Published in final edited form as:
Biochem J. 2013 October 1; 455(1): 47–56. doi:10.1042/BJ20130359.

AKAP79, PKC, PKA and PDE4 participate in a G_q-linked muscarinic receptor and adenylate cyclase 2 cAMP signalling complex

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Abstract

AC2 (adenylate cyclase 2) is stimulated by activation of G_q-coupled muscarinic receptors through PKC (protein kinase C) to generate localized cAMP in HEK (human embryonic kidney)-293 cells. In the present study, we utilized a sensitive live-cell imaging technique to unravel the proteins that play essential roles in a G_q-coupled muscarinic receptor-mediated cAMP signalling complex. We reveal that, upon agonist binding to the G_q-coupled muscarinic receptor, AKAP79 (A-kinase-anchoring protein 79) recruits PKC to activate AC2 to produce cAMP. The cAMP formed is degraded by PDE4 (phosphodiesterase 4) activated by an AKAP-anchored PKA (protein kinase A). Calcineurin, a phosphatase bound to AKAP79, is not involved in this regulation. Overall, a transient cAMP increase is generated from AC2 by G_q-coupled muscarinic receptor activation, subject to sophisticated regulation through AKAP79, PKC, PDE4 and PKA, which significantly enhances acetylcholine-mediated signalling.

Keywords

adenylate cyclase 2 (AC2); A-kinase-anchoring protein 79 (AKAP79); muscarinic receptor; phosphodiesterase 4 (PDE4); protein kinase A (PKA); protein kinase C (PKC)

INTRODUCTION

The muscarinic cholinergic receptor, a typical seven-transmembrane GPCR (G-protein-coupled receptor), binds the neurotransmitter acetylcholine and mediates numerous physiological functions. Among the five mammalian muscarinic receptors identified, the odd-numbered receptors (M_1, M_3 and M_5) couple with $G\alpha_{q/11}$, whereas the even-numbered receptors (M_2 and M_4) interact with G_i and G_o. The G_q-coupled M_3 receptor is the main muscarinic receptor endogenously expressed in HEK (human embryonic kidney)-293 cells and has been implicated in many physiological functions, including smooth muscle contraction, glandular secretion, food intake [1], and learning and memory [2].

A major outcome of activation of the G_q-coupled muscarinic receptor is to stimulate AC2 (adenylate cyclase 2) activity to produce cAMP [3-5]. In receiving and integrating signals from receptors coupled to G_s, G_i and G_q instead of a single pathway, AC2 was proposed to be a coincidence detector [3]. More recently, it has been recognized that such sophisticated

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AUTHOR CONTRIBUTION

Jia Shen performed the experiments. Jia Shen and Dermot Cooper designed the study and wrote the paper.

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regulatory options are organized through the recruitment of interacting partners such as AKAPs (A-kinase-anchoring proteins), PDEs (phosphodiesterases) and PKA (protein kinase A) to yield compartmentalized cAMP signalling [6]. Traditional cell population assays [7] cannot resolve the spatiotemporal regulation of activation of G_q-linked muscarinic receptor-mediated AC2 activity, whereas targeted single-cell sensors greatly enhance the possibility of addressing such potential complexity [8].

Earlier studies suggested that stimulation of AC2 by G_q-coupled receptor occurs through activation of PKC (protein kinase C), arising from the hydrolysis of PtdInsP_2 to yield DAG (diacylglycerol) [3,4]. In addition, immunoprecipitation experiments showed that AKAP79 associates with AC2 [9,10] and AKAP79 enhanced an inhibition by PKA of G_s-stimulation of AC2 [9], which already portends an important role of AKAP79 in AC2 regulation. AKAP79 has also been shown to assemble PKC to customize the regulation of various effector proteins including K^+ channels and TRPV (transient receptor potential vanilloid) cation channels TRPV1 and TRPV4 [11-13]. Those results led us to investigate the potential role of AKAP79 on G_q-linked muscarinic receptor-modulated AC2 activity.

In the present study, we utilized the plasma membrane-targeted FRET-based PM-Epac2 [8,14] sensor to investigate the proteins that are involved in the muscarinic receptor-mediated cAMP signalling complex in HEK-293 cells. Immunoprecipitation and single-cell imaging with AKAP79 overexpression, overexpression of an AKAP79 mutant lacking the PKC-binding site and AKAP79 knockdown demonstrated that AKAP79 potentiated G_q-linked muscarinic receptor-stimulated AC2 activity by anchoring PKC to phosphorylate and activate AC2. Furthermore, the cAMP generated by this activation is degraded by PDE4, whose activity is stimulated by PKA phosphorylation acting through AKAPs. Another protein that interacts with AKAP79, calcineurin, is not involved in this cAMP signalling complex.

EXPERIMENTAL

Constructs

Rat HA (haemagglutinin)–M_3 muscarinic receptor was kindly provided by Dr Jürgen Wess (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, U.S.A.). The constitutively active form of calcineurin was a gift from Dr Isaac Rabinovitz (Beth Israel Deaconess Medical Centre, Boston, MA, U.S.A.). The generation of the PM-Epac2 sensor [PM-Epac2 contains the SH4 (Src homology 4) motif of Lyn kinase, which allows the targeting of PM-Epac2 to the plasma membrane] and AKAP79–HA were described in [8] and [15] respectively. pSilencer vector, shRNA-AKAP79 and ΔPKC-AKAP79 (lacking the PKC-binding site, residues 31–51) were gifts from Professor John Scott (University of Washington, Seattle, MA, U.S.A.). AKAP79–YFP was from Professor Mark Dell'Acqua (University of Colorado Denver, Aurora, CO, U.S.A.).

Cell culture and transfection

HEK-293 cells (European Collection of Cell Culture, Porton Down, Wilts., U.K.) were grown in MEM (minimal essential medium) containing 10% (v/v) FBS, 100 μg/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine, and maintained in 5% CO_2/95% air at 37 °C. HEK-293 cells were plated on 25-mm-diameter poly-L-lysine-coated coverslips at 60% confluence 24 h before transfecting with 1-2 μg of the indicated constructs using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. For generating stable cell lines, the cells were selected with medium including 800 μg/ml G-418 for 4 days after transfection, and subsequently cultured in medium with 400 μg/ml G-418.

Measurement of cytosolic Ca^{2+} in cell populations

HEK-AC2 cells were loaded with 2 μM fura-2/AM (fura 2 acetoxymethyl ester) plus 0.02% pluronic F-127 for 40 min at 20 °C. After loading, cells were washed with Krebs buffer twice, divided into samples of 4×10^6 cells, and resuspended in 3 ml of Ca^{2+} -free Krebs buffer. The measurement of fluorescence emission ratios at 340 nm/380 nm was conducted in a PerkinElmer Life Science LS50B spectrofluorimeter (as described in [16]).

Measurement of cAMP in single cells

Fluorescence imaging of cells expressing PM-Epac2 [14] was conducted using an iXon + EMCCD camera (Andor) and an Optosplit (505DC; Cairn Research) to separate CFP and YFP emission images [8]. For dual-emission ratio imaging, cells were excited at 435 nm using a monochromator (Cairn Research) and 51017 filter set (Chroma) connected to a Nikon eclipse TE2000-S microscope (×40 objective). Emission images at 470 nm and 535 nm were collected every 3 s with a 250 ms integration time, and analysed by Metamorph imaging software (Molecular Devices). Single-cell FRET data were plotted as changes in background-subtracted 470 nm (CFP) against 530 nm (YFP) emission ratio relative to the maximal FRET obtained with a cocktail of 10 μM PGE_1 (prostaglandin E_1), 10 μM isoprenaline (isoproterenol), 10 μM forskolin and 100 μM IBMX (3-isobutyl-1-methylxanthine).

HA immunoprecipitation

HEK-293 cells expressing the indicated cDNA constructs were stimulated with or without 200 μM carbachol in HBS (Hepes-buffered saline) for 10 min, washed with PBS and lysed in solubilization buffer (50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 1 mM $MgCl_2$, 150 mM NaCl, 0.25% sodium deoxycholate, 1% Nonidet P-40 and protease inhibitors) by passing the cell suspension through a 21-gauge needle. The cell lysate was then centrifuged at 900 ***g*** for 5 min at 4 °C to remove debris before immunoprecipitation using anti-HA affinity-agarose beads (Roche). Lysates were rotated with 100 μl of pre-washed beads (50% slurry) for 4 h at 4 °C and washed twice with solubilization buffer, and bound proteins were eluted with solubilization buffer with 1% SDS and DNase. The eluted samples were subsequently incubated with Laemmli buffer at 37 °C for 30 min.

Western blotting

Proteins were electrophoretically separated by SDS/PAGE (8% gel), transferred on to nitrocellulose membranes and blocked with 5% (w/v) non-fat dried skimmed milk powder. Membranes were incubated with anti-AKAP79 (1:5000 dilution; Millipore), anti-HA (1:10000 dilution; Sigma), anti-tubulin (1:5000 dilution; Sigma) or anti-Myc (1:5000 dilution; Santa Cruz Biotechnology) antibodies in TBST (TBS with Tween 20) with 1% (w/v) non-fat dried skimmed milk powder. Membranes were then washed in TBST three times and incubated with anti-(mouse IgG) antibody linked to horseradish peroxidase (1:15000 dilution; Promega). Finally, the membranes were visualized with ECL Plus reagent (GE Healthcare) according to the manufacturer's instructions, and the immunoreactive bands were quantified by densitometry, using ImageJ software (NIH).

RESULTS

Overexpression of AKAP79 potentiates carbachol-mediated AC2 activity in HEK-293 cells

The aim of the present study was to explore potential protein partners that might be involved in muscarinic receptor-mediated stimulation of cAMP signalling. Earlier studies had shown that the muscarinic receptor agonist carbachol stimulated AC2 activity via the activation of PKC [3-5] and immunoprecipitation suggested that AC2 interacts directly with AKAP79

[9,10]. We therefore wished to investigate whether AKAP79 is involved in stimulation of AC2 activity by the muscarinic receptor agonist carbachol, since AKAP79 can bind PKC [16,17]. Initially, we confirmed the association between AC2 and AKAP79 by immunoprecipitation. HA–AC2 immune complexes were immunoprecipitated using an HA-specific antibody and probed for interaction with AKAP79 by an antibody that recognizes the C-terminal domain of AKAP79 in cells transfected with AKAP79–YFP, with or without co-expression of HA–AC2. AKAP79 was seen in the input of both AKAP79–YFP and AKAP79-YFP+HA–AC2-expressing cells, but was clearly dramatically enhanced in the pull-down of cells expressing HA–AC2, which confirmed the interaction between AC2 and AKAP79 in our experimental system (Figure 1A).

PM-Epac2, which combines the SH4 motif with Epac2-camps (exchange protein directly activated by cAMP 2-based cAMP sensor) [14] (Figure 1B), successfully targeted to the plasma membrane with or without AKAP79 co-expression (Figure 1C) and can therefore measure cAMP levels just underneath the membrane. AKAP79–YFP was also detected on the plasma membrane as expected [16] (Figure 1D). The overexpression of AKAP79 was confirmed by Western blotting (Figure 1E). Carbachol application yielded a transient cAMP response, as reported previously [5]. However, when carbachol-stimulated cAMP production was compared in HEK-293 cells stably expressing HA–AC2 with or without the transient overexpression of AKAP79, a significantly enhanced cAMP response was observed (Figures 1F and 1G). These results suggest a role for AKAP79 in the stimulation of AC2 by carbachol.

Knockdown of AKAP79 reduces carbachol stimulation of AC2 activity

To more firmly establish a role for AKAP79 in the action of carbachol on AC2 activity, the effect of AKAP79 knockdown was assessed, using shRNA directed against the endogenous AKAP79 of HEK-293 cells. Densitometries of Western blotted bands for AKAP79 indicated that the shRNA-AKAP79 depleted approximately 75% of endogenous AKAP79, compared with pSilencer (Figures 2A and 2B). The responsiveness of HA–AC2 cells treated with such shRNA-AKAP79 or pSilencer for 72 h to carbachol was compared and a clear decrease in the carbachol response was evident after AKAP79 knockdown (Figures 2C and 2D). This result underscored the likely importance of AKAP79 on AC2 regulation by PKC. Furthermore, to rule out the possibility that the diminished carbachol-mediated AC2 activity by shRNA-AKAP79 was due to ‘off-target’ effects, the shRNA-AKAP79-expressing HEK-AC2 cells were co-transfected with rat orthologue AKAP150 to investigate whether this co-transfection can rescue the shRNA-AKAP79 effects. Indeed, overexpression of AKAP150 reversed the effect of shRNA-AKAP79 and enhanced carbachol-mediated AC2 (Supplementary Figure S1 at http://www.biochemj.org/bj/455/bj4550047add.htm), confirming that the influence of shRNA-AKAP79 on carbachol-induced AC2 activity is due to knockdown of endogenous AKAP79.

Overexpression of ΔPKC–AKAP79 diminishes carbachol-mediated AC2 activity

Given that carbachol stimulates AC2 via phosphorylation mediated by PKC [5], that AC2 binds AKAP79 and that AKAP79 binds PKC, the likelihood that the foregoing effects were due to the binding of PKC by AKAP79, which could also bind AC2, was addressed more directly. ΔPKC–AKAP79 has the binding site (residues 31–51) for PKC deleted [17] (Figure 3A). The effect of overexpressing ΔPKC–AKAP79 was therefore tested. ΔPKC–AKAP79 can presumably compete with endogenous AKAP79 in binding to AC2, but not in recruiting PKC to AC2. Western blotting confirmed the overexpression of ΔPKC–AKAP79 (Figure 3B). Cells thus transfected yielded a curtailed stimulation by carbachol of AC2 activity. This experiment strongly supports the contention that AKAP79 tethers PKC to phosphorylate and activate AC2 upon carbachol stimulation.

AKAP79 interacts with activated muscarinic M_3 receptor

The M_3 receptor is the main muscarinic isoform expressed in HEK-293 cells. Consequently, we employed immunoprecipitation to examine the potential interaction between AKAP79 and M_3 receptor. The results showed a modest, but significant, co-immunoprecipitation of AKAP79–YFP with the M_3 receptor that had been stimulated by carbachol, but not by the unstimulated M_3 receptor (Figures 4A and 4B). We wondered whether co-expression of AC2 might potentiate the approximately 50% increase in M_3 receptor and AKAP79 association (Figure 4B). However, no relative increase was detected by co-expressing AC2 (Figures 4C and 4D). These data indicate that AKAP79 only associated with the activated M_3 receptor weakly and in an AC2-independent manner.

AKAP79 does not play a role in regulation of the PKC-insensitive mutant S490A/S543A-AC2

We have previously demonstrated that carbachol stimulation of AC2 activity is achieved by PKC phosphorylation on Ser^{490} and Ser^{543} of AC2; mutation of these two residues completely abrogated stimulation by carbachol and PKC [5]. Mutation of these two residues has no effect on the catalytic activity of AC2, since S490A/S543A-AC2 responds to PGE_1 stimulation and the phosphomimetic mutant S490D/S543D-AC2 shows enhanced basal, forskolin-mediated and PGE_1-mediated activities [5]. To establish the role that we envisaged of the recruitment by AC2-bound AKAP79 of PKC facilitating the stimulation by carbachol, we investigated the effect of overexpression of AKAP79, ΔPKC–AKAP79 and shRNA-AKAP79 on the non-phosphorylatable S490A/S543A-AC2. As expected, there was no stimulatory effect of carbachol on the non-phosphorylatable AC2, only a small inhibitory effect, which we had shown previously was due to $\beta\gamma$ subunits liberated upon G_q activation by carbachol [5]. Overexpressing any one of the AKAP79-related constructs has no effect on this carbachol-mediated cAMP decrease (Figure 5), showing no role for AKAP79 in the modulation of S490A/S543A-AC2 by carbachol or any non-specific effects caused by overexpressing the AKAP79 constructs.

PDE4 isoforms degrade cAMP produced by carbachol stimulation in HEK-293 cells

Previous studies suggested that transient cAMP kinetics (as seen in Figures 1F, 2C and 3C) reflect the balance between synthesis and degradation mediated by PDE, where the decline in the cAMP signal is due to PDE activity [18,19]. PDE3 and PDE4 are the dominant isoforms expressed in HEK-293 cells [20]. To clarify the roles of PDEs in the decline in cAMP, specific inhibitors of the PDE3 and PDE4 isoforms were used. The PDE4-specific inhibitor rolipram completely abolished the decline in cAMP decline, whereas the PDE3-specific inhibitor cilostamide caused only a general enhancement of the cAMP levels without eliminating the biphasic pattern (Figures 6A and 6B respectively). Thus PDE4 plays the major role in degrading the cAMP generated following AC2 stimulation by carbachol.

PKA-mediated phosphorylation through AKAP is involved in carbachol-mediated AC2 activity

Long isoforms of PDE4 can be activated by PKA-mediated phosphorylation [21] and these isoforms of PDE4 are commonly scaffolded to AKAPs (along with PKA). Since the results above implicated PDE4, we asked whether PKA inhibitors or AKAP disruptors would affect the carbachol-induced cAMP profile. Application of the PKA inhibitor KT5720 significantly attenuated the cAMP decline phase of the response to carbachol (Figures 7A and 7B), suggesting the involvement of PKA. To investigate whether the involvement of PKA is through AKAPs, the AKAP–PKA chemical interaction disruptor FMP-1 [22] was utilized. Pre-treatment with FMP-1 also prevented the cAMP decline phase; this finding indicated that AKAPs mediate the role of PKA in carbachol-mediated AC2 activity (Figures 7A and

7B). To ensure that effects on carbachol-mediated cAMP production in AC2-expressing cells by the PKA inhibitor is not due to any influence of PKA or AKAP disruption on carbachol-medicated Ca^{2+} release (which enhances the action of PKC [23]), the Ca^{2+} release triggered by carbachol in the absence and presence of KT5720 was examined. There was no action of KT5720 on Ca^{2+} release triggered by carbachol (Figure 7C), which supported a rather direct role of an AKAP-tethered PKA on PDE4 in the decline phase of the carbachol response.

Calcineurin is not involved in carbachol-mediated AC2 activity in HEK-293 cells

In addition to binding sites for PKA and PKC, AKAP79 contains a calcineurin (also known as protein phosphatase 2B)-binding site [17,24]. This situation raises the possibility that calcineurin tethered by AKAP79 could dephosphorylate AC2 after PKC phosphorylation and attenuate the overall response to carbachol. We therefore assessed the impact of the calcineurin inhibitor cyclosporin A and a constitutively active form of calcineurin, calcineurin-(1–390) on the carbachol response. The expression of calcineurin-(1–390) was confirmed by Western blotting (Figure 8A). However, neither the overexpression of calcineurin-(1–390) nor cyclosporin A treatment had any effect on the carbachol response (Figures 8B and 8C), suggesting no role for calcineurin in AC2 regulation. To rule out the possibility that the effect of calcineurin is obscured by PDE4 effects, we repeated the calcineurin experiments in the presence of the PDE4 inhibitor. Again, calcineurin-(1–390) and cyclosporin A did not influence the carbachol-mediated AC2 activity. Together, these results demonstrate that, although calcineurin might bind to AKAP79, it has no role in the carbachol stimulation of AC2 activity in the present paradigm.

DISCUSSION

The present study explores the molecular organization of our recently described stimulation of AC2 resulting from the activation by carbachol of PKC [5]. It has also been shown that stimulation of AC2 by G_q-coupled receptor is attributed to the activation of PKC [3,4]. Double mutation of PKC phosphorylation sites on AC2, i.e. S490A/S543A-AC2, completely abolished the carbachol-mediated AC2 activity, and pre-incubation with the PKC inhibitor chelerythrine chloride diminished this activity, confirming further that endogenous G_q-coupled muscarinic receptor activation-induced AC2 activity in HEK-293 cells is through the PKC pathway [5]. Given that PKC can be bound by AKAP79, we considered the possibility that a complex involving the AKAP might be involved in the effect of carbachol. Indeed, the results demonstrate that a signalling complex is involved wherein AKAP79 recruits PKC activated by the muscarinic agonist to phosphorylate serine on AC2 for localized generation of cAMP, which is then specifically degraded by the PKA-activated PDE4. AKAP79 has already been suggested to play a vital role in regulating the activity of numerous ACs, since it associates with AC2, AC3, AC5, AC6, AC8 and AC9 [9,15], and this association anchors PKA to inhibit AC2, AC5, AC6 and AC8 activity [9,15]. In the present study, we show that overexpression of AKAP79 potentiated carbachol-mediated-AC2 activity (Figure 1), whereas shRNA-AKAP79 knockdown reduced this activity (Figure 2), implicating AKAP79 in G_q-linked muscarinic receptor-mediated AC2 activity. The deletion of the PKC-binding site on AKAP79 reverses its ability to enhance carbachol-mediated AC2 activity (Figure 3), demonstrating that the capacity of AKAP79 tethering PKC to effector proteins is the role played by AKAP79 in carbachol elevation of AC2 activity. Overexpression of the non-PKC-binding form of AKAP79 presumably displaces any endogenous (PKC-binding) AKAP79 from AC2. Given that AKAP79 was successfully immunoprecipitated by AC2 in the present study and two other independent studies [9,10], and that AKAP79 and PKC interact and the PKC-binding site (residues 31–51) on AKAP79 was identified, it seems reasonable to expect that overexpression of ΔPKC–AKAP79 would

block the association of AC2 with PKC. These results extend the hypothesis that AKAP79 can recruit kinases or phosphatase to regulate target proteins [25,26].

Our immunoprecipitation data showed that AKAP79 associates with the activated muscarinic M_3 receptor, the main muscarinic isoform expressed in HEK-293 cells (Figure 4). The modest (50%) increase in AKAP79 immunoprecipitated by carbachol-mediated M_3 receptors compared with the negative control (Figures 4B and 4D) might lead to speculation that the association between AKAP79 and carbachol-stimulated M_3 receptor is indirect, or of low affinity. Since AKAP79 constitutively interacts with AC2 [10], the activation of the G_q-linked muscarinic receptor may just increase the affinity of the interaction between AKAP79 and its associated AC2 and PKC for the receptor. Previous studies have reported interactions of AKAP79 with the G_s-linked β_2-adrenergic and relaxin (RXFP-1) receptors [27]. Data of siRNA-AKAP knockdown indicated that the isoprenaline-triggered PKA-mediated phosphorylation of the β_2-adrenergic receptor was ablated by AKAP79 knockdown [20]. We may therefore speculate that the ability of AKAP79 to associate kinases with GPCRs may be a common thread to reinforce compartmentalization of GPCR-initiated pathways, including those coupled to G_q. In this regard, upon activation of G_q-linked muscarinic receptor by acetylcholine, AKAP79 positions activated PKC to phosphorylate and close M-current KCNQ channels, producing an increase in membrane excitability [25,28]. These results, together with our data, underline roles for AKAP79 in enhancing G_q-linked muscarinic receptor anchoring of PKC to phosphorylate downstream targets.

The selective ability of the PDE4 inhibitor rolipram (Figure 6) and PKA inhibitor KT5720 (Figure 7) to attenuate the cAMP decline phase shows that these components of the cAMP signalling complex contribute to the dynamics of cAMP changes. Thus this system is a further example of a PDE4 activated by anchored PKA phosphorylation negatively regulating cAMP levels and thereby compartmentalizing sub-plasmalemmal cAMP signals [10,29-31]. Although PDEs do not seem to necessarily interact with AKAP directly, PDE4D5 has been suggested to be an essential component of the AKAP79-based complex [20,32,33]. The efficacy of the chemical disruptor of AKAP79–PKA FMP-1 at inhibiting the cAMP decline phase, like KT5720, establishes the dependency of the PKA effect of PKA on organization by an AKAP, again underlining the role of AKAPs in organizing the strict spatial and temporal control on cAMP signalling and signal specificity [26,34]. Since AKAP79 also contains calcineurin-binding sites, the possibility that calcineurin is involved in carbachol-mediated AC2 activity was investigated by using a constitutively active form of calcineurin, calcineurin-(1–390) and the calcineurin inhibitor cyclosporin A. As the residues 30–98 and 311–336 on calcineurin are crucial for AKAP79–calcineurin interaction [35], calcineurin-(1–390) would still possess the ability to be anchored by AKAP79 to AC2. Cyclosporin A is widely used to analyse the effect of calcineurin. Therefore both approaches should be efficacious, and the ineffectiveness of both approaches on carbachol-mediated AC2 activity indicates that calcineurin is unlikely to be involved in G_q-linked muscarinic receptor and AC2 signalling complex.

Taken together, high-resolution single-cell imaging techniques, as applied in the present study, allow the dissection and identification of the residents of a G_q-linked muscarinic receptor-mediated AC2 signalling complex. The evidence seems clear that AKAP79, PDE4, PKC and PKA are elegantly organized to generate a transient and localized cAMP response for transducing compartmentalized downstream signalling by this complex. Specifically, PKC targeted to AKAP79 initiated the on-rate of cAMP production upon G_q-linked muscarinic receptor activation. Subsequently, the locally generated cAMP was quickly removed by PDE4 activated by PKA. This discovery sheds light on the G_q-linked

muscarinic receptor regulation in physiological systems, and provides a platform for the development of novel drugs to modulate G_q-coupled muscarinic receptor-regulated events.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

FUNDING

This work was supported by the Wellcome Trust [grant number RG 31760] and the Newton Trust. D.M.F.C. is a Royal Society Wolfson Research Fellow.

Abbreviations used

AC2	adenylate cyclase 2
AKAP	A-kinase-anchoring protein
GPCR	G-protein-coupled receptor
HA	haemagglutinin
HEK	human embryonic kidney
IBMX	3-isobutyl-1-methylxanthine
PDE	phosphodiesterase
PGE_1	prostaglandin E_1
PKA	protein kinase A
PKC	protein kinase C
SH4	Src homology 4
TBST	TBS with Tween 20
TRPV	transient receptor potential vanilloid

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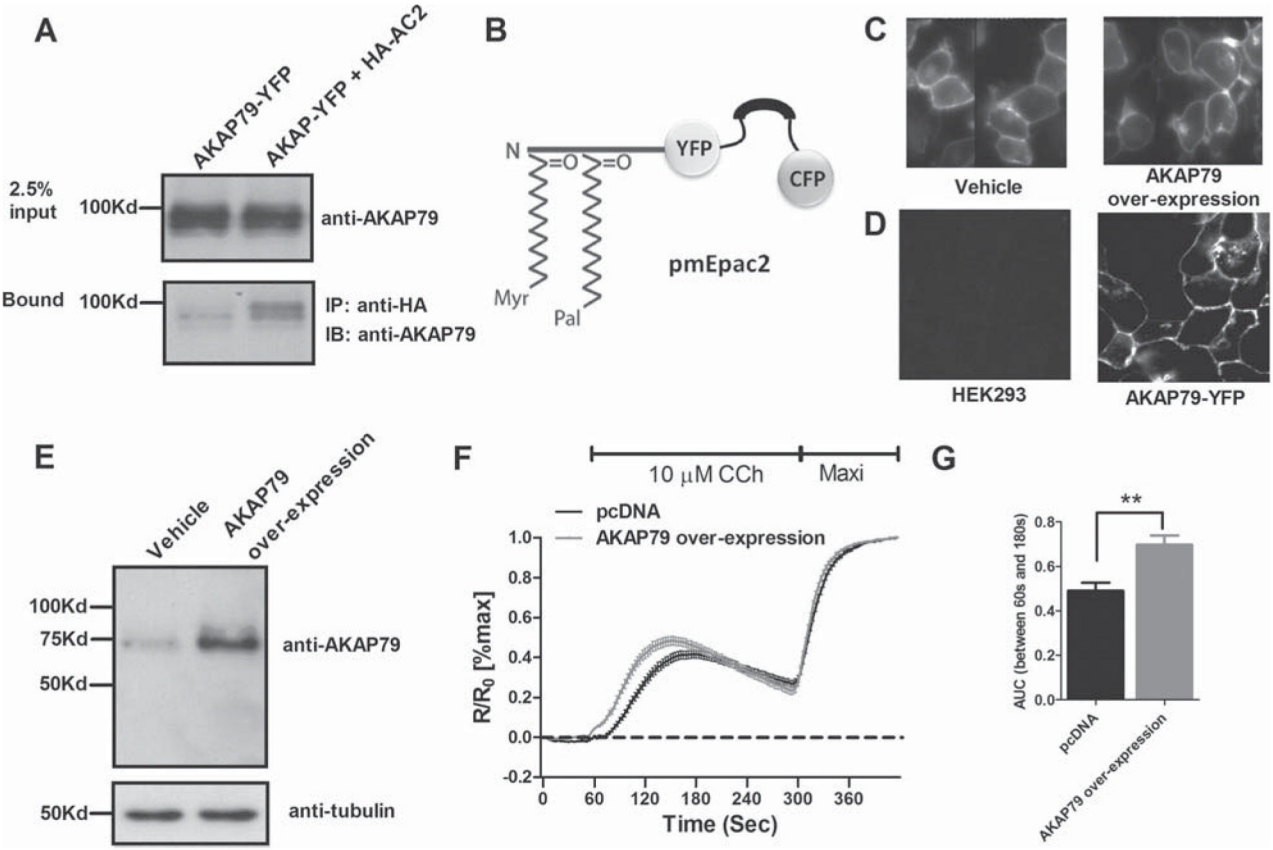

Figure 1. Effects of overexpression of AKAP79 on carbachol-stimulated AC2 activity

(**A**) Co-immunoprecipitation of AKAP79–YFP by HA–AC2 and pcDNA in HEK-293 cells using HA–agarose beads. Molecular masses are indicated in kDa. IB, immunoblot; IP, immunoprecipitation. (**B**) Schematic representation of the PM-Epac2 sensor. Myr, myristate; Pal, palmitate. (**C**) The expression and localization of the PM-Epac2 biosensor in HEK-293 cells with or without co-expressing AKAP79–YFP. Expression of the sensors was detected by recording the CFP emission of the sensor. (**D**) Fluorescence images of untransfected HEK-293 cells and cells transiently expressing AKAP79–YFP. No non-specific fluorescence was detected in untransfected cells, whereas AKAP79–YFP was localized on the plasma membrane in AKAP79–YFP-transfected cells. (**E**) Western blotting showing overexpression of AKAP79. The blot was re-probed with anti-tubulin antibody to confirm equal protein loading. Molecular masses are indicated in kDa. (**F**) Carbachol (CCh)-mediated cAMP production in HEK-AC2 cells with pcDNA3 (n = 86) or AKAP79 (n = 95) co-expression assessed by FRET-based PM-Epac2. Cells were stimulated with 10 μM carbachol at 60 s for 4 min and followed by a maximal stimulation (10 μM forskolin, 10 μM isoprenaline and 100 μM IBMX) at 300 s for 2 min. Data are plotted as relative FRET ratio changes with R_0 taken at 0 s, and R/R_0 is then normalized to the maximum signal response. (**G**) The AUC (area under the curve) between 60 and 180 s from (**F**) for 10 μM carbachol. $^{**}P < 0.01$.

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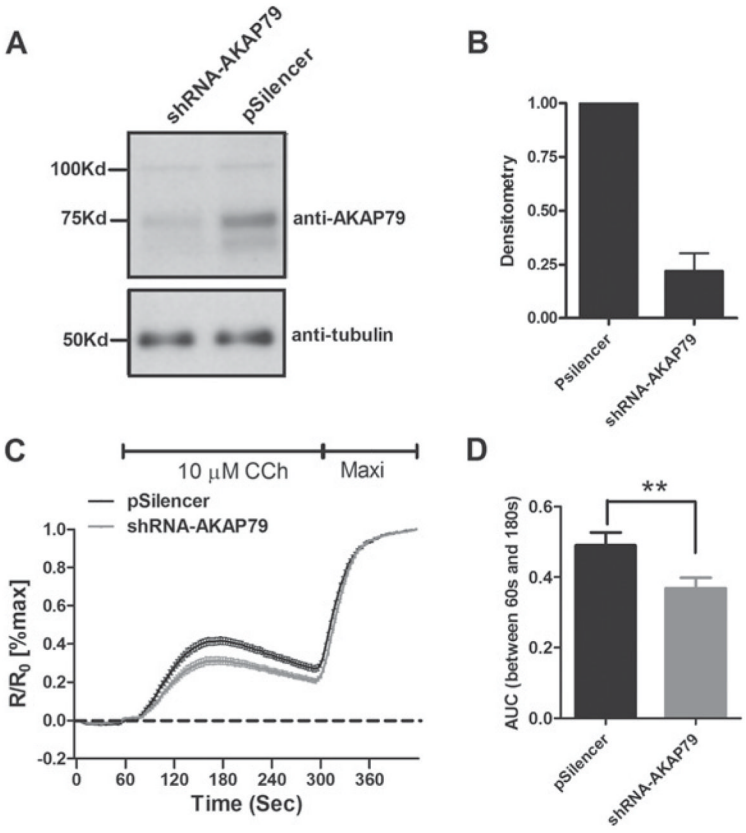

Figure 2. Effects of shRNA-AKAP79 knockdown on carbachol-stimulated AC2 activity

(**A**) Western blotting shows the knockdown of AKAP79 by shRNA-AKAP79 compared with pSilencer (control). The anti-tubulin antibody was used to confirm equal loading. Molecular masses are indicated in kDa. (**B**) Quantification of the knockdown by densitometries as means ± S.E.M. from four repeats. (**C**) Carbachol (CCh)-induced cAMP increase in HEK-AC2 cells transfected with pSilencer (n = 80) or shRNA-AKAP79 (n = 83), assessed by PM-Epac2. Cells were stimulated with 10 μM carbachol at 60 s for 4 min, followed by a maximal stimulation (Maxi: 10 μM forskolin, 10 μM isoprenaline and 100 μM IBMX) at 300 s for 2 min. Data are plotted as relative FRET ratio changes with R_0 taken at 0 s, and R/R_0 is then normalized to the maximum signal response. (**D**) The AUC (area under the curve) between 60 and 180 s from (**C**) for 10 μM carbachol. **P < 0.01.

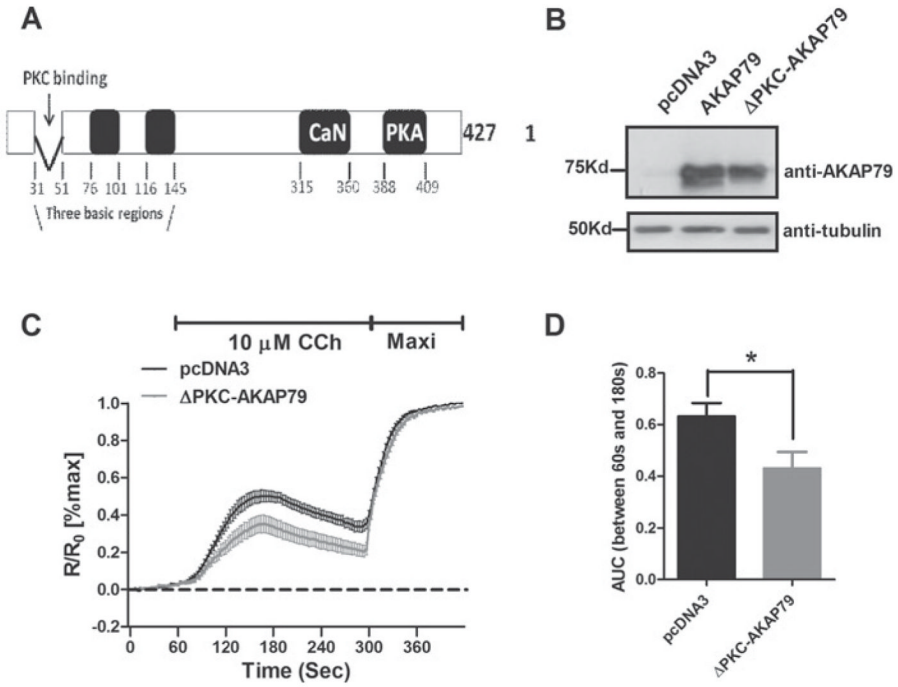

Figure 3. Effects of overexpression of ΔPKC–AKAP79 on carbachol-stimulated AC2 activity

(**A**) Structure of ΔPKC–AKAP79 with binding domains for its interaction partners labelled. CaN, calcineurin. (**B**) Western blotting demonstrates the expression of ΔPKC–AKAP79 with anti-tubulin antibody used as loading control. Molecular masses are indicated in kDa. (**C**) Carbachol (CCh)-induced cAMP production in HEK-AC2 cells transfected with pcDNA3 (*n* 57) or ΔPKC-AKAP79 (*n* = 56) assessed by PM-Epac2. Cells were stimulated with 10 μM carbachol at 60 s for 4 min and followed by a maximal stimulation (Maxi: 10 μM forskolin, 10 μM isoprenaline and 100 μM IBMX) at 300 s for 2 min. Data are plotted as relative FRET ratio changes with R_0 taken at 0 s, and R/R_0 is then normalized to the maximum signal response. (**D**) The AUC (area under the curve) between 60 and 180 s from (**C**) for 10 μM carbachol. *$P < 0.1$.

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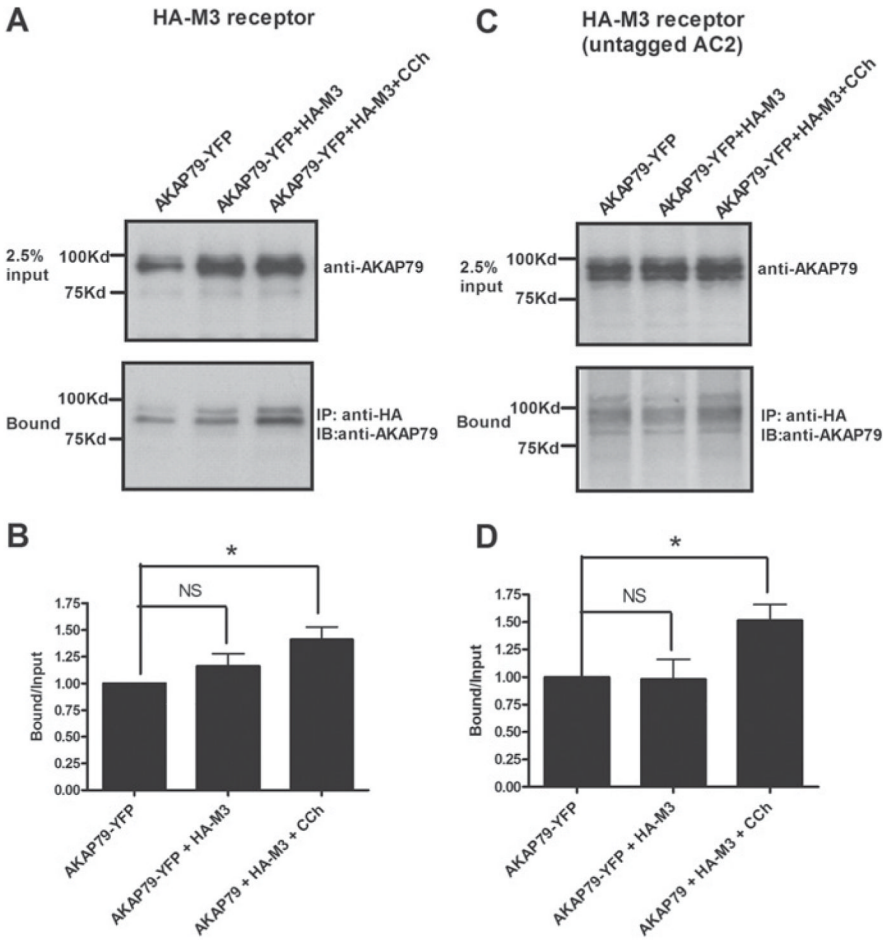

Figure 4. Interaction between muscarinic M_3 receptor and AKAP79

(**A**) Co-immunoprecipitation using HA–agarose beads of AKAP79–YFP from HEK-293 cells transfected with pcDNA, or HA–M_3 stimulated or not with carbachol, as indicated. Molecular masses are indicated in kDa. IB, immunoblot; IP, immunoprecipitation. (**B**) Average densitometry of the immunoprecipitation in (**A**). (**C**) Co-immunoprecipitation of AKAP79–YFP by pcDNA, unstimulated HA–M_3 and carbachol-stimulated HA–M_3 co-transfected with wild-type AC2 in HEK-293 cells using HA–agarose beads. (**D**) Average densitometry for immunoprecipitation of (**C**). *P < 0.1; NS, not significant. CCh, carbachol

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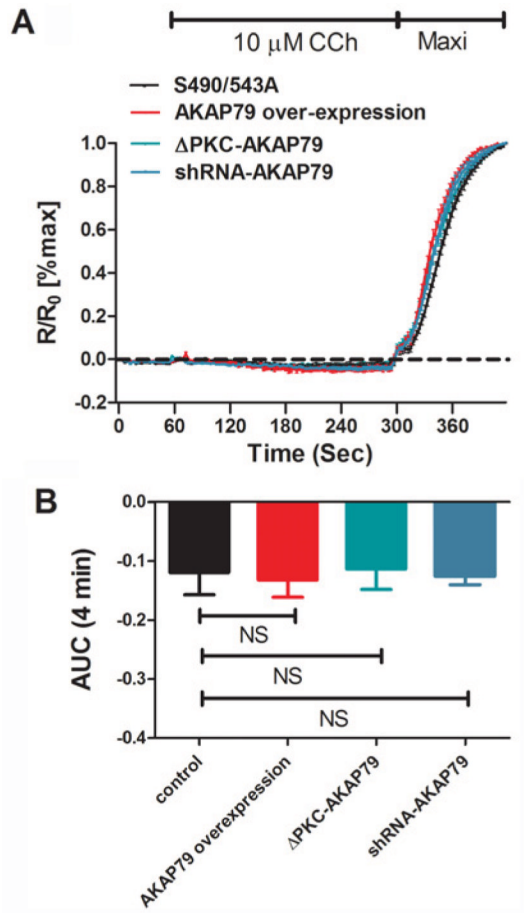

Figure 5. Role of AKAP79 on carbachol-stimulated PKC-insensitive mutant S490A/S543A-AC2

(**A**) Carbachol (CCh)-induced cAMP change in HEK-293 cells stably expressing S490A/S543A-AC2 co-transfected with pcDNA3 (n = 43), AKAP79 (n = 45), ΔPKC-AKAP79 (n = 47) and shRNA-AKAP79 (n = 42) assessed using PM-Epac2. Cells were stimulated with 10 μM carbachol at 60 s for 4 min and followed by a maximal stimulation (Maxi: 10 μM forskolin, 10 μM isoprenaline and 100 μM IBMX) at 300 s for 2 min. Data are plotted as relative FRET ratio changes with R_0 taken at 0 s, and R/R_0 is then normalized to the maximum signal response. (**B**) The 4 min AUC (area under the curve) from (**A**) for 10 μM carbachol. NS, not significant.

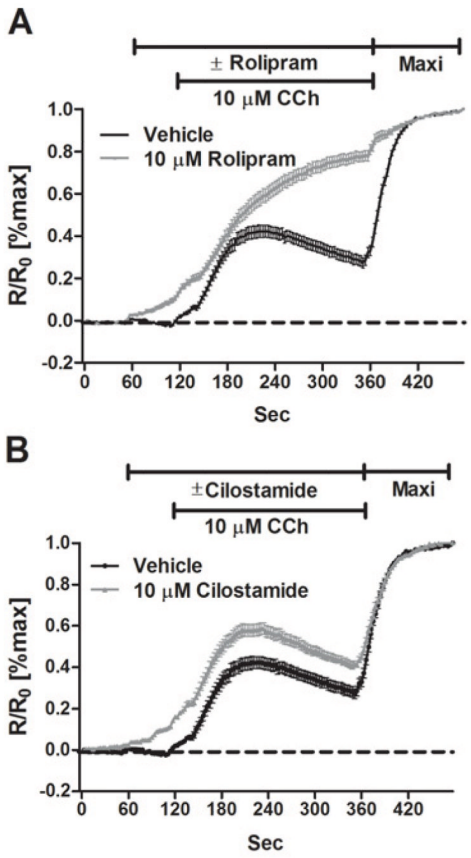

Figure 6. Exploring the PDE isoform which specifically degrades the carbachol-elevated cAMP from AC2

Carbachol (CCh)-induced cAMP increase in HEK-AC2 cells in the absence (n = 57) or presence of PDE4-specific inhibitor rolipram (n = 49) or cilostamide (n = 58) assessed using PM-Epac2. HEK-AC2 cells were incubated with 10=μM rolipram (**A**) or 10μM cilostamide (**B**) at 60 s, and then stimulated with 10 μM carbachol with 10 μM rolipram (**A**) or 10 μM cilostamide (**B**) before saturating the sensor by a maximal stimulation (Maxi: 10 μM forskolin, 10 μM isoprenaline and 100 μM IBMX) at 300 s for 2 min. Data are plotted as relative FRET ratio changes with R_0 taken at 0 s, and R/R_0 is then normalized to the maximum signal response.

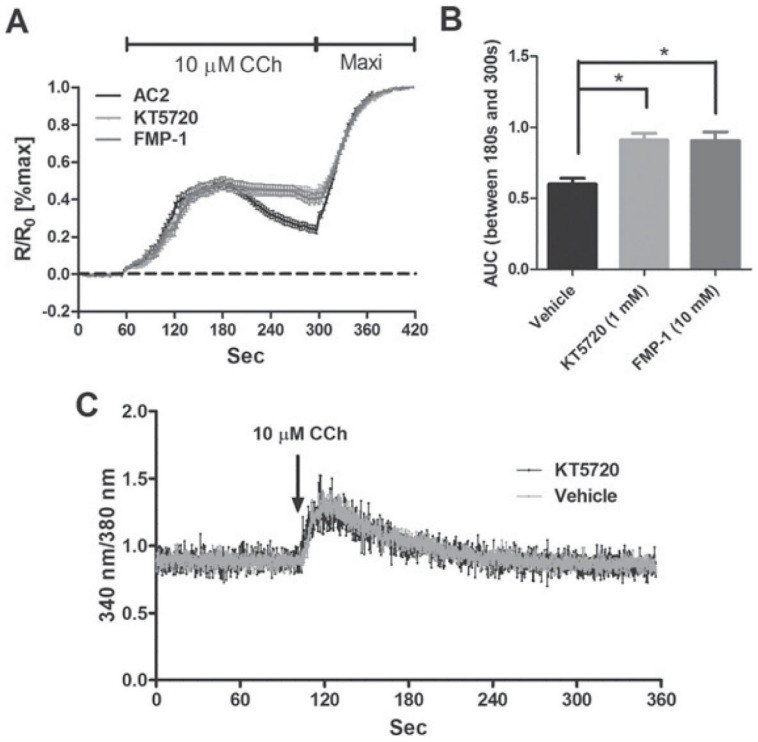

Figure 7. Involvement of PKA in carbachol-mediated AC2 activity

(**A**) HEK-AC2 cells were pre-incubated with vehicle (n = 41), 1 μM KT5720 (n = 43) or FMP-1 (n = 43) for 10 min and then stimulated with 10 μM carbachol (CCh) at 60 s for 4 min before saturating the sensor by a maximal stimulation (Maxi: 10 μM forskolin, 10 μM isoprenaline and 100 μM IBMX) at 300 s for 2 min. Data are plotted as relative FRET ratio changes with R_0 taken at 0 s, and R/R_0 is then normalized to the maximum signal response. (**B**) The AUC (area under the curve) between 180 s and 300 s from (**A**). *P < 0.1. (**C**) HEK-AC2 cells were incubated with 100 μM EGTA in Krebs buffer, and stimulated with 10 μM carbachol (CCh) at 100 s. Data are plotted as 340/380 nm ratio change.

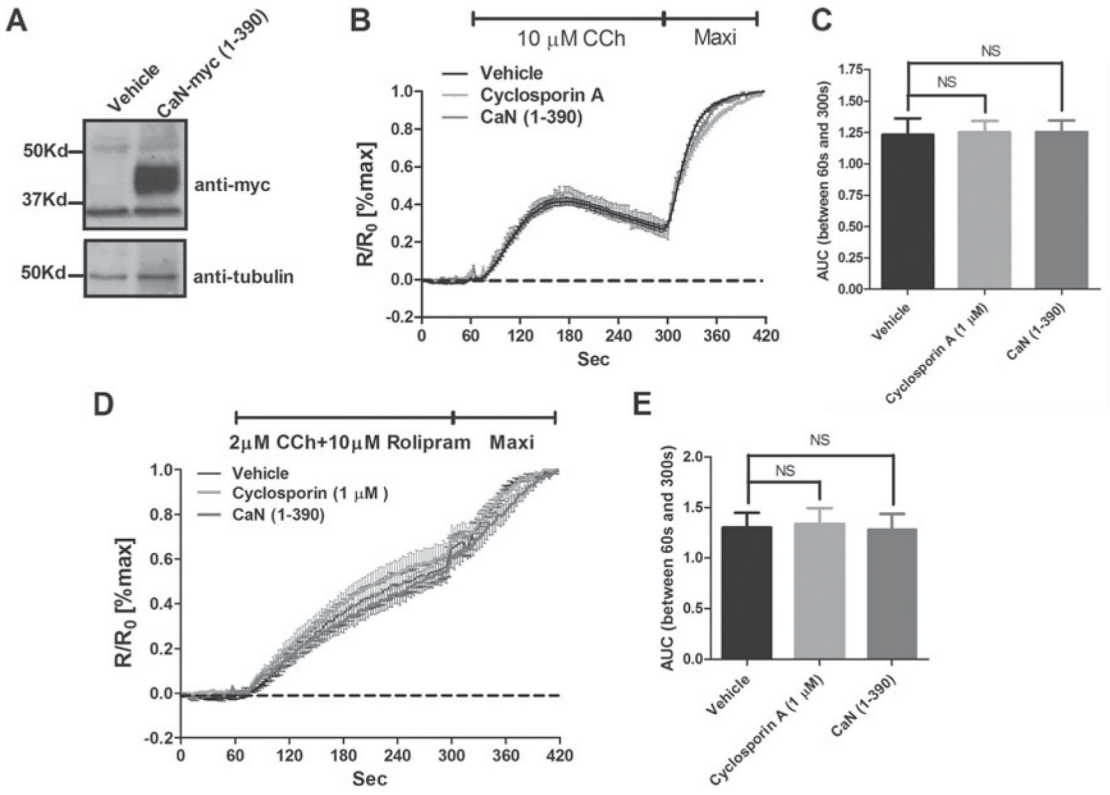

Figure 8. Identifying whether calcineurin is involved in the carbachol effects

(**A**) Western blotting establishes the expression of constitutively active calcineurin, calcineurin-(1–390) [CaN-myc (1-390)]. The lower blot shows anti-tubulin immunoreactivity as a loading control. Molecular masses are indicated in kDa. (**B**) Carbachol (CCh)-mediated cAMP production in HEK-AC2 cells transfected with (n = 56) or without (n = 41) calcineurin-(1–390) [CaN (1-390)] after pre-incubating with vehicle or cyclosporin A (n = 35). Data are plotted as relative FRET ratio changes with R_0 taken at 0 s, and R/R_0 is then normalized to the maximum signal response. (**C**) The AUC (area under the curve) between 60 s and 300 s from (**B**). NS, not significant. (**D**) Carbachol (CCh)- and rolipram-induced cAMP change in HEK-AC2 cells transfected with or without calcineurin-(1–390) [CaN (1-390)] after pre-incubating with vehicle or cyclosporin A. Data are plotted as relative FRET ratio changes with R_0 taken at 0 s, and R/R_0 is then normalized to the maximum signal response. (**E**) The AUC (area under the curve) between 60 s and 300 s from (**D**). NS, not significant. After pre-incubating with vehicle (n = 42) or 1 μM cyclosporin A (n = 44) for 10 min, HEK-293 cells transfected with (n = 40) or without (n = 44) calcineurin-(1–390) stimulated with 10 μM carbachol (**B** and **C**) or 2 μM carbachol + 10 μM rolipram (**D** and **E**) at 60 s for 4 min and followed by a maximal stimulation (Maxi: 10 μM forskolin, 10 μM isoprenaline and 100 μM IBMX) at 300 s for 2 min.