9 nmoles $cAMP \cdot mg^{-1} \cdot min^{-1}$) and this was further enhanced 1.7-fold by 10 µM SDPA (13.4 nmoles $cAMP \cdot mg^{-1} \cdot min^{-1}$). An SDPA concentration-response curve yielded an EC_{50} of 1.2 µM, i.e. similar to those established in HEK293-expressed mAC isoforms (Fig. 6A; compare to Fig. 1 and Appendix figure 3). The data demonstrated that the SDPA effect of GPLs on mAC activities was not due to peculiar membrane properties of the cultured HEK293 cells and supported the suggestion that the effects of GPLs are of more general physiological relevance.

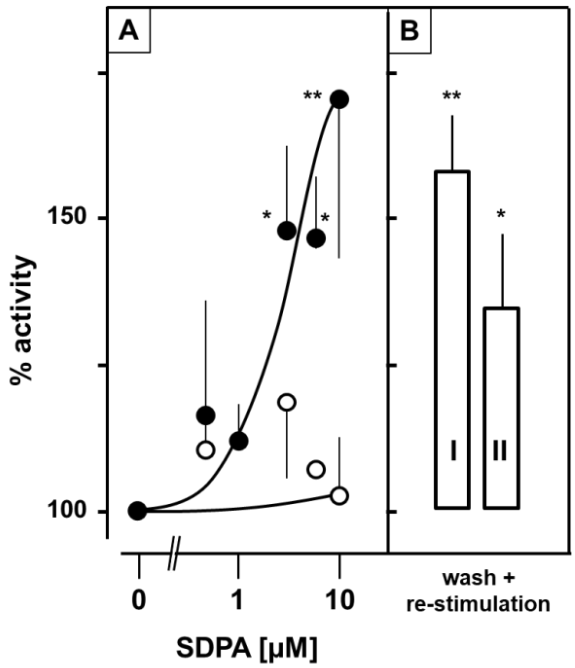


Figure 6. 1-Stearoyl-2-docosahexaenoyl-phosphatidic acid concentration-dependently potentiates Gsα activated adenylyl cyclase activity in brain cortical membranes from mouse. (A) 600 nM Gsα was used to activate mACs in cortical membranes (solid circles: 100 % Gsα-activated activity is 7.9 ± 1.9 nmol cAMP•mg^{-1}•min^{-1}); open circles: basal activity (in absence of Gsα) is 0.3 ± 0.2 nmol cAMP•mg^{-1}•min^{-1}. n = 6. (B) Reversibility of SDPA action. Cortical brain membranes were incubated for 15 min without (I) and with (II) 10 µM SDPA. Membranes were then collected by centrifugation, and re-assayed. I: control membranes + 600 nM Gsα + 10 µM SDPA; II: membranes incubated with 10 µM SDPA, collected and re-assayed with 600 nM Gsα + 10 µM SDPA. Error bars denote S.E.M., *: $p< 0.05$; **: $p< 0.01$ (n=6).

The next question was whether SDPA acts directly from the extracellular site via the membrane-receptor domain of mAC3 or is a cytosolic effector. We used HEK293 cells transfected with mAC3. 10 µM isoproterenol increased cAMP levels from 0.8 to 2.2 pmol/mg within 15 min. SDPA up 50 µM did not affect the isoproterenol stimulation (data not shown). These unequivocal

data virtually excluded that SDPA acted via extracellular binding sites (receptors) or via an efficient and rapid uptake system. The data indicated a cytosolic site for the action of GPLs.

Is SDPA a ligand?

GPLs are common building blocks of cell membranes. The major constituents of the inner leaflet are phosphatidylserines and phosphatidylethanolamines, whereas the predominant lipids of the outer leaflet are phosphatidylcholine and sphingomyelin. Phosphatidic acids are indispensable, yet minor membrane components (Kooijman & Burger, 2009). The potentiation by SDPA of Gsα-activated mAC3 could be due to a lack of SDPA in the vicinity of the membrane-imbedded mACs. Added SDPA might be incorporated into the membrane or inserted into hydrophobic pockets of mACs resulting in an irreversible reordering of mAC domains. Alternatively, SDPA may bind reversibly to the cyclase in a transient manner. Under these latter circumstances the biochemical effect should be reversible. Using mouse cortical brain membranes, we attempted to dissect these possibilities. We incubated membranes for 15 min at 37°C with 10 µM SDPA. The membranes were then collected at 100,000 g and washed once. The pre-treated membranes were susceptible to Gsα stimulation and concomitant potentiation by SDPA like naïve cortical membranes (Fig. 6B). Furthermore, the supernatant of a 50 µM SDPA preincubation was used to potentiate the Gsα activation in naïve membranes, i.e. SDPA was not significantly incorporated into the membrane preparation. The data support the notion that SDPA, and most likely other GPLs, serve as intracellular regulatory effectors for cellular mACs.

Discussion

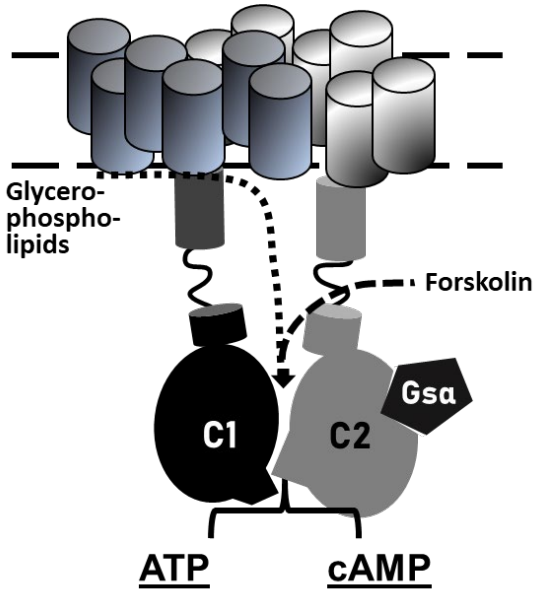
Our results were contrary to the hypothesis at the outset because we expected to find an mAC inhibitory input. Most surprisingly we identified SDPA and other GPLs as positive effectors of

mAC activities. Obviously, we have discovered a new system of intracellular mAC regulation. At this state our findings open more questions than can be answered with this initial report.

We used HEK293 cells permanently transfected with mACs. HEK293 cells express considerable endogenous AC3 and 6 activities (Soto-Velasquez, Hayes et al., 2018). These endogenous mAC activities appear to be negligible in this context. First, upon transfection of mAC isoforms we observed very different basal AC activities virtually excluding that 'contaminating' endogenous AC activities affected our results (see Appendix Table 2 for a list of basal activities in transfected HEK293 cells). Second, we tested HEK293 cells in which mACs 3 and 6 were knocked out (Soto-Velasquez et al., 2018). Upon mAC3 transfection SDPA similarly potentiated Gsα-activated mAC3 activity. Because these engineered cells proliferated rather slowly they were not used routinely.

Diacylglycerols' and PA are lipid second messengers that regulate physiological and pathological processes, e.g. phosphatidic acids were reported to effect ion channel regulation and SDPA to act on the serotonin transporter in the brain (Lu, Murakami et al., 2020, Robinson, Rohacs et al., 2019). So far, the specificity of fatty acyl residues and head groups in these lipids was not explored. Here, we observed a striking exclusivity of fatty-acyl esters at positions 1- and 2- of the glycerol backbone supporting a specific effector-mAC interaction. Usually fatty acyl substitutions are regulated because they impart specific biophysical and biochemical properties (Hishikawa, Valentine et al., 2017). We demonstrated that the combined fatty acyl ligands 1-stearoyl-2-docosahexaenoyl are more or less exclusive for the actions of SDPA. Even seemingly minor changes caused substantial changes in activity and efficacy, e.g. a change from stearoyl to palmitoyl at glycerol position 1 (Fig. 3). This argues for a cooperative interaction between the stearoyl- and docosahexaenoyl carbon chains. Apparently, such interactions are substantially diminished when only one of the two acyl residues is altered. Notably, di-docosahexaenoyl- and

399 di-arachidonoyl-phosphatidic acids (DDPA and DAPA) had mostly lost the capability to promote
400 AC3 activity (Fig. 3). A particularly interesting point is the preference for 2-docosahexaenoyl
401 acylation in the GPLs. Docosahexaenoic acid is an essential omega-3 fatty acid that cannot be
402 synthesized by humans. It is part of a balanced diet and sold as a nutraceutical. A specific
403 transporter for uptake of this fatty acid, packaged as a lysophosphatidic acid, has been thoroughly
404 studied (Cater, Chua et al., 2021, Nguyen, Ma et al., 2014). Docosahexaenoic acid is needed for
405 normal brain development and cognitive functions, a role in depression and Alzheimer's disease
406 is discussed (Duan, Song et al., 2021, Heras-Sandoval, Pedraza-Chaverri et al., 2016, Nguyen et
407 al., 2014, Zhu, Tan et al., 2015). So far, mACs have not yet been noticed in metabolic
408 disturbances caused by a lack of docosahexaenoic acid. The data presented here provides
409 evidence that docosahexaenoic acid stimulates the cAMP generating system.
410



411

412 **Figure 7.** Scheme of a 2X6TM-adenylyl cyclase with regulatory input from Gsα , binding to the
413 C2 catalytic domain, forskolin, binding to a degenerated second substrate-binding site (Guo et al.,

2001, Tesmer & Sprang, 1998), and glycerophospholipids, here proposed to enter and bind at the membrane anchor-receptor and extending towards the catalytic dimer.

Examination of head group specificity displayed different patterns of mAC susceptibility and activity (Fig. 5). Notably, mAC isoforms 1 and 2 were not significantly affected by any of the GPLs assayed. This may be due to a general insensitivity for GPLs or that we did not identify the suitable bioactive GPLs. We did not examine the specificity of acyl substitution at the glycerol backbone in SDPS, SDPE, SDPG and SDPC because of the specificity of the stearic-/docosahexaenoic acid couple in SDPA. We tested 1-stearoyl-2-arachidonoyl-phosphatidyl choline and the corresponding phosphatidyl-ethanolamine. Biological activity was absent with mACs 3, 5, 7, and 9, bolstering the assertion that fatty acyl specificity is stringent in these GPLs as well. Presently we cannot completely exclude that GPLs acylated by different couples of acid substituents at the 1- and 2-positions might possess equal or better effector properties. In view of the large variety of GPLs this cannot be tested with a reasonable effort. Currently, we consider such a possibility as remote. We do not know how GPL's mechanistically potentiate AC activity in a synergistic interaction together with Gsα. The scheme in Fig. 7 is intended to illustrate an approximation of the potential interaction sites in relation to Gsα and forskolin (see below). Another question which is not answered in this study concerns the intracellular origin of GPLs, how their biosynthesis and release is regulated and tied into the cAMP regulatory system. Despite being water insoluble, an efficient traffic of phospholipids in cells exists, e.g. between locations of uptake and biosynthesis, to and from low-density-, high-density- and very low density lipoproteins, and the diversity of membrane-enclosed organelles such as mitochondria, nucleus, endoplasmic reticulum, endosomes, lysosomes, and the plasma membrane itself. Thus, lipid trafficking is a permanent cellular process connected to diverse signaling systems (Hishikawa et

al., 2017). Part of the biosynthetic pathways for phosphatidic acid is the hydrolysis of GPLs with choline, ethanolamine or serine as headgroups by phospholipase D which generates phosphatidic acids. (Jang, Lee et al., 2012). Chemically, GPLs are excellently suited to serve as mAC effectors because termination of SDPA signaling is easily accomplished by phospholipase C. The relationship between SDPA and forskolin is debatable. The agents do not cooperatively interact at mAC proteins. Certainly, the structural changes caused by either agent promote the interactions between AC and Gsα. Yet this is no proof that such changes are identical or even similar.

A critical observation was the potentiation by SDPA of Gsα-activated mAC activity in mouse brain cortical membranes. mAC3 has been reported to be abundantly expressed in brain (Ludwig & Seuwen, 2002, Sanabra & Mengod, 2011). The efficacy of SDPA was comparable to that determined in mAC3-HEK293 membranes. This demonstrated that the effect of GPLs observed in HEK293 expressed AC isoforms is of physiological significance. Our approach has then discovered intracellular processes which in conjunction with the established canonical GPCR/Gsα-regulation of mACs add a new dimension of mAC regulation. Currently, we cannot exclude the possibility that other GPLs exist which have an inhibitory input. Actually, thermodynamic considerations would argue in favor of such a possibility. Whether this is realized as a biological mechanism remains an open possibility. Presently, many important questions remain unanswered, such as how are intracellular GPL levels regulated, which of the intracellular GPLs have access to the membrane-delimited ACs, are GPL concentrations persistently or acutely adjusted in a cell, e.g. by stress, diet, diurnal or seasonal effects or by peculiar disease states? In other word, are we dealing with a long-term regulation of the Gsα-sensitivity of the cAMP generating system or with coordinated short term signaling events? Answering these medically relevant questions remains a formidable challenge in the future.

Materials and methods

The genes of the human AC isoforms 1 – 9 cloned into the expression plasmid pcDNA3.1+/C-(K)-DYK were purchased from GenScript and contained a C-terminal flag-tag. Creatine kinase was purchased from Sigma, restriction enzymes from New England Biolabs or Roche Molecular. All chemicals were from Avanti Lipids and Sigma-Merck. The constitutively active GsαQ227L point mutant was expressed and purified as described earlier (Diel, Klass et al., 2006, Graziano, Freissmuth et al., 1989, Graziano et al., 1991). Forskolin was a gift from Hoechst, Frankfurt, Germany. Human serum (catalog # 4522 from human male AB plasma) and fetal bovine serum were from Gibco, Life Technologies, Darmstadt, Germany (catalog #: 10270; lot number: 42Q8269K).

Plasmid construction and Protein Expression

ACIC1_ACIIC2 was generated in pQE60 with NcoI/HindIII restrictions sites according to Tang et al. (Tang & Gilman, 1995). The construct boundaries were: $MRGSH_6$-HA-hAC1-$C1_{M268–R482}$-AAAGGMPPAAAGGM -hAC2-$C2_{R822-S1091}$. HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum at 37°C with 5% CO2. Transfection of HEK293 cells with single mAC plasmids was with PolyJet (SignaGen, Frederick, MD, USA). Permanent cell lines were generated by selection for 7 days with G418 (600 µg/mL) and maintained with 300 µg/mL G418 (Baldwin, Li et al., 2019, Cumbay & Watts, 2004, Soto-Velasquez et al., 2018). For membrane preparation cells were tyrpsinized and collected by centrifugation (3,000xg, 5 min). Cells were lysed and homogenized in 20 mM HEPES, pH 7.5, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT, and one tablet of cOmplete, EDTA-free (for 50 mL), 250 mM sucrose by 20 strokes in a potter homogenizer. Debris was removed by centrifugation for 5 min at 1,000 x g, membranes were then collected by centrifugation at 100,000 x g for 60 min at

0°C, resuspended and stored at -80°C in 20 mM MOPS, pH 7.5, 0.5 mM EDTA, 2 mM $MgCl_2$. Expression was checked by Western blotting.

Membrane preparation from mouse brain cortex was according to (Schultz & Schmidt, 1987, Seth et al., 2020).

Adenylyl cyclase assay

AC activities were determined in a volume of 10 µl using 1 mM ATP, 2 mM $MgCl_2$, 3 mM creatine phosphate, 60 µg/ml creatine kinase, 50 mM MOPS, pH 7.5 using cAMP assay kit from Cisbio (Codolet, France) according to the supplier's instructions. For each assay a cAMP standard curve was established (Seth et al., 2020). Lipids were dissolved in 100% ethanol or DMSO at high concentrations and acutely diluted in 20 mM MOPS pH 7.5 at concentrations which limited organic solvent in the assay at maximally 1%.

Lipidomic analysis

Lipids were extracted from MonoQ purified aqueous fractions by methyl-*tert*-butyl ether / methanol as described (Matyash et al., 2008) after adjusting their pH to 1.0 and 6.0, respectively. The collected extracts were dried under vacuum, and re-dissolved in 500µl of water / acetonitrile 1:1 (v/v). Lipids were analyzed by LC-MS/MS on a Xevo G2-S QTof (Waters) mass spectrometer interfaced to Agilent 1200 liquid chromatograph. Lipids were separated on a Cortecs C18 2.7 µm beads; 2.1 mm ID x 100 mm (Waters) using a mobile phase gradient: solvent A: 50% aqueous acetonitrile; solvent B: 25% of acetonitrile in isopropanol; both A and B contained 0.1% formic acid (v/v) and 10 mM ammonium formate. The linear gradient was delivered with flow rate of 300 µl /min in 0 min to 12 min from 20% to 100 % B; from 12 min to 17 min maintained at 100% B, and from 17 min to 25min at 20% B. Mass spectra were acquired within the range of *m/z* 50 to m/z 1200 at the mass resolution of 20 000 (FWHM). The chromatogram was searched against web-accessible XCMS compound database at

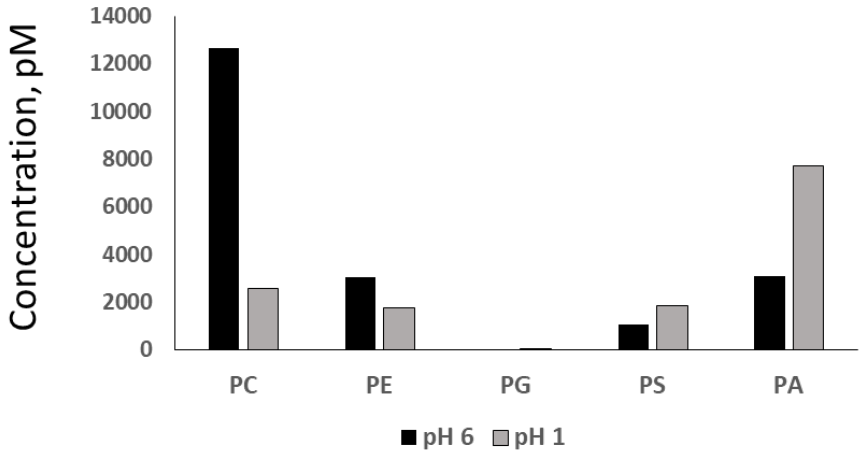
509 https://xcmsonline.scripps.edu/landing_page.php?pgcontent=mainPage. Lipids were quantified
510 using Skyline 21.1.0.278 software using synthetic lipid standards (Vvedenskaya et al., 2021)
511 spiked into the analyzed fractions prior lipid extraction.
512

513 ## Data analysis and statistical analysis

514 All incubations were in duplicates or triplicates. Usually, S.E.M values are given. Data analysis
515 was with GraphPad prism 8.1.2.
516

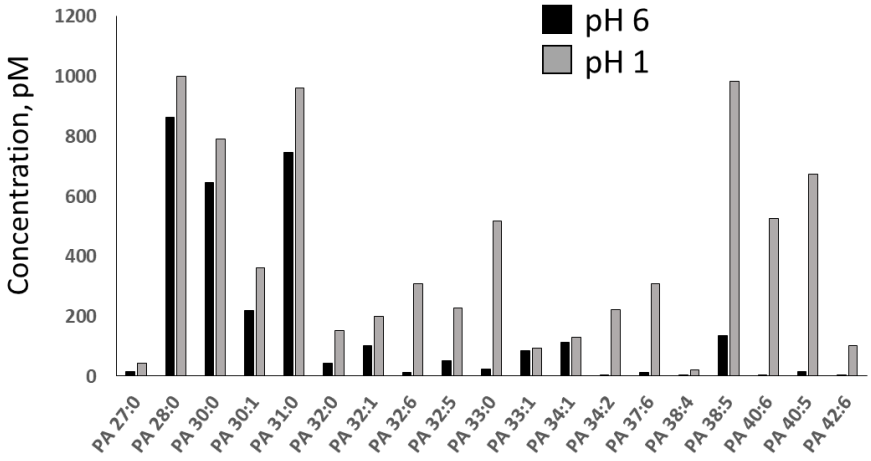
Supplemental Material

Appendix Figure 1



Lipid class composition of MTBE / methanol extracts. MonoQ-purified fractions were extracted at pH 1.0 and pH 6.0. Expectantly, the extract recovered under acidic conditions was enriched with PA. Y-axis: total abundance of lipid classes, pmol/L (n=2)

Appendix Figure 2



Molecular composition of PA species extracted by MTBE / methanol from the fractions with pH 6.0 and pH 1.0. Acidic extraction increased the recovery of PA by more than 2-fold and also enriched the extract with the molecular species comprising long polyunsaturated fatty acid moieties. Y-axes: molar abundance of lipid species, in pmol/L (n=2)

Appendix Table 1:

List of lipids tested:

from Avanti lipids:

- 131303P Cerebrosides
- 131305P Sulfatides
- 800818C-1-stearoyl-2-arachidonoyl-sn-glycerol
- 800819 --stearoyl-2-docosahexaenoyl-sn-glycerol
- 830855C 1,2-dipalmitoyl-sn-glycero-3-phosphate
- 840051P L-α-phosphatidylcholine (Egg, Chicken)
- 840055C L-α-phosphatidylcholine (Liver, Bovine)
- 840065C 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phospho-L-serine
- 840101C L-α-phosphatidic acid (Egg, Chicken) (sodium salt)
- 840859C 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphate (sodium salt)
- 840860C 1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphate (sodium salt)
- 840862C 1-stearoyl-2-linoleoyl-sn-glycero-3-phosphate (sodium salt)
- 840863C 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphate (sodium salt)
- 840864C 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphate (sodium salt)
- 840875C 1,2-dioleoyl-sn-glycero-3-phosphate (sodium salt)
- 840885C 1,2-dilinoleoyl-sn-glycero-3-phosphate (sodium salt)
- 840886C 1,2-diarachidonoyl-sn-glycero-3-phosphate (sodium salt)
- 840887C 1,2-didocosahexaenoyl-sn-glycero-3-phosphate (sodium salt)
- 850469C 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine
- 850472C 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine
- 850804C 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphoethanolamine
- 850806C 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphoethanolamine
- 850852C 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N,N-dimethyl
- 857130P 1-oleoyl-2-hydroxy-sn-glycero-3-phosphate (sodium salt)
- 857328P 1-oleoyl-sn-glycero-2,3-cyclic-phosphate (ammonium salt)
- 860053P total ganglioside extract (Brain, Porcine-Ammonium Salt)
- 860492 Sphingosine-1-phosphate; D-erythro-sphingosine-1-phosphate

- LIPOID (Heidelberg) has donated the following lipids:

- 30. 556200Lipoid PC 14:0/14:0; 1,2-Dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC)
- 31. 556300Lipoid PC 16:0/16:0;1,2-Dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC)
- 32. 556500 Lipoid PC 18:0/18:0; 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC)
- 33. 556600 Lipoid PC 18:1/18:1; 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC)
- 34. 556400 Lipoid PC 16:0/18:1; 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)
- 35. 557100 Lipoid PC 22:1/22:1; 1,2-Dierucoyl-sn-glycero-3-phosphocholine (DEPC)
- 36. 566300 Lipoid PA 16:0/16:0; 1,2-Dipalmitoyl-sn-glycero-3-phosphate, mono-sodium salt (DPPA-Na)
- 37. 567600 Lipoid PS 18:1/18:1; 1,2-Dioleoyl-sn-glycero-3-phosphoserine, sodium salt (DOPS-Na)
- 38. 560200 Lipoid PG 14:0/14:0; 1,2-Dimyristoyl-sn-glycero-3-phospho-rac-glycerol-Na (DMPG)
- 39. 560300 Lipoid PG 16:0/16:0; 1,2-Dipalmitoyl-sn-glycero-3-phospho-rac-glycerol-Na (DPPG)
- 40. 560400 Lipoid PG 18:0/18:0; 1,2-Distearoyl-sn-glycero-3-phospho-rac-glycerol-Na (DSPG)
- 41. 565600 Lipoid PE 14:0/14:0; 1,2-Dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE)
- 42. 565300 Lipoid PE 16:0/16:0; 1,2-Dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE)
- 43. 565400 Lipoid PE 18:0/18:0; 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (DSPE)
- 44. 565600 Lipoid PE 18:1/18:1; 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)

602 Appendix table 2:

603

604 Effect of bulk lipids on Gsα-activated AC3 activity.

605

606 The molecular weights given by the vendor is list below the lipid class.

607 Numbers indicate ranges

608

609

mAC activities in HEK293 cell membranes transfected with human mAC3		
hAC3, N=2-9	**nmol cAMP•mg^{-1}•min^{-1} (range)**	
	+0.6 µM Gsα	**+10 µM Lipid**
Egg L-α-phosphatidic acid (MW 706)	0.88/0.85	1.56/1.94
Egg L-α-phosphatidylcholine (MW 770)	0.87/0.92	0.80/0.86
Liver L-α-phosphatidylcholine (MW 787)	0.3	0.34
Brain Ganglioside (MW 1563)	0.32/0.20	0.16/0.37
Brain Sulfatides (MW 898)	0.25/0.23	0.05/0.08
Brain Cerebrosides (MW 792)	0.27	0.28

610

611

Appendix Table 3 (basal and Gsα-activated mAC activities)

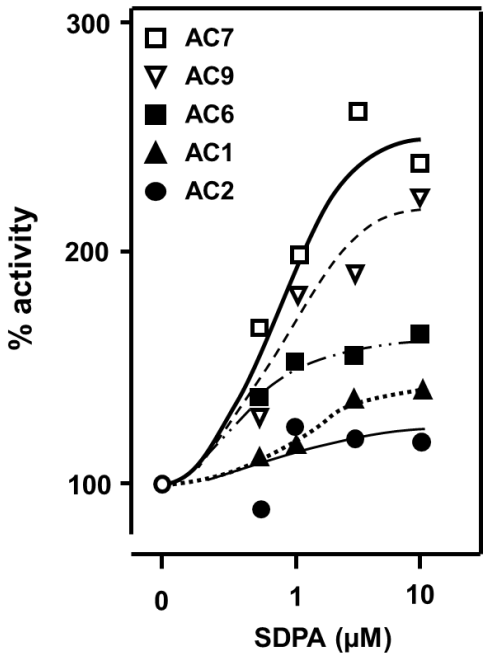
mAC activities in HEK293 cell membranes transfected with human mAC isoforms

nmol cAMP•mg^{-1}•min^{-1}

	basal activity	+ 0.6 µM Gsα	
HEK293 AC1	0.16	0.71	(4-fold)
HEK293 AC2	0.34	5.17	(15-fold)
HEK293 AC3	0.03	0.55	(16-fold)
HEK293 AC4	0.02	0.2	(9-fold)
HEK293 AC5	0.07	2.46	(37-fold)
HEK293 AC6	0.08	1.41	(18-fold)
HEK293 AC7	0.03	0.19	(7-fold)
HEK293 AC8	0.15	1.08	(7-fold)
HEK293 AC9	0.03	1.87	(71-fold)

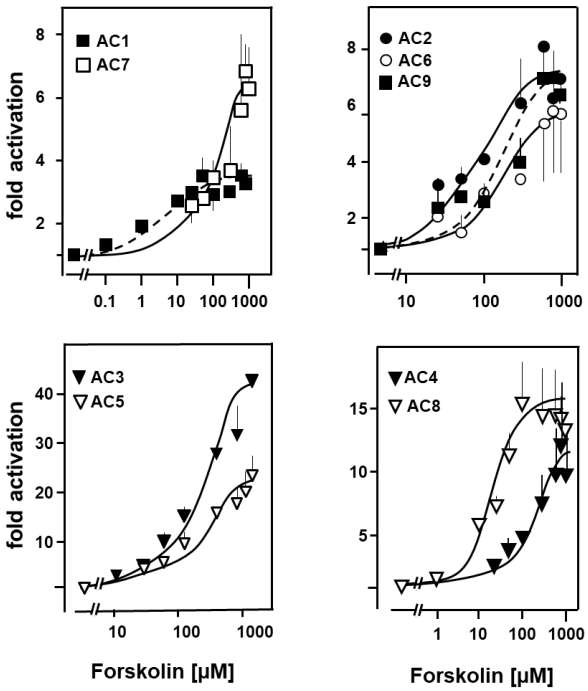
n= 7-12

Appendix Figure 3



Concentration-response curves for SDPA potentiation of mAC isoforms 7, 9, 6, 1, and 2. Basal and Gsα-activated activities are listed in Appendix table 3.

655 **Appendix Figure 4:**



AC	EC_{50} [µM]
AC1	2
AC2	91
AC3	240
AC4	233
AC5	233
AC6	479
AC7	513
AC8	21
AC9	417

656

657

658 Forskolin concentration-response curves for the nine human mAC isoforms expressed in

659 HEK293 cells. Error bars denote S.E.M. The calculated EC50 concentrations are listed at right.

660

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Max-Planck-Gesellschaft		Anubha Seth Sherif Elsabbagh

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Author contributions

AS, ML, AS, acquisition of data, analysis and interpretation of data; SE, transfection and culture of permanent HEK293 cell lines with human adenylyl cyclases , AS, ST, lipidomic analysis and interpretation of data, JES, conception and design, analysis and interpretation of data, design of figures, writing manuscript.

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