Muscarinic receptors stimulate AC2 by novel phosphorylation sites, whereas Gβγ subunits exert opposing effects depending on the G-protein source

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INTRODUCTION

cAMP (cyclic AMP) impacts on many aspects of the lives of eukaryotic cells to regulate numerous fundamental physiological functions [1,2]. The precise control of cAMP synthesis and subsequent physiological processes occurs in a cell type-specific manner, and reflects the repertoire of AC (adenyl cyclase) isozymes as well as the influence of regulatory molecules [3–6]. A total of nine membrane-bound ACs have been identified [7,8], which can be distinguished as those that are subject to regulation by Ca2+ and/or calmodulin and those that are not [8]. The closely related AC2, AC4 and AC7 share an insensitivity to Ca2+ and/or calmodulin and those that are not [8]. The closely related AC2, AC4 and AC7 share an insensitivity to Ca2+ (intracellular Ca2+ concentration) rises with a reported regulation by PKC (protein kinase C; except AC4) and stimulation by the βγ subunits of Gi-proteins [7,9]. This potential sensitivity opens the door to the regulation of these enzymes by GPCRs (G-protein-coupled receptors) that do not exclusively act via G-protein α subunits. This sensitivity coupled with their expression in bronchial smooth muscle, hippocampus, pituitary cortex, spinal cord and olfactory system etc. [10–12] renders them important physiological and therapeutic targets.

AC2 was first reported to be activated by phorbol esters, presumed to be acting via PKC-mediated phosphorylation, in 1993 [13–16]. Early studies also suggested that stimulation of AC2 by Gq-coupled receptors occurred by the activation of PKC as a result of the diacylglycerol production that accompanies PIP2 (phosphatidylinositol 4,5-bisphosphate) hydrolysis [17,18]. Using a chimaera of AC1 and AC2, a PKC-responsive region was suggested in the C-terminus of AC2, although it lacked any potential PKC phosphorylation sites, which hinted at an intermediary or facilitatory role for this domain [19]. Subsequently, Pfeuffer and colleagues performed studies on this putative phosphorylation and regulation of AC2, and suggested that Thr1057 (in the C-terminal region) might be the significant PKC phosphorylation site on AC2 [20,21]. In the present study, we have re-addressed the regulation of AC2 by PKC, Gq-coupled muscarinic receptors and Gi-coupled SST (somatostatin) receptors using site-directed mutagenesis incorporating previous and newly predicted phosphorylation sites and single-cell methods. Surprisingly, the T1057A mutant was as responsive to PKC activation as was the wild-type AC2. Therefore other potential phosphorylation sites were considered. Single mutations at two of these potential sites, Ser490 or Ser543, significantly diminished the PKC-stimulated AC2 activity, whereas a double phosphorylation-deficient mutation of these two sites (S490/543A) completely abrogated the sensitivity of AC2 to activation by PKC. A phosphomimetic mutant (S490/543D) was unresponsive to PMA (phorbol myristic acid), but displayed an increased basal activity. These results collectively demonstrate that Ser490 and Ser543 are the significant phosphorylation targets of PKC. Stimulation of AC2 by the Gβγ-linked muscarinic receptor was assessed in single-cell assays. The stimulation observed appeared to occur via these same sites, and to be mediated by PKC activation. Mutation of these sites abrogated the stimulation by CCh (carbachol) and revealed that the Gβγ liberated from CCh binding to muscarinic receptors exerts an inhibitory effect on AC2 activity. By contrast, the consequence of Gβγ released from the Gβγ-linked SST receptor was a stimulation of activity. Thus AC2...
EXPERIMENTAL

Materials

Mouse monoclonal anti-HA (haemagglutinin; H3663) and anti-α-tubulin antibodies (T9026) were from Sigma. Sources for all of the other materials used in the present study have been described previously [22,23].

Constructs

Epac2-camps was from Dr Martin Lohse (University of Würzburg, Würzburg, Germany) [23,24]. Rat HA–M3 muscarinic receptor was kindly provided by Dr Jurgen Wess [NIDDK (National Institute of Diabetes and Digestive and Kidney Diseases), Bethesda, MD, U.S.A.). Gα (transducin) was a gift from Professor Heidi Hamm (Vanderbilt University, Nashville, TN, U.S.A.). Wild-type rat AC2 was subcloned into pcDNA3 vector with an N-terminal HA tag. The potential PKC phosphorylation sites of AC2 were identified using NetPhos 2.0 [25], GPS 2.1 [26], PPSP 1.0 [27] and PhoScan [28]. Any potential PKC phosphorylation site residues in AC2 were substituted to alanine or aspartic acid residues using the Phusion™ high-fidelity polymerase kit (Finnzymes) following the manufacturer’s instructions, and by site-specific mutagenesis according to the QuikChange protocol (Stratagene). All of the AC2 constructs were verified by DNA sequencing.

Cell culture and transfection

HEK (human embryonic kidney)-293 cells (European Collection of Cell Cultures, Porton Down, U.K.) were cultured in MEM (minimal essential medium) supplemented with 10% (v/v) fetal bovine serum, 100 μg/ml streptomycin, 100 units/ml penicillin and 2 mM L-glutamine, and maintained at 37°C (5% CO2). HEK-293 cells were plated on to either 90-mm dishes or 25-mm poly-L-lysine-coated coverslips at 60% confluence 1 day prior to transfection with 1–2 μg of the appropriate constructs using Lipofectamine™ 2000 (Invitrogen) following the manufacturer’s instructions.

To produce cells stably expressing AC2 constructs, HEK-293 cells were plated at ~60% confluence 1 day prior to transfection with 2 μg of constructs. The transfected cells were selected 48 h later by replacing the medium with one containing 800 μg/ml G-418 (Formedium). After selection, cells were maintained in medium with 400 μg/ml G-418.

Cell population measurement of cytosolic Ca2+

HEK-293 cells were incubated with 2 μM fura-2/AM (fura 2 acetoxymethyl ester) and 0.02 % pluronic F-127 for 40 min in the dark at room temperature (20°C), washed twice with Krebs buffer, aliquoted into samples with 4 × 106 cells, and finally resuspended in 3 ml of Ca2+-free Krebs buffer. Fluorescence emission ratios at 340 nm/380 nm were then measured in a PerkinElmer Life Sciences LS50B spectrofluorimeter as described previously [30].

Cell population measurement of cAMP accumulation

Measurement of cAMP accumulation was based on the method of Evans et al. [29]. Transfected cells in 24-well plates were labelled with [2-3H]adenine (1.5 μCi/well) in MEM at 37°C for 90 min. Cells were pre-incubated with 100 μM IBMX (3-isobutyl-1-methylxanthine) for 10 min prior to a 10–15 min assay with the reagents indicated. 3H-nucleotides were separated by Dowex/alumina chromatography, and the cAMP accumulation was normalized as the percentage conversion of [3H]ATP into [3H]cAMP as described previously [30]. To assess the effects of PMA, cells expressing the various constructs were pre-incubated with 100 μM IBMX for 10 min prior to stimulating with 100 nM PMA and 100 nM FSK (forskolin) for 10 min.

Western blotting

Crude membranes of HEK-293 cells were prepared as described previously [31]. A 20 μg aliquot of crude membrane proteins were electrophoretically separated by SDS/PAGE (8 % gel), blotted on to nitrocellulose membranes and probed using anti-HA (1:5000 dilution; Figure 1D, i, upper panel) and anti-tubulin (1:5000 dilution; Figure 1D, i, lower panel) antibodies. The blots were visualized with ECL Plus reagent (GE Healthcare) according to the manufacturer’s guide and the immunoreactive bands were quantified by densitometry using ImageJ (http://rsbweb.nih.gov/ij/).

Single-cell Epac2-camps FRET (fluorescence resonance energy transfer) measurements

Measurement of cAMP in single HEK-293 cells expressing Epac2-camps [24] was performed using an iXon+ EMCCD (electron multiplying charge-coupled device) camera (Andor) and an Optosplit (505DC; Cairn Research) to separate CFP (cyan fluorescent protein; 470 nm) and YFP (yellow fluorescent protein; 535 nm) emission images as described previously [22], and analysed using Metamorph imaging software (Molecular Devices). Emission images at 470 nm and 535 nm were collected every 3 s with a 250 ms integration time. FRET data were plotted as the changes in background-subtracted 470 nm (CFP) compared with 535 nm (YFP) emission ratio relative to the maximum FRET ratio obtained with a cocktail of 10 μM FSK, 10 μM PGE1 (prostaglandin E1), 10 μM ISO (isoproterenol) and 100 μM IBMX. The 4 min AUC (area under the curve) was calculated using GraphPad Prism.

RESULTS

Identification of regulatory PKC phosphorylation sites

Previous studies had indicated that phosphorylation of AC2 by PKC alters the sensitivity of AC2 to different G-proteins, thereby changing the ability of AC2 to integrate signals from multiple hormonal inputs [16,17,32,33]. In an initial attempt to explore the regulation of AC2 by PKC, a single point mutation was introduced at the PKC phosphorylation site proposed earlier (T1057A mutant [20]) and the response to the phorbol ester PMA was compared with the wild-type AC2 in whole-cell-population cAMP accumulation assays (Figure 1A). A priming concentration of FSK (100 nM) was included to enhance the effect of PMA. Unexpectedly, this mutant showed robust PKC stimulation by PMA, comparable with that seen in the wild-type. The inference that Thr1057 is not the major determinant of PKC-mediated AC2 regulation led us to consider other potential PKC phosphorylation sites.

A total of six potential serine/threonine phosphorylation sites in AC2, schematized in Figure 1(B), were predicted and consequently substituted with an alanine residue to preclude their phosphorylation. When expressed in HEK-293 cells and subjected to co-addition of 100 nM PMA (a PKC activator) and 100 nM
Regulation of AC2 by PKC and G-protein-coupled receptors

Figure 1 Screening potential PKC phosphorylation mutants of AC2

(A) Cell population cAMP data testing the effects of 100 nM PMA on the wild-type AC2 compared with the point mutant T1057A in the presence of 100 μM IBMX and 100 nM FSK in a 10 min assay. Results are means ± S.D. for at least 3 independent experiments. (B) Schematic representation of AC2 domains and positions of the potential PKC phosphorylation sites. (C) The effect of mutating each potential phosphorylation site on PKC-stimulated AC2 activity. The numbers at the top of each column represent fold stimulation between 100 nM FSK alone and the co-addition of 100 nM FSK and 100 nM PMA. Results are means ± S.D. for 3 independent experiments. Assays were performed as in (A) and the results are representative of 3 experiments with similar results. (D) i, Western blotting to establish expression levels of the wild-type AC2 and its mutants. The lower blot shows anti-tubulin immunoreactivity as a loading control. #, Glycosylated AC2 monomers; ##, non-glycosylated monomeric AC2. ii, the histogram represents the densitometries of AC2-specific bands relative to those of tubulin, normalized to the AC2 control. Results are means ± S.E.M. for 3 separate experiments.

FSK, four of the mutants, T218A, S654A, S871A and T1057A, displayed a similar (8–10-fold) stimulation relative to 100 nM FSK alone, as did the wild-type AC2. In contrast, the stimulatory efficacy of PMA was severely curtailed to 1.6- and 2.2-fold respectively in S490A- and S543A-transfected cells (Figure 1C). These data clearly indicate that mutation of Ser490 or Ser543 in the C1b domain diminishes PKC-mediated AC2 stimulation (Figure 1C).

To address the possibility that the results from the cAMP accumulation assays were being distorted by differential protein expression, AC2 expression was assessed by Western blotting with a monoclonal mouse HA-specific antibody that recognised the N-terminal HA epitope tag (Figure 1D). AC2 constructs were detected as double bands, which most probably represented both the glycosylated (140 kDa) and non-glycosylated (120 kDa) forms of AC2, as described by Bol et al. [21] (Figure 1D, i). Equal protein loading was confirmed using an α-tubulin antibody (Figure 1D, i, lower panel). Quantification of the densitometries of HA-specific bands, relative to the tubulin signals, revealed similar protein levels among all the AC2 constructs (Figure 1D, ii). Consequently, any varied PMA-stimulated AC2 activity reflects the specific mutation rather than differing protein expression. Thus Ser490 and Ser543 are prime candidates to mediate the effect of PMA on AC2.

Double mutation abolished PKC-mediated AC2 activity

The above findings prompted us to investigate the two potential PKC phosphorylation sites (Ser490 and Ser543) in greater detail. We generated both a phosphorylation-deficient S490/543A mutant and a negatively charged phosphomimetic S490/543D mutant, and assessed their effects on PKC-mediated activation. A higher basal cAMP accumulation was observed in the phosphomimetic S490/543D mutant, compared with wild-type AC2 and the phosphorylation-deficient S490/543A mutant, which is consistent with the phosphomimetic S490/543D mutant mimicking a PMA-stimulated AC2 state at rest (Figure 2A). Wild-type AC2 increased cAMP in a dose-dependent manner following PMA stimulation, which contrasted with the complete lack of effect of PMA on both the phosphorylation-deficient S490/543A and phosphomimetic S490/543D mutants. This observation suggests that the double mutation at Ser490 and Ser543 abolishes PMA-stimulated activity, and renders the protein completely insensitive to PKC-mediated activation. Furthermore, the phosphomimetic S490/543D mutation mimics the phosphorylation state of the wild-type AC2, and makes the protein insensitive to further activation by PKC.

The expression of all of the AC2 constructs was again assessed by Western blotting. A higher expression level of phosphorylation-deficient S490/543A was detected, compared with the wild-type AC2 and the phosphomimetic S490/543D mutant (Figure 2B), relative to the tubulin loading control (Figure 2B, ii). Thus lower protein expression (or increased degradation) does not account for the lack of response to PMA of the phosphorylation-deficient S490/543A construct. Given that the expression levels of the wild-type AC2 and phosphomimetic S490/543D were similar (Figure 2B), the increased basal activity of the phosphomimetic S490/543D mutant seemed likely to be due to pseudo-phosphorylation, rather than increased
HEK-293 cells transfected with the wild-type AC2, the dose–response curves revealed clear synergism between PMA- and FSK-mediated cAMP increase, but only an additive response between PMA and PGE1 stimulation (Figure 3B). In parallel experiments, no further increment in response to PMA was seen in either the phosphorylation-deficient S490/543A or phosphomimetic S490/543D mutants at any FSK or PGE1 concentration (Figures 3C and 3D).

Muscarnic receptor agonist CCh-mediated AC2 activity at the single cell level

A stimulation of AC2 by PKC might be anticipated to arise from the activation of Gα-coupled receptors, as has been previously postulated [17,18]. We wished to assess the significance of the phosphorylation sites we had identified using the more physiological stimulus of the muscarinic Gα-linked receptor agonist, CCh, which can trigger diacylglycerol-dependent PKC activation [34]. The cAMP accumulation assay revealed a significant augmentation in response to 500 μM CCh, but this was extremely modest in comparison with that evoked by PMA (Figure 4A). In the phosphomimetic S490/543D mutant there was no significant increment in cAMP accumulation in response to 500 μM CCh (Figure 4A). This was not due to a lack of protein expression, as the phosphomimetic S490/543D mutant was strongly stimulated by FSK.

Owing to the very small increase in AC2-stimulated cAMP in response to CCh, subsequent experiments utilized a more sensitive single cell analysis using an Epac2-camps FRET-based sensor for cAMP, as we have used previously [23]. In real-time cAMP measurements in both the presence and absence of extracellular Ca2+, cells stably-expressing AC2 showed a rapid increase in cAMP in response to CCh stimulation, and this increase reached maximal levels within 2 min (Figure 4B). In the presence of extracellular Ca2+, the CCh-stimulated cAMP response was potentiated and sustained, probably due to the increased potential for Ca2+-stimulated PKC activation. For ease of interpretation, however, subsequent real-time cAMP measurements were performed in the absence of Ca2+.

We explored the responsiveness of AC2 mutants to stimulation by CCh. Cells expressing either the wild-type AC2 or the T1057A mutant exhibited a comparable and rapid enhancement in cAMP in response to CCh (Figures 4C and 4D). By contrast, there was no cAMP response to CCh in untransfected HEK-293 cells and even a decreased response in cells expressing phosphorylation-deficient S490/543A or phosphomimetic S490/543D. These results established that, as with PMA, Ser490 and Ser543, rather than Thr1057, mediated the effects of CCh on AC2 activity (Figures 4C and 4D). In order to confirm that the changes in FRET that we were recording actually reflected changes in cAMP, the experiments were repeated using a version of the sensor that could not bind cAMP (Supplementary Figure S1 at http://www.BiochemJ.org/bj/447/bj4470393add.htm). As we expected based on previous use of this sensor [23] no changes in cAMP were detected.

By calibrating the Epac2-camps sensors according to a previously described method [23,35], it was possible to approximately compare the absolute cAMP concentrations achieved by the various AC2 mutants. Stimulation with 10 μM CCh (at 60 s) transiently elicited a cAMP concentration of ∼1.3 μM in cells stably expressing AC2 or T1057A (Figure 4E). The basal cAMP concentration in untransfected HEK-293 cells was calculated as ∼250 nM, whereas the concentration in the AC2-, phosphorylation-deficient S490/543A- and

Regulation of the wild-type AC2, phosphorylation-deficient (S490/543A) and phosphomimetic (S490/543D) mutants by FSK and PGE1

A significant feature of the stimulatory effects of PMA on AC2 is a synergistic potentiation by FSK or Gα [13,14]. To explore whether Ser490 and Ser543 also played a role in the synergistic response, dose-dependent stimulation with FSK or PGE1 (acting via Gα) was employed to analyse the co-stimulatory effects of FSK or Gα on PMA-mediated AC2 activation.

There was little if any impact of either FSK or PGE1 on cAMP accumulation in response to PMA treatment of untransfected HEK-293 cells (Figure 3A). By contrast, in
Figure 3  Regulation of phosphorylation-deficient S490/543A and phosphomimetic S490/543D by FSK and PGE₁

Untransfected cells (A) and cells expressing wild-type AC2 (B), phosphorylation-deficient S490/543A (C) and phosphomimetic S490/543D (D) were assayed with increasing concentrations of FSK or PGE₁ for 10 min in the presence and absence of PMA (as indicated) after pre-incubating with 100 μM IBMX for 10 min. Results are means ± S.D. for at least 3 independent experiments.

Thr₁⁰⁵⁷-expressing cells was greater (∼500 nM). Interestingly, the basal cAMP concentration in the phosphomimetic S490/543D-expressing cells was much greater ∼1 μM (Figure 4E). The comparison between the basal and maximal CCh-mediated cAMP concentration change in the AC2 construct-expressing cells confirmed that the CCh-mediated response in AC2 construct-expressing cells is statistically significant in terms of absolute cAMP concentrations (Figure 4F). These cAMP concentration estimations confirmed that the double mutations on Ser⁴⁹⁰ and Ser⁵⁴³ did not affect catalytic activity of AC2, and the phosphomimetic S490/543D mutation possesses a higher basal activity that is refractory to further stimulation by PKC.

CCh-mediated cAMP production occurs via activation of muscarinic receptors

To confirm that the CCh-mediated cAMP changes in cells expressing AC2 constructs is due to activation of muscarinic receptor, the non-selective competitive muscarinic receptor antagonist, atropine, was employed along with CCh. Atropine did not affect cAMP levels of untransfected HEK-293 cells
Figure 4 Stimulation of wild-type and mutant AC2 constructs by the muscarinic receptor agonist CCh at the single-cell level using the FRET-based sensor Epac2-camps

(A) cAMP accumulation was measured in untransfected cells and cells transiently expressing AC2, phosphorylation-deficient S490/S493A or phosphomimetic S490/S493D in response to 500 μM CCh, 100 nM PMA or 10 μM FSK for 10 min in the presence of 100 μM IBMX. Results are means ± S.D. for 3 independent experiments. (B) Stimulation of AC2 stable cells expressing Epac2-camps with 10 μM CCh in the presence of 1 mM Ca2+ or 100 μM EGTA for 4 min before saturating the sensor (Max STIM) by 100 μM IBMX, 10 μM FSK, and 10 μM Iso at 300 s for 2 min. Data are normalized to the maximal signal containing the cocktail to saturate the sensor (n = 43–53). (C) CCh-induced changes in the cAMP response in untransfected HEK-293 cells and cells stably expressing AC2, T1057A, phosphorylation-deficient S490/S493A or phosphomimetic S490/S493D were monitored. Cells were incubated with 10 μM CCh at 60 s for 4 min prior to saturating the probe at 300 s for 2 min. Results are means ± S.E.M. (n = 30–60). **P < 0.01 and ***P < 0.001 using Student’s t test. NS, not significant.

(Figure 5A). Conversely, atropine completely abolished the CCh-induced cAMP increase in cells expressing the wild-type AC2 and T1057A mutant (Figures 5B and 5C), confirming that activation of muscarinic receptors is responsible for CCh-mediated AC2 activity. It is also important to note that the CCh-induced cAMP decline in cells expressing S490/S493A or S490/S493D was also abrogated by atropine (Figures 5D and 5E), which confirmed that those cAMP decrease also were mediated by muscarinic receptors.

Overexpressed M3 receptor enhanced the effects of CCh

To achieve an increased response to CCh, the endogenous M3 receptor of HEK-293 cells was heterologously overexpressed in HEK-293 cells and cells stably expressing AC2 constructs. To confirm that overexpressing M3 receptor (as confirmed by Western blotting, Figure 6D) might enhance CCh-mediated activity, CCh-induced Ca2+ release was compared between cells transfected with or without the M3 receptor. Indeed, overexpressing the M3 receptor enhanced CCh-mediated Ca2+ release in the wild-type HEK-293 and AC2-stable cells (Figures 6A and 6B). However, when cAMP was measured using FRET-based sensors, CCh saturated the Epac2 sensor in the wild-type HEK-293 cells overexpressing the M3 receptor (and also in the other AC2 stable cells, Supplementary Figure S2 at http://www.BiochemJ.org/bj/447/bj4470393add.htm), indicating that very high cAMP concentration was produced by endogenous

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ACs upon CCh stimulation, so that the sensitive cAMP measurement was not appropriate with M3 overexpression. Such experiments were therefore pursued by the less sensitive cAMP population assay. Clearly overexpressing the M3 receptor enhanced the cAMP accumulation in all cell types (Figures 6C). Notably the increased CCh-mediated cAMP accumulation by overexpressing the M3 receptor in cells expressing the PKC phosphorylation-deficient mutant S490/543A and the PKC phosphomimetic mutant S490/543D is higher than the increased CCh-mediated cAMP accumulation by overexpressing M3 receptor in HEK-293 cells (Figure 6C), suggesting that the increased cAMP accumulation is due to the activation of PKC-inactivated AC2 mutants. This suggestion is reinforced by the PKC inhibitor CC (chelerythrine chloride) attenuating only the responsiveness of phosphorylation-deficient S490/543A and phosphomimetic S490/543D (Figure 6C). This observation shows that the responsiveness of phosphorylation-deficient S490/543A and phosphomimetic S490/543D is independent of the PKC pathways and suggests that in M3 receptor-overexpressing cells CCh not only activates the PKC pathway, but also possibly Gsα. It seems not inconceivable that overexpressed M3 receptors could also couple (heterodimerically) with Gs-coupled receptors to yield Gs-coupled signalling.

**CCh-stimulated AC2 activity is mediated through PKC**

To establish that CCh-stimulated AC2 activity is mediated by PKC, we examined the effect of the PKC inhibitor CC [36].
CC did not affect untransfected HEK-293 cells (Figure 7A); however, the magnitude of the CCh response in HE-K293 cells stably expressing AC2 or T1057A was significantly decreased after treatment with the PKC inhibitor, which confirmed the involvement of PKC in the CCh-mediated stimulation of AC2 activity (Figures 7B and 7C). However, the CCh-induced decrease in the cAMP response in the phosphorylation-deficient S490/543A or phosphomimetic S490/543D stably expressing cells was unaffected by CC treatment, which implies that the residual decreased response does not involve PKC (Figures 7D and 7E).

Gβγ associated with Gα-linked muscarinic receptors exerts an inhibitory effect on AC2 activity that is overwhelmed by PKC activation

CCh occupancy of muscarinic receptors not only induces PKC activation, but also liberates G-protein Gβγ subunits. The direct binding of the Gβγ subunits released from activated Gα-coupled receptor has been proposed to enhance the activity of the Ca2+-insensitive AC subgroup (AC2, AC4 and AC7) [37,38]. However, to test for any effect of Gβγ released following activation of Gα, by CCh-stimulation of muscarinic receptors, the Gβγ inhibitor gallein [39] was used. Gallein did not affect untransfected HEK-293 cells (Figure 8A) nor was there any effect on the increased cAMP response induced by CCh in cells expressing AC2 or T1057A (Figures 8B and 8C). However, a CCh-induced decrease in cAMP was seen in cells expressing the phosphorylation-deficient S490/543A and phosphomimetic S490/543D, which was reversed by pre-incubation with gallein (Figures 8D and 8F). To further confirm the apparent Gβγ effect, the Gβγ-sequestering protein Gα (transducin) was overexpressed to inhibit the activation by Gβγ. Like the Gβγ inhibitor gallein, overexpression of Gα precludes the CCh-mediated cAMP decrease in cells expressing the phosphorylation-deficient mutant S490/543A or phosphomimetic mutant S490/543D (Supplementary Figure S3). These findings suggest that the Gβγ released following activation of a Gα-coupled receptor inhibits AC2 activity, in contrast to the Gβγ released following Gβγ activation. The fact that this inhibitory activity was not apparent in cells expressing the wild-type AC2 suggests that the inhibitory response is usually overwhelmed by PKC activation.

Gβγ released from Gα-coupled SST receptor stimulates AC2 activity

To confirm the proposals from the earlier literature and to contrast the effects of Gβγ released from activation of the Gα-coupled SST receptor with Gβγ released from activation of Gα-coupled receptors, the effect of SST was examined on the various AC2 constructs in the presence or absence of gallein at the single-cell level. SST (1 μM) significantly stimulated AC2, T1057A and the phosphorylation-deficient mutant S490/543A activity, whereas the untransfected cells showed no effect (Figures 9A–9D). Furthermore, a larger effect of SST on the phosphomimetic mutant than on the wild-type AC2 was observed (Figures 9B and 9D). Moreover, a larger effect of SST on the phosphomimetic mutant and the phosphorylation-deficient mutant S490/543A activity, whereas the untransfected cells showed no effect (Figures 9A–9D). Furthermore, a larger effect of SST on the phosphomimetic mutant than on the wild-type AC2 was observed (Figures 9B and 9D). Moreover, a larger effect of SST on the phosphomimetic mutant than on the wild-type AC2 was observed (Figures 9B and 9D).

DISCUSSION

As a potential target for Gα, Gβγ, and Gγ-coupled receptors, AC2 is a locus at which information from multiple hormonal inputs...
Regulation of AC2 by PKC and G-protein-coupled receptors

Figure 7 Effects of the PKC inhibitor CC on CCh-stimulated cAMP activity in single cells

Untransfected HEK-293 cells (A) and cells stably expressing wild-type AC2 (B), T1057A (C), phosphorylation-deficient S490/543A (D) and phosphomimetic S490/543D (E) were stimulated with 10 μM CCh at 60 s for 4 min with or without 1 μM CC pre-treatment for 30 min (n = 20–36). The time course of cAMP production is shown in the left-hand panel (means ± S.E.M.) and the 4 min AUC (calculated from the left-hand panel) is on the right-hand side (means ± S.D.). **P < 0.01 using Student’s t test. NS, not significant. Max STIM, saturation of the sensor.

could be integrated to modulate cAMP levels. Although AC2 has no potential PKA (protein kinase A) phosphorylation sites and no reported sensitivity to PKA, there is substantial evidence that PKC phosphorylation can regulate AC2 activity [13,16], although a recent re-evaluation [9] suggests that the implied Gβγ-coupled receptor stimulation of AC2 attributed to PKC or Gβγ is by no means established in the literature. In the present study we re-examined both the regulation of AC2 by PKC and its susceptibility to regulation by Gβγ liberated from activation of either Gz- or Gz-coupled receptors.

Single-site mutations at Ser490 or Ser543 attenuated, but did not completely abolish, AC2 regulation by PKC (Figure 1C). However simultaneous mutation of both sites produced an AC2 variant that was insensitive to PMA stimulation (Figure 2A), which suggested that phosphorylation of both Ser490 or Ser543 is required to achieve the maximal stimulatory effect of PKC. Given that Ser490 or Ser543 are exclusively conserved in PKC-stimulated AC2 and AC7 [40], but absent from the PKC-insensitive AC4 [41], it is tempting to speculate that phosphorylation of these residues by PKC is conserved in both AC2 and AC7.

The present data conflict with the conclusions of an earlier study [20], which identified Thr1057 as the PKC phosphorylation site in AC2. In that study phorbol ester-mediated enhancement of cAMP in extended cell population assays was reportedly reduced by 60-80 % in the T1057A mutant, compared with wild-type AC2, leading the authors to conclude that Thr1057 was the residue through which PKC exerted its effect. However, decreased levels of expression of the T1057A mutant compared with the wild-type AC2 would explain an apparent decreased response to PMA and, indeed, the expression level of AC2 appeared considerably higher.
than that of T1057A in the stable cell lines used in the earlier study [20].

The finding that PKC-mediated stimulation of AC2 occurs via phosphorylation of Ser⁴⁹⁰ or Ser⁵⁴³ at the C₁b domain underscores the importance of this region in determining the activity of AC2. The C₁b domain is highly divergent in length and sequence among different AC isoforms, and might be expected to be a potential phosphorylation target for isoform-specific kinases [38,42]. For instance, phosphorylation of Ser⁴⁹⁰ or Ser⁵⁴³ at the C₁b domain of AC1 by Ca²⁺/calmodulin-dependent kinase IV alters calmodulin-mediated activation of AC1 without affecting the basal catalytic activity [43]. In addition, PKA phosphorylation of the C₁b domain of AC6 at Ser⁶⁷⁴ inhibits low affinity stimulation by Gₛₐ [44]. Therefore phosphorylation of C₁b domains in different ACs by kinases may represent a general mechanism for modulation of AC activity. The presence of Thr¹⁵⁵⁷ within the highly conserved C-terminal catalytic C₂a domain underscores its unattractiveness as a candidate for AC2 regulation by PKC.

To explore the actions of receptor agonists that were expected to regulate AC2 we turned to more sensitive single live cell assays. In the case of the muscarinic agonist, CCh, a clear stimulation of activity was seen that reflected the activation of PKC and which was eliminated in the phospho-deficient and phosphomimetic AC2 mutants. However preclusion of the actions of PKC (by mutagenesis of AC2) allowed an inhibitory effect of Gₜ, arising from the activation of Gₛₐ, to be manifest, although we cannot rule out the possibility that the mutation on Ser⁴⁹⁰ or Ser⁵⁴³ actively permitted the responsiveness of AC2 to the effects of Gₜ. This inhibitory effect by Gₜ adds a further regulatory nuance to the actions of a Gₛₐ-coupled receptor on AC2 regulation, which might
The involvement of G\(\beta\gamma\) in AC2 activity induced by activation of G\(_i\)-coupled SST receptor

Untransfected HEK-293 cells (A) and cells stably expressing AC2 (B), T1057A (C), phosphorylation-deficient S490/543A (D) and phosphomimetic S490/543D (E) were incubated with 1 \(\mu\)M SST for 4 min with or without 10 \(\mu\)M gallein pre-treatment for 30 min (n = 44–77). The left-hand panels indicates the time course of cAMP generation (means ± S.E.M.) and the right-hand panels are the corresponding 4 min AUC (means ± S.D.). *P < 0.05 and **P < 0.01 using Student’s t test. NS, not significant.

be manifest in situations where PKC is not available to modulate activity.

Since it has already been established that G\(_i\)-coupled receptors can activate AC2, although in the presence of activated G\(_\alpha\) [32,45], we wished to compare the actions of G\(\beta\gamma\) released from G\(_i\)-coupled muscarinic receptor to those of G\(\beta\gamma\) liberated by SST receptor activation. Strikingly the opposite consequence for AC2 activity (stimulation) was seen upon activation of the endogenous SST receptors in HEK-293 cells without G\(_\alpha\). Thus we can envisage a range of responses for AC2 depending on the complement of receptors and protein kinases in its environment.

The unique PFAHL motif (within the C1b domain) spanning amino acids 493–509 is indispensable for G\(\beta\gamma\)-mediated activity of AC2 [46]. The importance of the C1b domain in PKC-stimulated AC2 regulation is further supported by the finding that this region suppresses catalytic activity by maintaining AC2 in the basal state [47]. Furthermore, according to earlier circular dichroism data, the C1b domain can adopt various ligand-dependent stable conformations that affect the catalysis rate by changing the structural conformation of the C1a–C2a catalytic core [48]. Therefore theoretical considerations as well as the present and earlier studies collectively indicate that AC2 utilizes its C1b domain (containing Ser\(^{490}\) or Ser\(^{543}\)) in response to PKC-mediated stimulation, which undergoes conformational changes upon phosphorylation to increase the catalytic rate by releasing the non-stimulated state of AC2. Interestingly, a recent study [49] confirmed the initial G\(\beta\gamma\) PFAHL motif, and also identified another two G\(\beta\gamma\)-interaction sites, one of which is also encompassed by the C1b domain. The authors suggested that the C1b domain may act as the main G\(\beta\gamma\)-binding face, again
implying that this region may be essential for AC2 to integrate coincident signals from G<sub>i</sub>, G<sub>q</sub> and G<sub>a</sub>. Given that the direct binding of Gβγ from the G<sub>i</sub>-coupled receptor on AC2 has a stimulatory effect, the inhibitory effect of Gβγ from muscarinic receptor seems likely to be due to indirect effects involving other signalling pathways.

In summary, the present study establishes that AC2 activity is stimulated by PKC either in response to PMA or Gq-coupled signalling pathways.

REFERENCES

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AUTHOR CONTRIBUTION

Jia Shen, Sebastian Wachten, Michelle Halls and Katy Everett contributed to the experiments; and Jia Shen and Dermot Cooper wrote the paper.
Regulation of AC2 by PKC and G-protein-coupled receptors


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SUPPLEMENTARY ONLINE DATA

Muscarinic receptors stimulate AC2 by novel phosphorylation sites, whereas Gβγ subunits exert opposing effects depending on the G-protein source

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Figure S1 Changes in the CFP/YFP response of the cells

Changes in the CFP/YFP response (R/R₀) of HEK-293 cells (A) or the indicated AC2 constructs (B-E) stable cells transiently transfected with global (‘dead’) Epac²⁹⁷E in response to 10 μM CCh. Maximal change in R/R₀ (Maxi) was sought using 100 μM IBMX, 10 μM FSK and 10 μM Iso at 300 s for 2 min. Results are means ± S.E.M.

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Figure S2  Effects of overexpressing the M3 receptor on CCh-mediated cAMP activity in single cells

Untransfected HEK-293 cells (A) and cells stably expressing wild-type AC2 (B), T1057A-AC2 (C), phosphorylation-deficient mutant S490/543A-AC2 (D) and phosphomimetic S490/543A-AC2 (E) transfected with or without the M3 receptor were stimulated with 10 μM CCh at 60 s for 4 min. Results are means ± S.E.M.
Figure S3  Effects of overexpressing Gtα (transducin) on CCh-mediated cAMP activity in single cells

Untransfected HEK-293 cells (A) and cells stably expressing wild-type AC2 (B), T1057A-AC2 (C), phosphorylation-deficient mutant S490/543A-AC2 (D) and phosphomimetic S490/543A-AC2 (E) transfected with or without Gtα were stimulated with 10 μM CCh at 60 s for 4 min. The time course of cAMP production is shown in the left-hand panel and the 4 min AUC (calculated from the left-hand panel) is on the right-hand side. Results are means ± S.D. *P < 0.05 and **P < 0.01 using Student's t test. Max STIM, saturation of the sensor; NS, not significant.

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