Distinct glycerophospholipids potentiate Gsα-activated adenylyl cyclase activity

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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). Lipids extracted from fetal bovine serum at pH 1 inhibited several mAC activities. Guided by a lipidomic analysis we tested glycerophospholipids as potential ligands. Contrary to expectations 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 glycerol positions 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 appears 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. Unexpectedly, SDPA did not affect cAMP generation in HEK293 cells stimulated by isoproterenol, PGE_2 and adenosine, virtually excluding a role as an extracellular ligand and, instead, suggesting an intracellular role. In summary, we discovered a new dimension of intracellular AC regulation by chemically defined glycerophospholipids.

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

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-bound 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 accepted regulation is indirect and includes *(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). Secondarily, calmodulin, Ca^{2+} ions, $G_{\beta\gamma}$ and phosphorylation are cytosolic effectors. In contrast, we recently have assigned a direct regulatory role mediated by the membrane domains of mACs acting 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)* on 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 lipophilic 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 fractionated 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

In exploratory experiments, 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 residues were dissolved in DMSO and tested against human mAC isoforms 1, 2, 3, 5, 6, 7, 8, and 9 in membrane preparations from HEK293 cells transfected with the respective ACs (Table 1). The pH 1 extract inhibited Gsα-activated AC 1, 2, 5, 6, and 7 to different extents. The pH 6 and pH 14 extracts appeared to enhance Gsα-activated AC isoforms 2, 3, 8, and 9 (table 1).

Table 1.

Lipid Extraction of FBS with Chloroform / Methanol

% Gsα activated Adenylyl Cyclase Activity

	pH 14	pH 6	pH 1
hAC1	99±5	91±5	25±1
hAC2	176±8	158±4	149±9
hAC3	183±40	175±41	31±11
hAC5	98±7	123±12	50±7
hAC6	91±4	89±2	57±4
hAC7	140±6	131±4	79±6
hAC8	192±11	193±7	115±4
hAC9	201±22	251±12	140±12

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 extracts were added. Basal AC activities were in the order of the table: 0.11, 0.43, 0.02, 0.4, 0.16, 0.02, 0.33, and 0.04 nmol $cAMP \cdot mg^{-1} \cdot min^{-1}$, respectively. 600 nM Gsα-activated activities were 0.49, 1.31, 0.36, 2.23, 0.71, 0.25, 3.16 and 1.67 nmol $cAMP \cdot mg^{-1} \cdot min^{-1}$. n = 4 to 12.

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 previous data we expected potential ligands which inhibit Gsα-activated mAC activities and concentrated on lipids present in the pH 1 fraction (Seth et al., 2020). Apart from several minor 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. Egg

131 phosphatidic acids significantly stimulated, whereas other bulk lipids such as egg and liver
132 phosphatidylcholine, brain gangliosides, sulfatides and cerebrosides had no significant effects.
133 The lipidomic analysis showed that highly unsaturated fatty acids such as arachidonic acid and
134 docosahexaenoic acid are prominent acyl substituents in phosphatidic acids (Appendix Fig. 2).
135 These acyl residues are only minor components in the tested egg or liver phosphatidic acids.
136 Therefore, we assayed commercially available synthetic GPLs containing polyunsaturated fatty
137 acids as acyl substituents. The general structure of glycerophospholipids is shown below (see
138 Appendix Table 1 for a complete list of lipids examined in this study).
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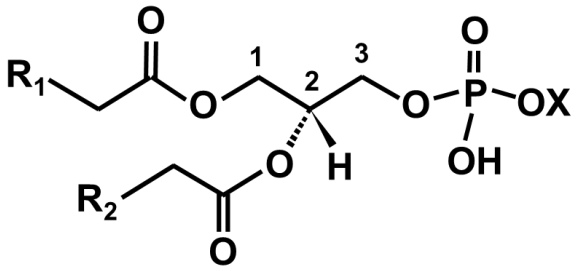

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141 Basic structure of glycerophospholipids: $R_1$and R_2 are fatty acyl residues esterified at glycerol
142 positions 1 and 2; X can be a proton H^+ as in phosphatidic acid, choline (phosphatidylcholine),
143 serine (phosphatidylserine), glycerol (phosphatidylglycerol), or ethanolamine
144 (phosphatidylethanolamine).
145
146
147 The assays used membranes containing human mAC isoforms expressed in HEK293 cells. The
148 mACs were activated by 600 nM of a constitutively active Gsα (Q227L, here termed Gsα)
149 because we expected to characterize an inhibitory input (Graziano, Freissmuth et al., 1991, Seth
150 et al., 2020). Most surprisingly, we discovered that 1-stearoyl-2-docosahexaenoyl-phosphatidic
151 acid (SDPA) potentiated mAC3 up to 7-fold above the 16-fold activation already exerted by 600
152 nM Gsα alone (Fig. 1). The EC_{50} of SDPA was 0.9 µM. In the absence of Gsα 10 µM SDPA had

153 no significant effect (Fig. 1). As far as the synergism between Gsα-activated mAC3 is concerned
154 the effect of SDPA was reminiscent of the known cooperativity between forskolin and Gsα
155 activated mACs (Dessauer et al., 2017).
156 Does the action of SDPA require a membrane-anchored AC holoenzyme or is the activity of a
157 Gsα-activated C1/C2 catalytic dimer potentiated as well? We produced a soluble active AC
158 construct connecting the catalytic C1 domain of mAC1 and the C2 domain of mAC2 by a flexible
159 linker (Tang & Gilman, 1995). The construct was expressed in *E. coli* and purified via its His_6-
160 tag. It was activated 12-fold by Gsα (from 12 to 150 pmol cAMP•mg^{-1}•min^{-1}). SDPA up to 10
161 µM did not affect basal activity and failed to significantly enhance Gsα-activated activity of the
162 chimera. We tentatively conclude that the SDPA action requires membrane anchoring of mACs.
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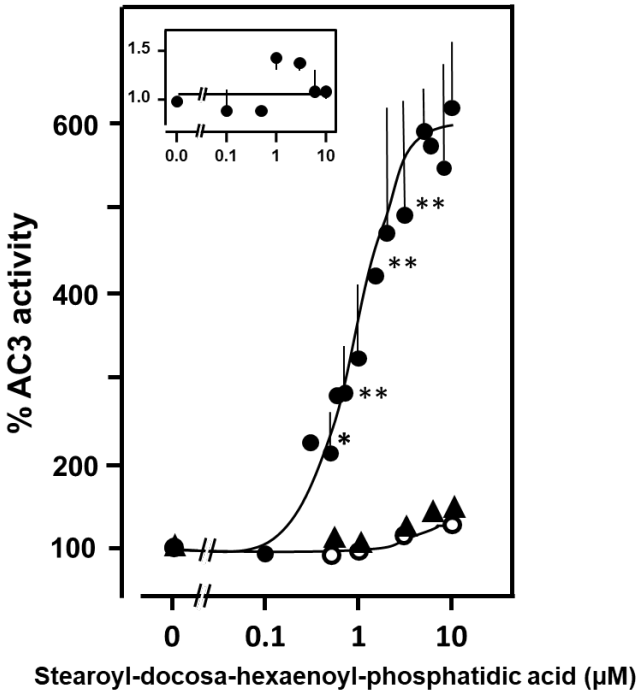

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168 **Figure 1**. 1-Stearoyl-2-docosahexaenoyl-phosphatidic acid concentration-dependently
169 potentiates mAC3 activated by 600 nM Gsα (filled circles). 100 % Gsα-activated mAC3 activity
170 corresponded to 707 ± 187 pmoles cAMP•mg^{-1}•min^{-1}. Basal mAC3 activity is not significantly
171 affected by SDPA (open circles; 100 % basal activity corresponds to 34 pmoles cAMP•mg^{-1}•min^{-}
172 ^{1}). Triangles: Effect of SDPA on the C1-C2 soluble AC construct activated by 600 nM Gsα
173 (Basal activity was 12 pmol cAMP•mg^{-1}•min^{-1}. 600 nM Gsα activated activity was 150 pmol
174 cAMP•mg^{-1}•min^{-1} corresponding to 100 %). Insert: Activity of the mycobacterial AC Rv1625c is
175 unaffected by SDPA (activity was 23 nmoles cAMP•mg^{-1}•min^{-1}). Data were normalized to
176 respective 100 % activities. Significances in a two-tailed t-test: *: $p<0.05$; **: $p<0.01$ compared
177 to 100% activity. For clarity, not all significances are marked. N = 4-6; error bars denote
178 S.E.M.'s.
179

180 We investigated whether SDPA affects the activity of a Gsα-insensitive membrane-bound
181 bacterial AC. We used the mycobacterial AC Rv1625c, a monomeric progenitor of mammalian
182 mACs, which has a hexahelical membrane domain and is active as a dimer (Guo, Seebacher et
183 al., 2001). The activity of the Rv1625c holoenzyme was unaffected by SDPA (Fig. 1 insert). The
184 particular intrinsic properties of the mammalian membrane domains in conjunction with Gsα-
185 activation may be required to confer SDPA sensitivity.
186 Next, we examined which kinetic parameters are affected by SDPA. For mAC3, the enzymatic
187 reaction rates ± SDPA were linear with respect to protein concentration and time up to 30 min.
188 The Km for substrate ATP (0.1 mM) was unaffected. The most striking effect of SDPA was the
189 increase in Vmax (from 4 to 8 nmol cAMP•mg^{-1}•min^{-1}). Concentration-response curves for Gsα
190 in the presence of different SDPA concentrations showed that the affinity of mAC3 for Gsα was
191 significantly increased (Fig. 2). Most likely, Gsα and SDPA act at distinct sites of the protein and
192 potentiation by SDPA is due to concerted structural interactions, reminiscent of the cooperativity
193 between Gsα and forskolin (Dessauer et al., 2017).

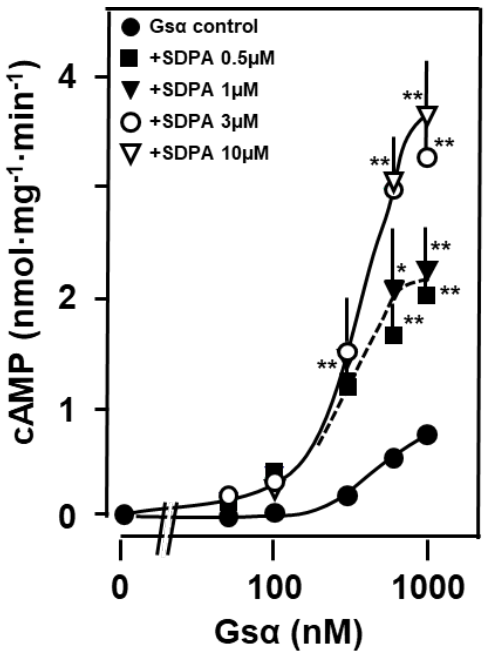
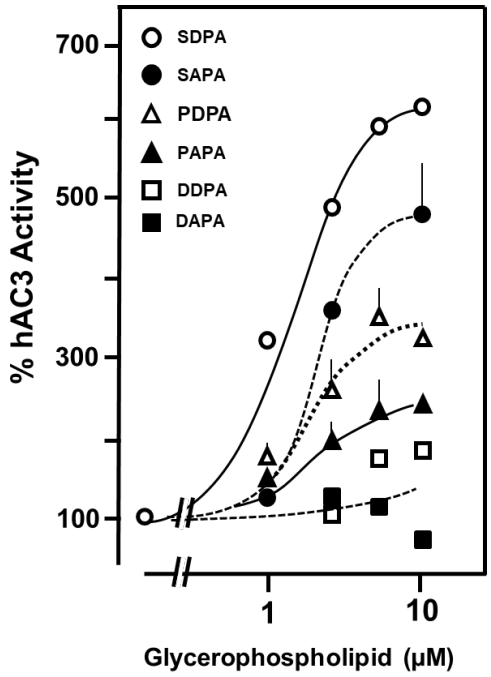


Figure 2. SDPA increases the affinity of mAC3 for Gsα. The EC_{50} concentration for Gsα in the absence of SDPA was 518 nM and in the presence it was 336 ± 29 nM ($p<0.02$; n=5-6). Basal AC3 activity was 27 ± 21 pmoles cAMP•mg^{-1}•min^{-1}; 1000 nM Gsα increased mAC3 activity to 791± 128 pmoles cAMP•mg^{-1}•min^{-1}). Significances: *: $p<0.05$; **: $p<0.01$ compared to corresponding activities without SDPA. n = 5-6; error bars denote S.E.M.. 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. At physiological pH it carries about 1.5 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 substituents in phosphatidic acid (Fig. 3).



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

223

224

225 10 μM 1-Stearoyl-2-arachidonoyl-phosphatidic acid (SAPA) potentiated Gsα-activated mAC3
226 about 5-fold (EC_{50} = 4.8 μM; Fig. 3). Exchanging the stearic acid at position 1 by a palmitic acid,
227 i.e. 1-palmitoyl-2-docosahexaenoyl-phosphatidic acid (PDPA) reduced activity by about 50 %
228 compared to SDPA (EC_{50} = 1.3 μM). Strikingly, the corresponding 1-palmitoyl-2-arachidonoyl-
229 phosphatidic acid (PAPA) lost about 70% of activity compared to SDPA (Fig. 3), highlighting
230 the structural contribution of the 1-fatty acyl substituent to biochemical activity. The importance
231 of the substituent at position 1 was further emphasized when assaying 1, 2-di-docosahexaenoyl-

232 phosphatidic acid (DDPA). The efficiency was reduced by 80 % compared to SDPA (Fig. 3). The
233 EC_{50} for DDPA was 1.4 μM. Even more drastic was the absence of an effect using 1, 2-di-
234 arachidonoyl-phosphatidic acid (DAPA; Fig. 3). Expectedly then, 1-stearoyl-2-linoleoyl-
235 phosphatidic acid was inactive (not shown). The data show a remarkable positional specificity for
236 the 1- and 2-acyl substituents of the glycerol backbone and indicate a specific and concerted
237 interaction between the fatty acyl esters. The specificity of fatty acyl-substitution also strongly
238 indicated that SDPA is not acting in its property as a general membrane GPL because other
239 phosphatidic acids should be equally suitable as membrane lipids. Further, the peculiar
240 biochemical properties of SDPA in its relation with AC isoforms suggest that the negative
241 charges of phosphatidic acid are not sufficient to determine specificity, but that the lipid
242 substitutions on position 1- as well as 2- probably are equally important.

243 **Head group specificity of glycerophospholipids**

244 The next question is whether 1-stearoyl-2-docosahexaenoyl-GPLs with different head groups
245 might affect Gsα-activated mAC3. First, we replaced the phosphate head group in SDPA by
246 phosphoserine generating SDPS. This greatly reduced potentiation of Gsα-activated mAC3
247 activity (2.8-fold potentiation; Fig. 4). A concentration-response curve of SDPS with mAC3
248 showed that the EC_{50} concentration was similar to that of SDPA (1.2 vs 0.9 μM; n=6-9; n.s.), but
249 its efficacy is significantly lower suggesting that identical binding sites are involved.
250 We further used 1-stearoyl-2-docosahexaenoyl-ethanolamine (SDPE), 1-stearoyl-2-
251 docosahexaenoyl-phosphatidylglycerol (SDPG) and 1-stearoyl-2-docosahexaenoyl-
252 phosphatidylcholine (SDPC; Fig. 4). In this order, efficacy to enhance the Gsα-activated mAC3
253 declined, with SDPC having no significant effect (Fig. 4). The surprising specificity of the 1- and
254 2-fatty acyl-substituents of the glycerol backbone was emphasized once again when we used 1-
255 stearoyl-2-arachidonoyl-phosphatidyl-ethanolamine (SAPE) and 1-stearoyl-2-arachidonoyl-

256 phosphatidylcholine. In both instances biochemical activity was lost (not shown). Consequently,
257 we did not further probe GPLs with differing fatty acyl combinations at the glycerol 1- and 2-
258 positions because, as demonstrated, changes in acyl substitutions resulted in considerable
259 reduction or loss of biological activity (see Fig 3).
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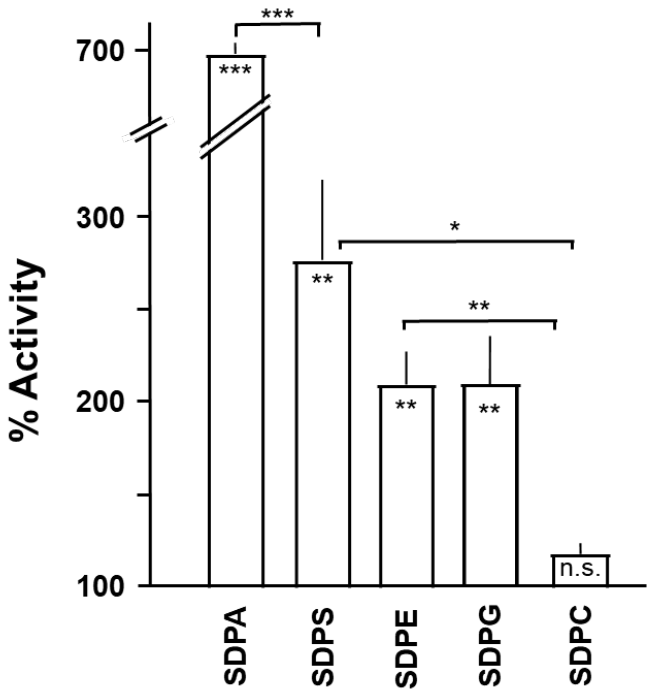

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263
264 **Figure 4.** Head group specificity of glycerophospholipids enhancing Gsα-activated mAC3
265 activity. Basal mAC3 activity was 0.03. Gsα-stimulated activity was 0.56 nmol cAMP•mg^{-1}•min^{-}
266 ^{1} (corresponding to 100 %). Concentration of lipids was 10 µM. Error bars denote S.E.M..
267 Significances: *: $p< 0.05$; **: $p< 0.01$; ***: $p<0.001$; n = 3-9.
268

269 **Effect of glycerophospholipids on Gsα-activated adenylyl cyclase isoforms**

270 So far, we examined only the mAC3 isoform which showed a particularly high synergism
271 between Gsα and SDPA. 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).

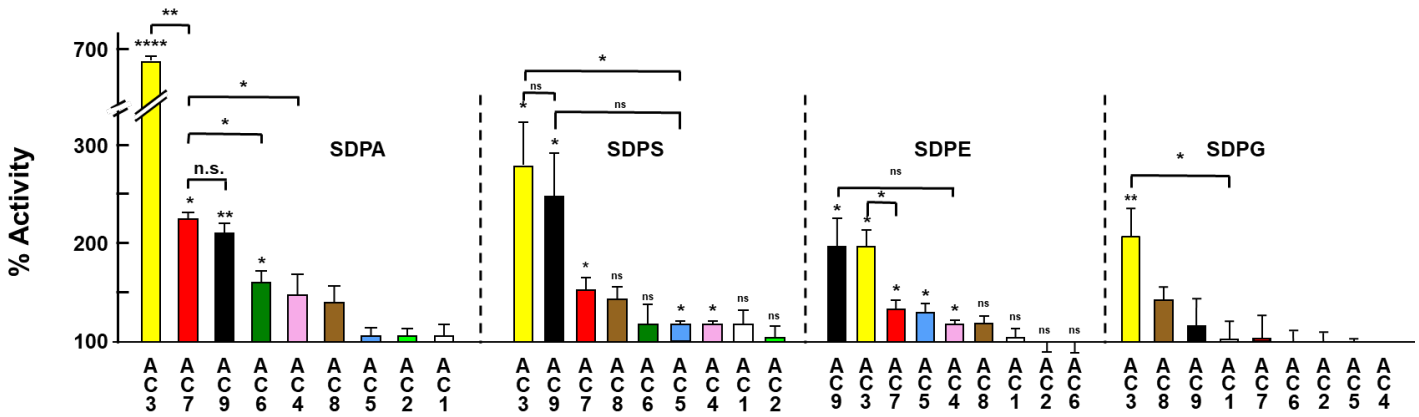

Figure 5. Effect of 10 µM of glycerophospholipids on various mAC isoforms activated by 600 nM Gsα. Basal activities and Gsα-activated activities are listed in Appendix Table 2). Error bars denote S.E.M.. Significances: *: p< 0.05; **: p< 0.01; ***: p<0.001; ****: p<0.0001 compared to Gsα-activated activity (set at 100 %). n = 3-9.

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 suggesting 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 four more stearoyl-2-docosahexaenoyl-GPLs (Fig. 5). 10 µM SDPS potentiated mAC3 and mAC9.

292 Smaller, yet significant effects were measured with mACs 7, 8, 5, and 6 (Fig. 5). 10 µM SDPE
293 significantly potentiated Gsα-activated mAC isoforms 9, 3, 7, 5, and 4 (in this order). 10 µM
294 SDPG significantly enhanced only mAC3 activity (Fig. 5). Compared to the seven-fold effect of
295 SDPA on mAC3 these effects were small, yet, in mammalian biology such enhancements in
296 mAC activity may well have profound physiological consequences. Up to 20 µM SDPC which is
297 a major constituent of the outer leaflet of membranes had no effect on any mAC isoform (not
298 shown). Taken together, the data then demonstrate the capacity of chemically defined GPLs to
299 enhance or potentiate the activation of Gsα-activated mACs. We can virtually exclude
300 coincidental and unspecific effects of the amphiphilic phospholipids because mAC isoforms were
301 affected differentially. The results strongly suggest that a defined conformational space must
302 exist at mACs which allows specific interactions with GPLs. Presently, the molecular details of
303 the binding mode remain unknown.

304 **Relationship between SDPA and forskolin**

305 SDPA failed to activate basal mAC3 activity and only potentiates Gsα-activated mAC3 activity
306 (Fig. 1). The plant diterpene forskolin stimulates basal as well as Gsα-activated mAC activities
307 (Dessauer et al., 2017, Tesmer, Sunahara et al., 1997, Zhang, Liu et al., 1997), i.e. the effects of
308 SDPA and forskolin are only partly similar. Forskolin stimulates mACs expressed in Sf9 cells to
309 rather different extents and with discrepant potencies, e.g. the EC_{50} concentrations for AC1 (0.7
310 µM) and AC2 (8.7 µM) differ more than 12-fold (Pinto, Papa et al., 2008). We established
311 forskolin concentration-response curves for all mAC isoforms expressed in HEK293 cells under
312 identical experimental conditions using Mg^{2+} as divalent cation, a comprehensive study which is
313 lacking so far (Appendix Figure 4).

314 Stimulations at 1 mM forskolin were between 3-fold for mAC1 and remarkable 42-fold for
315 mAC3. The EC_{50} concentrations ranged from 2 µM (mAC1) to 512 µM forskolin (mAC7;

316 Appendix Figure 4). We also observed forskolin activation of mAC9 although the current
317 consensus regarding this isoform is that it is forskolin insensitive. The latter conclusion is based
318 on experiments with mAC9 expressed in insect Sf9 cells using Mn^{2+} as a cation (Dessauer et al.,
319 2017). Another report described forskolin activation of mAC9 when expressed in HEK293
320 (Premont, Matsuoka et al., 1996), in line with our data (Appendix Figure 4). We examined
321 potential interactions between forskolin and SDPA using mAC3 activated by 600 nM Gsα. Up to
322 10 μM, SDPA did not significantly affect forskolin stimulation. We reason that the absence of
323 interactions or cooperativity between forskolin and SDPA suggests that both agents affect mAC
324 regions which exclude mutual cooperative interactions. Nevertheless, considering the structural
325 dissimilarity of forskolin and SDPA and the obvious lack of a molecular fit an identical binding
326 site for both lipophilic agents is rather unlikely. On the other hand, both agents do interact with
327 distantly-binding Gsα in a cooperative manner.
328
329 **SDPA enhances Gsα-stimulated cAMP formation in mouse brain cortical membranes**
330 Above we tested GPLs with individual mAC isoforms. At this point the question is whether
331 SDPA would potentiate mAC activity in membranes isolated from mammalian organs. Several
332 mAC isoforms are expressed in any given tissue and cell, yet the ratios of isoform expression
333 differ. In mouse brain cortex all mAC isoforms with the exception of mAC4 are expressed
334 (Ludwig & Seuwen, 2002, Sanabra & Mengod, 2011). Depending on the expression ratios we
335 might expect at least a moderate potentiation of the Gsα-activated AC activity by SDPA. In
336 mouse cortical membranes the basal AC activity of 0.3 nmoles $cAMP \cdot mg^{-1} \cdot min^{-1}$ was unaffected
337 by 10 μM SDPA (Fig. 6A). 600 nM Gsα stimulated AC activity 20-fold (7.9 nmoles $cAMP \cdot mg^{-1} \cdot min^{-1}$)
338 and this was further enhanced 1.7-fold by 10 μM SDPA (13.4 nmoles $cAMP \cdot mg^{-1} \cdot min^{-1}$).
339 An SDPA concentration-response curve yielded an EC_{50} of 1.2 μM, i.e. similar to those

340 established in HEK293-expressed mAC isoforms (Fig. 6A; compare to Fig. 1 and Appendix
341 Figure 3). The data demonstrated that the SDPA effect of GPLs on mAC activities was not due to
342 peculiar membrane properties of the cultured HEK293 cells and supported the suggestion that the
343 effects of GPLs are of more general physiological relevance.
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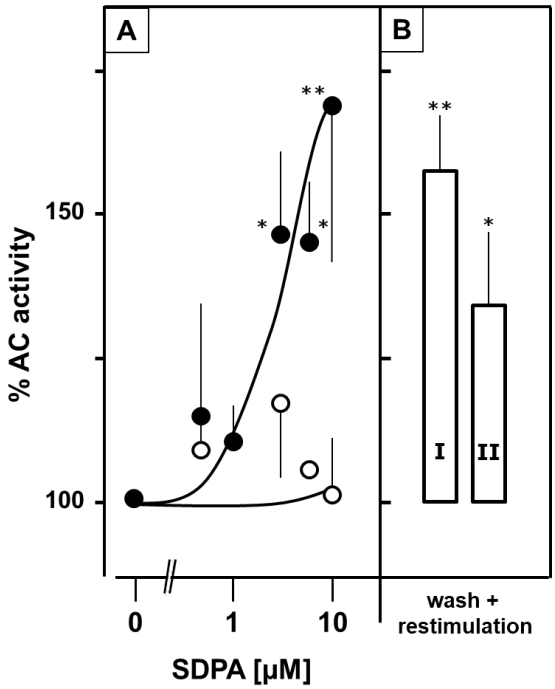

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346 **Figure 6. 1-Stearoyl-2-docosahexaenoyl-phosphatidic acid (SDPA) concentration-**
347 **dependently potentiates Gsα activated adenylyl cyclase activity in brain cortical membranes**
348 **from mouse.** (A) 600 nM Gsα was used to activate mACs in cortical membranes (solid circles:
349 100 % Gsα-activated activity is 7.9 ± 1.9 nmol cAMP•mg^{-1}•min^{-1}); open circles: basal activity (in
350 absence of Gsα) is 0.3 ± 0.2 nmol cAMP•mg^{-1}•min^{-1}. n = 6. (B) Reversibility of SDPA action.
351 Cortical brain membranes were incubated for 15 min without (I) and with (II) 10 µM SDPA.
352 Membranes were then collected by centrifugation, and re-assayed + 600 nM Gsα and 10 µM
353 SDPA. Error bars denote S.E.M., *: $p< 0.05$; **: $p< 0.01$ (n=6).
354
355
356 The next question was whether SDPA acts directly from the extracellular site via the membrane-
357 receptor domain of hAC3 or is a cytosolic effector. We used HEK293 cells transfected with
358 hAC3. 2.5 µM isoproterenol increased cAMP levels from 0.06 to 0.24 pmol/10^4 cells within 45

359 min (6-fold stimulation). Including 10 μM SDPA in the medium did not affect isoproterenol
360 stimulation. Similarly, we used 10 μM adenosine and 1 μM prostaglandin E_2. Addition of SDPA
361 did not enhance intracellular cAMP generation (see Appendix Table 3). These unequivocal data
362 virtually excluded that SDPA acted via extracellular binding sites (receptors) or via an efficient
363 and rapid uptake system. In fact, although SDPA is a GPL, it is unlikely that it can pass into
364 intact cells. First, SDPA has a negatively charged headgroup which at the physiological pH of
365 incubations is dissociated. Second, SDPA might slide into the outer leaflet of the membrane, but
366 it would require a flippase for incorporation into the inner leaflet and potential release into the
367 cytosol. Considering that the inner leaflet of the plasma membrane usually has a negative surface
368 charge due to the predominance of phosphatidylserine we consider this as unlikely. Third, we did
369 not observe significant incorporation of SDPA into brain cortical membranes (see below). Thus,
370 the data tentatively suggest a cytosolic site for the action of GPLs.
371
372 **Is SDPA a ligand?**
373 GPLs are common building blocks of cell membranes. Major constituents of the inner leaflet are
374 phosphatidylserines and phosphatidylethanolamines, whereas the predominant lipids of the outer
375 leaflet are phosphatidylcholine and sphingomyelin. In many tissues docosahexaenoic acid is a
376 major acyl substituent in membrane GPLs (Hishikawa, Valentine et al., 2017). Phosphatidic acids
377 are indispensable, yet minor membrane components (Kooijman & Burger, 2009). The
378 potentiation by SDPA of Gsα-activated mAC3 could be due to a lack of SDPA in the vicinity of
379 the membrane-imbedded mACs. Added SDPA might be incorporated into the membrane or
380 inserted into hydrophobic pockets close to mACs resulting in an irreversible reordering of mAC
381 domains. Alternatively, SDPA may bind reversibly to the cyclase in a transient manner. Under
382 these latter circumstances the biochemical effect should be reversible. Using mouse cortical brain

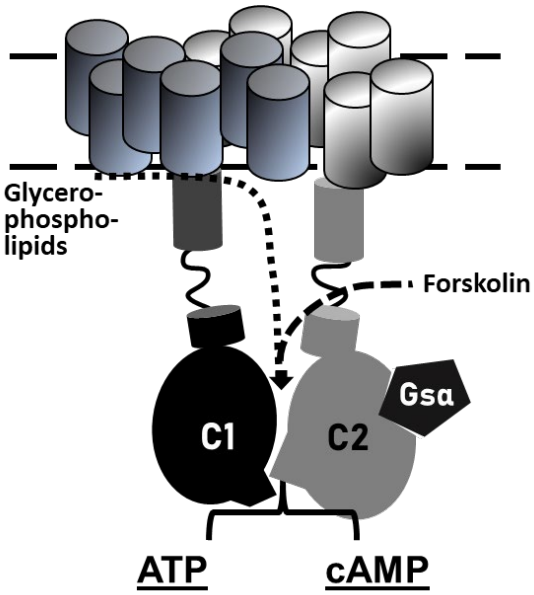
383 membranes, we attempted to dissect these possibilities. We incubated membranes for 15 min at
384 37°C with 10 µM SDPA. The membranes were then collected at 100,000 g and washed once. The
385 pre-treated membranes were susceptible to Gsα stimulation and concomitant potentiation by
386 SDPA like naïve cortical membranes (Fig. 6B). Furthermore, the supernatant of a 50 µM SDPA
387 preincubation was used to potentiate the Gsα activation in naïve membranes, i.e. SDPA was not
388 significantly incorporated into the membrane preparation. The data support the notion that SDPA,
389 and most likely other GPLs, serve as intracellular effectors for mACs.
390

391 **Discussion**

392 Our results were contrary to the hypothesis at the outset because we expected to find an mAC
393 inhibitory input. Most surprisingly we identified SDPA and other GPLs as positive effectors of
394 mAC activities. Obviously, we have discovered a new system of intracellular mAC regulation. At
395 this state our findings open more questions than can be answered with this initial report.
396 We used HEK293 cells permanently transfected with mACs. HEK293 cells express considerable
397 endogenous AC3 and 6 activities (Soto-Velasquez, Hayes et al., 2018). These endogenous mAC
398 activities appear to be negligible in this context. First, upon transfection of mAC isoforms we
399 observed very different basal AC activities virtually excluding that 'contaminating' endogenous
400 AC activities affected our results (see Appendix Table 2 for a list of basal activities in transfected
401 HEK293 cells). Second, we tested HEK293 cells in which mACs 3 and 6 were knocked out
402 (Soto-Velasquez et al., 2018). Upon mAC3 transfection SDPA similarly potentiated Gsα-
403 activated mAC3 activity. Because these engineered cells proliferated rather slowly they were not
404 used routinely.
405 Diacylglycerols' and PA are lipid second messengers that regulate physiological and pathological
406 processes, e.g. phosphatidic acids were reported to effect ion channel regulation and SDPA to act

407 on the serotonin transporter in the brain (Lu, Murakami et al., 2020, Robinson, Rohacs et al.,
408 2019, Shin & Loewen, 2011). So far, the specificity of fatty acyl residues and head groups in
409 these lipids was not explored. Here, we observed a striking exclusivity of fatty-acyl esters at
410 positions 1- and 2- of the glycerol backbone supporting a specific effector-mAC interaction.
411 Usually fatty acyl substitutions are regulated because they impart specific biophysical and
412 biochemical properties (Hishikawa et al., 2017). We demonstrated that the combined fatty acyl
413 ligands 1-stearoyl-2-docosahexaenoyl are more or less exclusive for the actions of SDPA. Even
414 seemingly minor changes caused substantial changes in activity and efficacy, e.g. a change from
415 stearoyl to palmitoyl at glycerol position 1 (Fig. 3). This argues for a specific steric interaction
416 between the flexible stearoyl- and docosahexaenoyl carbon-chains. Acyl chain substitutions
417 might then impair specific protein-ligand interactions, e.g. by a shrinkage of the binding surface.
418 Apparently, such interactions are substantially diminished when only one of the two acyl residues
419 is altered. Notably, di-docosahexaenoyl- and di-arachidonoyl-phosphatidic acids (DDPA and
420 DAPA) had mostly lost the capability to promote AC3 activity (Fig. 3). A particularly interesting
421 point is the preference for 2-docosahexaenoyl acylation in the GPLs. Docosahexaenoic acid is an
422 essential omega-3 fatty acid that cannot be synthesized at adequate quantities in infants or seniors
423 (Qiu, 2003). Therefore it is widely sold as a nutraceutical and should be included into a balanced
424 diet. Docosahexaenoic acid is particularly abundant in membrane lipids in the retina (about 60 %
425 of all lipids contain docosahexaenoic acid), testes, brain, heart and skeletal muscle (Hishikawa et
426 al., 2017) and a sodium-dependent symporter for uptake of this fatty acid, packaged as a
427 lysophosphatidic acid, has been characterized and its structure was elucidated by cryo-EM (Cater,
428 Chua et al., 2021, Nguyen, Ma et al., 2014, Wood, Zhang et al., 2021). Docosahexaenoic acid is
429 needed for normal brain development and cognitive functions, a role in depression, aging and
430 Alzheimer's disease is discussed (Duan, Song et al., 2021, Heras-Sandoval, Pedraza-Chaverri et

431 al., 2016, Hishikawa et al., 2017, Nguyen et al., 2014, Zhu, Tan et al., 2015). So far, mACs have
432 not yet been noticed in metabolic disturbances caused by a lack of docosahexaenoic acid. The
433 data presented here provides evidence that docosahexaenoic acid is involved in stimulating the
434 cAMP generating system.
435



436

437 **Figure 7.** Tentative scheme of a 2X6TM-adenylyl cyclase with regulatory input from Gsα,
438 binding to the C2 catalytic domain, forskolin, binding to a degenerated second substrate-binding
439 site (Guo et al., 2001, Tesmer & Sprang, 1998), and glycerophospholipids, here proposed to enter
440 and bind at the membrane anchor-receptor and extending towards the catalytic dimer.
441

442 Examination of head group specificity displayed different patterns of mAC susceptibility and
443 activity (Fig. 5). Notably, mAC isoforms 1 and 2 were not significantly affected by any of the
444 GPLs assayed. This may be due to a general insensitivity for GPLs or that we did not identify the
445 suitable bioactive GPLs. We did not examine the specificity of acyl substitution at the glycerol
446 backbone in SDPS, SDPE, SDPG and SDPC because of the specificity of the
447 stearic/docosahexaenoic acid couple in SDPA. We tested 1-stearoyl-2-arachidonoyl-phosphatidyl
448 choline and the corresponding phosphatidyl-ethanolamine. Biological activity was absent with

449 mACs 3, 5, 7, and 9, bolstering the assertion that fatty acyl specificity is stringent in these GPLs
450 as well. Presently we cannot completely exclude that GPLs acylated by different couples of acid
451 substituents at the 1- and 2-positions might possess equal or better effector properties. In view of
452 the large variety of GPLs this cannot be tested with a reasonable effort. Currently, we consider
453 such a possibility as remote. We do not know how GPL's mechanistically potentiate AC activity
454 in a synergistic interaction together with Gsα. The tentative scheme in Fig. 7 is intended to
455 illustrate an approximation of potential interaction sites in relation to Gsα and forskolin. The
456 precise nature of such interactions requires structural details (in progress).
457 Another question which is not answered in this study concerns the intracellular origin of GPLs,
458 how their biosynthesis and release is regulated and tied into the cAMP regulatory system. Despite
459 being water insoluble, an efficient traffic of phospholipids in cells exists, e.g. between locations
460 of uptake and biosynthesis, to and from low-density-, high-density- and very low density
461 lipoproteins, and the diversity of membrane-enclosed organelles such as mitochondria, nucleus,
462 endoplasmic reticulum, endosomes, lysosomes, and the plasma membrane itself. Thus, lipid
463 trafficking is a continuous cellular process connected to diverse signaling systems (Hishikawa et
464 al., 2017, Shin & Loewen, 2011). Part of the biosynthetic pathways for phosphatidic acid is the
465 hydrolysis of GPLs with choline, ethanolamine or serine as headgroups by phospholipase D
466 which generates phosphatidic acids (Jang, Lee et al., 2012). Chemically, GPLs are excellently
467 suited to serve as mAC effectors because termination of SDPA signaling is easily accomplished
468 by phospholipase C. The relationship between SDPA and forskolin is debatable. The agents do
469 not cooperatively interact at mAC proteins. Certainly, the structural changes caused by either
470 agent promote the interactions between AC and Gsα. Yet this is no proof that such changes are
471 identical or even similar.

472 A critical observation was the potentiation by SDPA of Gsα-activated mAC activity in mouse
473 brain cortical membranes. mAC3 has been reported to be abundantly expressed in brain (Ludwig
474 & Seuwen, 2002, Sanabra & Mengod, 2011). The efficacy of SDPA was comparable to that
475 determined in mAC3-HEK293 membranes. This demonstrated that the effect of GPLs observed
476 in HEK293 expressed AC isoforms is of physiological significance. Our approach has then
477 discovered intracellular processes which in conjunction with the established canonical
478 GPCR/Gsα-regulation of mACs add a new dimension of mAC regulation. Currently, we cannot
479 exclude the possibility that other GPLs exist which have an inhibitory input. Actually,
480 thermodynamic considerations would argue in favor of such a possibility. Whether this is realized
481 as a biological mechanism remains an open possibility. Presently, many important questions
482 remain unanswered, such as how are intracellular GPL levels regulated, which of the intracellular
483 GPLs have access to the membrane-delimited ACs, are GPL concentrations persistently or
484 acutely adjusted in a cell, e.g. by stress, diet, diurnal or seasonal effects or by peculiar disease
485 states? In other word, are we dealing with a long-term regulation of the Gsα-sensitivity of the
486 cAMP generating system or with coordinated short term signaling events? Answering these
487 medically relevant questions remains a formidable challenge in the future.
488

489 **Materials and methods**

490 The genes of the human AC isoforms 1 – 9 cloned into the expression plasmid pcDNA3.1+/C-
491 (K)-DYK were purchased from GenScript and contained a C-terminal flag-tag. Creatine kinase
492 was purchased from Sigma, restriction enzymes from New England Biolabs or Roche Molecular.
493 All chemicals were from Avanti Lipids and Sigma-Merck. The constitutively active GsαQ227L
494 point mutant was expressed and purified as described earlier (Diel, Klass et al., 2006, Graziano,
495 Freissmuth et al., 1989, Graziano et al., 1991). Forskolin was a gift from Hoechst, Frankfurt,

496 Germany. Human serum (catalog # 4522 from human male AB plasma) and fetal bovine serum
497 were from Gibco, Life Technologies, Darmstadt, Germany (catalog #: 10270; lot number:
498 42Q8269K).

499 **Plasmid construction and Protein Expression**

500 ACIC1_ACIIC2 was generated in pQE60 with NcoI/HindIII restrictions sites according to Tang
501 et al. (Tang & Gilman, 1995). The construct boundaries were: MRGSH$_6$-HA-hAC1-C1$_{M268–R482}$-
502 AAAGGMPPAAAGGM -hAC2-C2$_{R822-S1091}$. HEK293 cells were maintained in Dulbecco's
503 modified Eagle's medium (DMEM) containing 10% fetal bovine serum at 37°C with 5% CO2.
504 Transfection of HEK293 cells with single mAC plasmids was with PolyJet (SignaGen, Frederick,
505 MD, USA). Permanent cell lines were generated by selection for 7 days with G418 (600 µg/mL)
506 and maintained with 300 µg/mL G418 (Baldwin, Li et al., 2019, Cumbay & Watts, 2004, Soto-
507 Velasquez et al., 2018). For membrane preparation cells were tyrpsinized and collected by
508 centrifugation (3,000xg, 5 min). Cells were lysed and homogenized in 20 mM HEPES, pH 7.5, 1
509 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT, and one tablet of cOmplete, EDTA-free (for 50 mL), 250
510 mM sucrose by 20 strokes in a potter homogenizer. Debris was removed by centrifugation for 5
511 min at 1,000 x g, membranes were then collected by centrifugation at 100,000 x g for 60 min at
512 0°C, resuspended and stored at -80°C in 20 mM MOPS, pH 7.5, 0.5 mM EDTA, 2 mM $MgCl_2$.
513 Expression was checked by Western blotting.
514 Membrane preparation from mouse brain cortex was according to (Schultz & Schmidt, 1987,
515 Seth et al., 2020). For each preparation three cerebral cortices were dissected and homogenized in
516 4.5 ml cold 48 mM Tris-HCl, pH 7.4, 12mM $MgC1_2$, and 0.1 mM EGTA with a Polytron hand
517 disperser (Kinematica AG, Switzerland). The homogenate was centrifuged for 15min at 12,000g
518 at 4°C and the pellet was washed once with 5 mL 1 mM potassium bicarbonate. The final
519 suspension in 2 mL 1 mM $KHCO_3$ was stored in aliquots at -80°C.

520 **Adenylyl cyclase assay**

521 AC activities were determined in a volume of 10 µl using 1 mM ATP, 2 mM $MgCl_2$, 3 mM
522 creatine phosphate, 60 µg/ml creatine kinase, 50 mM MOPS, pH 7.5 using the cAMP assay kit
523 from Cisbio (Codolet, France) according to the supplier's instructions. For each assay a cAMP
524 standard curve was established (Seth et al., 2020). Lipids were dissolved in 100% ethanol or
525 DMSO at high concentrations and acutely diluted in 20 mM MOPS pH 7.5 at concentrations
526 which limited organic solvent in the assay at maximally 1%. Up to 2 % neither ethanol nor
527 DMSO had any effect on AC activities.

528

529 **Lipidomic analysis**

530 Lipids were extracted from MonoQ purified aqueous fractions by methyl-*tert*-butyl ether /
531 methanol as described (Matyash et al., 2008) after adjusting their pH to 1.0 and 6.0, respectively.
532 The collected extracts were dried under vacuum, and re-dissolved in 500µl of water / acetonitrile
533 1:1 (v/v). Lipids were analyzed by LC-MS/MS on a Xevo G2-S QTof (Waters) mass
534 spectrometer interfaced to Agilent 1200 liquid chromatograph. Lipids were separated on a
535 Cortecs C18 2.7 µm beads; 2.1 mm ID x 100 mm (Waters) using a mobile phase gradient: solvent
536 A: 50% aqueous acetonitrile; solvent B: 25% of acetonitrile in isopropanol; both A and B
537 contained 0.1% formic acid (v/v) and 10 mM ammonium formate. The linear gradient was
538 delivered with flow rate of 300 µl /min in 0 min to 12 min from 20% to 100 % B; from 12 min to
539 17 min maintained at 100% B, and from 17 min to 25min at 20% B. Mass spectra were acquired
540 within the range of *m/z* 50 to m/z 1200 at the mass resolution of 20 000 (FWHM). The
541 chromatogram was searched against web-accessible XCMS compound database at
542 https://xcmsonline.scripps.edu/landing_page.php?pgcontent=mainPage. Lipids were quantified

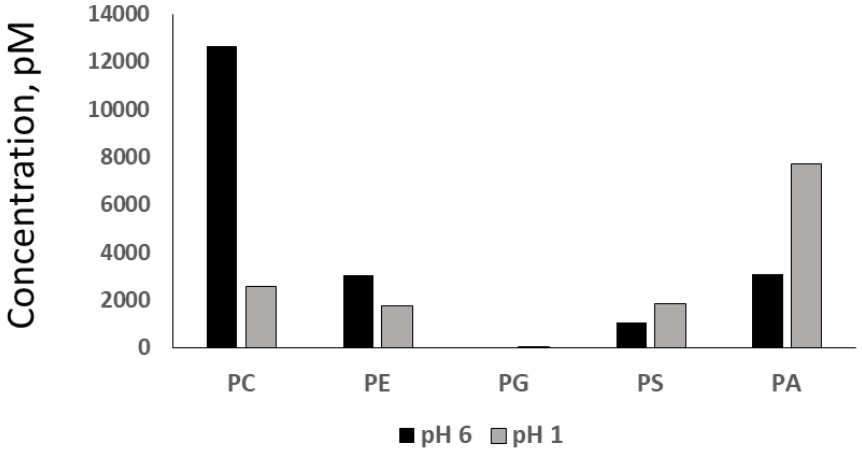
543 using Skyline 21.1.0.278 software using synthetic lipid standards (Vvedenskaya et al., 2021)
544 spiked into the analyzed fractions prior lipid extraction.
545
546 **Data analysis and statistical analysis**
547 All incubations were in duplicates or triplicates. For easier presentations data were normalized to
548 respective controls and n and S.E.M values are indicated in all figures. Data analysis was with
549 GraphPad prism 8.1.2 using a two-tailed t-test.
550

551 **Supplemental Material**

552

553 Appendix Figure 1

554



555

556

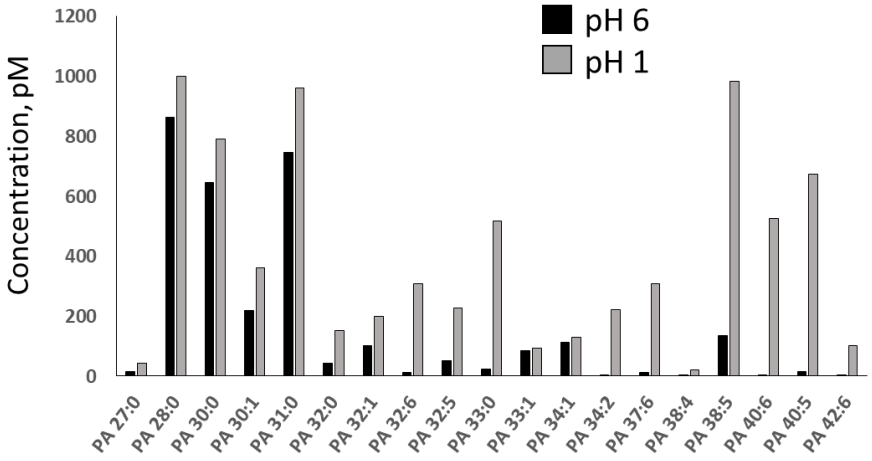
557 Lipid class composition of MTBE / methanol extracts. MonoQ-purified fractions were extracted
558 at pH 1.0 and pH 6.0. Expectantly, the extract recovered under acidic conditions was enriched
559 with PA. Y-axis: total abundance of lipid classes, pmol/L (n=2).

560

561 Appendix Figure 2

562

563



564

565

566 Molecular composition of PA species extracted by MTBE / methanol from the fractions with pH
567 6.0 and pH 1.0. Acidic extraction increased the recovery of PA by more than 2-fold and also
568 enriched the extract with the molecular species comprising long polyunsaturated fatty acid
569 moieties. Y-axes: molar abundance of lipid species, in pmol/L (n=2).

570 **Appendix Table 1:**
571
572 List of lipids tested:
573
574 from Avanti lipids:
575
576 - 131303P Cerebrosides
577 - 131305P Sulfatides
578 - 800818C-1-stearoyl-2-arachidonoyl-sn-glycerol
579 - 800819 --stearoyl-2-docosahexaenoyl-sn-glycerol
580 - 830855C 1,2-dipalmitoyl-sn-glycero-3-phosphate
581 - 840051P L-α-phosphatidylcholine (Egg, Chicken)
582 - 840055C L-α-phosphatidylcholine (Liver, Bovine)
583 - 840065C 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phospho-L-serine
584 - 840101C L-α-phosphatidic acid (Egg, Chicken) (sodium salt)
585 - 840859C 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphate (sodium salt)
586 - 840860C 1-palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphate (sodium salt)
587 - 840862C 1-stearoyl-2-linoleoyl-sn-glycero-3-phosphate (sodium salt)
588 - 840863C 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphate (sodium salt)
589 - 840864C 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphate (sodium salt)
590 - 840875C 1,2-dioleoyl-sn-glycero-3-phosphate (sodium salt)
591 - 840885C 1,2-dilinoleoyl-sn-glycero-3-phosphate (sodium salt)
592 - 840886C 1,2-diarachidonoyl-sn-glycero-3-phosphate (sodium salt)
593 - 840887C 1,2-didocosahexaenoyl-sn-glycero-3-phosphate (sodium salt)
594 - 850469C 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphocholine
595 - 850472C 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine
596 - 850804C 1-stearoyl-2-arachidonoyl-sn-glycero-3-phosphoethanolamine
597 - 850806C 1-stearoyl-2-docosahexaenoyl-sn-glycero-3-phosphoethanolamine
598 - 850852C 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N,N-dimethyl
599 - 857130P 1-oleoyl-2-hydroxy-sn-glycero-3-phosphate (sodium salt)
600 - 857328P 1-oleoyl-sn-glycero-2,3-cyclic-phosphate (ammonium salt)
601 - 860053P total ganglioside extract (Brain, Porcine-Ammonium Salt)
602 - 860492 Sphingosine-1-phosphate; D-erythro-sphingosine-1-phosphate
603
604

- LIPOID (Heidelberg) 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)

636 **Appendix Table 2**

637

638 **mAC activities in HEK293 cell membranes**
639 **transfected with human mAC isoforms**

640

641

642 nmol cAMP•mg^{-1}•min^{-1}

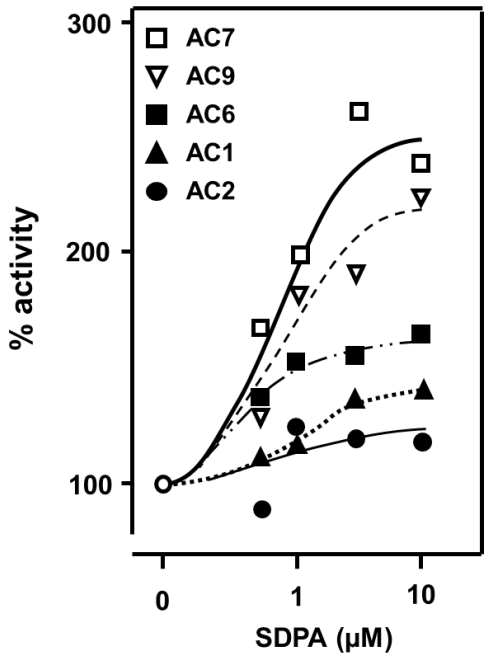
643

	basal activity	+ 0.6 µM Gsα	
HEK293	0.02	0.19	(10-fold)
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)
HEK293ΔAC3,6	0.006	0.06	(10-fold)

(n= 5-12)

670

671 **Appendix Figure 3**



672

673

674 Concentration-response curves for SDPA potentiation of mAC isoforms 7, 9, 6, 1, and 2. Basal
675 and Gsα-activated activities are listed in Appendix table 2. n=2-5.
676

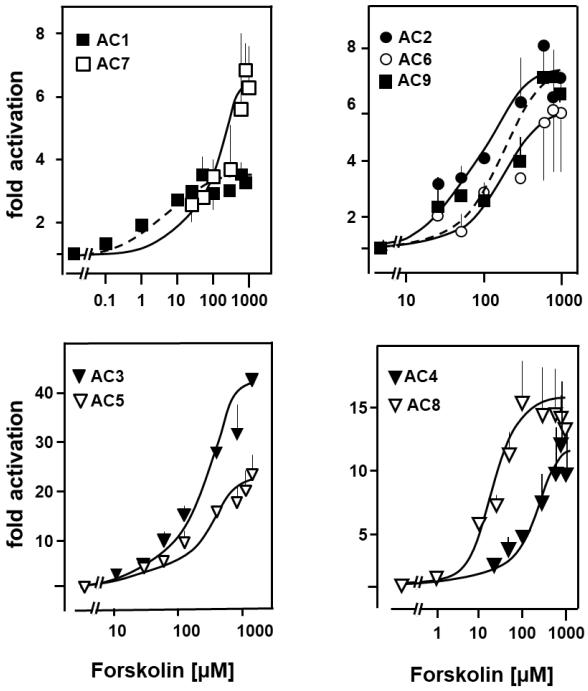
677

678

679
680

681 **Appendix Figure 4:**

682



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

683

684

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

686 HEK293 cells. Error bars denote S.E.M. The calculated EC_{50} concentrations are listed at right. n

687 = 2-4.

688

689

690 **Appendix Table 3**
691
692 With hAC3 transfected HEK293 cells in a 396 well plate were incubated and stimulated at 37°C
693 for 45 min by adenosine, isoproterenol and prostaglandin E_2 ± 10 µM SDPA.
694
695 n = 3 to 4, mean ± S.E.M. Incubations were stopped by addition of detection and lysis buffer of
696 the cAMP assay kit (10 µl/well; Cisbio).
697
698
699
700
701

	pMoles cAMP/ 10^4 cells
basal	**0.06 ± 0.02**
SDPA, 10 µM	**0.02 ± 0.01**
2.5 µM isoproterenol	**0.24 ± 0.03**
2.5 µM isoproterenol + 10 µM SDPA	**0.25 ± 0.03**
1 µM prostaglandin E_2	**0.10 ± 0.01**
1 µM prostaglandin E_2 + 10 µM SDPA	**0.11 ± 0.02**
10 µM adenosine	**0.17 ± 0.07**
10 µM adenosine + 10 µM SDPA	**0.07 ± 0.01**

712
713
714
715 [Please note that isolated HEK293 membrane preparations did not respond to adenosine,
716 isoproterenol or PGE_2]
717

Acknowledgements

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Competing interests: None.

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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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