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Differential dopamine receptor subtype regulation of adenylyl cyclases in lipid rafts in human embryonic kidney and renal proximal tubule cells

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Abstract

Dopamine D_1-like receptors (D_1R and D_5R) stimulate adenylyl cyclase (AC) activity, whereas the D_2-like receptors (D_2, D_3 and D_4) inhibit AC activity. D_1R, but not the D_5R, has been reported to regulate AC activity in lipid rafts (LRs). We tested the hypothesis that D_1R and D_5R differentially regulate AC activity in LRs using human embryonic kidney (HEK) 293 cells heterologously expressing human D_1 or D_5 receptor (HEK-hD_1R or HEK-hD_5R) and human renal proximal tubule (hRPT) cells that endogenously express D_1R and D_5R. Of the AC isoforms expressed in HEK and hRPT cells (AC3, AC5, AC6, AC7, and AC9), AC5/6 was distributed to a greater extent in LRs than non-LRs in HEK-hD_1R (84.5±2.3% of total), HEK-hD_5R (68.9±3.1% of total), and hRPT cells (66.6±2.2 % of total) ($P<0.05$, n=4/group). In HEK-hD_1R cells, the D_1-like receptor agonist fenoldopam (1μM/15 min) increased AC5/6 protein (+17.2±3.9 % of control) in LRs but decreased it in non-LRs (-47.3±5.3 % of control) ($P<0.05$, vs. control, n=4/group). By contrast, in HEK-hD_5R cells, fenoldopam increased AC5/6 protein in non-LRs (+67.1±5.3% of control, $P<0.006$, vs. control, n=4) but had no effect in LRs. In hRPT cells, fenoldopam increased AC5/6 in LRs but had little effect in non-LRs. Disruption of LRs with methyl-β-cyclodextrin decreased basal AC activity in HEK-D_1R (-94.5±2.0 % of control) and HEK-D_5R cells (-87.1±4.6 % of control) but increased it in hRPT cells (6.8±0.5-fold). AC6 activity was stimulated to a greater extent by D_1R than D_5R, in agreement with the greater colocalization of AC5/6 with D_1R than

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D_5R in LRs. We conclude that LRs are essential not only for the proper membrane distribution and maintenance of AC5/6 activity but also the regulation of D_1R- and D_5R-mediated AC signaling.

Keywords

Adenylyl cyclase; dopamine receptor; lipid rafts; signal transduction

1. Introduction

Adenylyl cyclases (ACs) catalyze the conversion of intracellular ATP to adenosine 3, 5-cyclic monophosphate (cAMP) which mediates the actions of many hormones and neurotransmitters (1-4). To date, 10 mammalian ACs have been cloned and characterized (3, 5). They are grouped into 4 major subfamilies: group 1 is comprised of Ca^{2+}-stimulated AC1, AC3, and AC8; group 2 is comprised of Gβγ-stimulated AC2, AC4, and AC7; group 3 is comprised of Ca^{2+} -inhibited AC5 and AC6; and group 4 has one member, forskolin-insensitive AC9 (3-5). All of these nine ACs are stimulated by the GTP-bound α subunit of G protein (3, 5). G protein-coupled receptors (GPCRs) regulate AC activity through G protein subunits (6-9). However, the tenth AC, a splice variant in the testis (sAC), lacks a membrane spanning domain and is stimulated by calcium but not by G proteins (3, 5). Each AC isoform has a specific pattern of tissue/organ distribution and a specific pattern of regulation by G proteins, calcium/calmodulin, and protein kinases (1-5).

Dopamine receptors are classically divided into two groups: D_1- and D_2-like receptors, based on their interaction with AC (6-8). The D_1-like receptors comprised of the D_1R and D_5R receptor subtypes stimulate AC through Gαs whereas the D_2-like receptors comprised of the D_2, D_3, and D_4 receptor subtypes inhibit AC (7, 8). We have reported that all AC mRNAs and proteins are found in the rat kidney except AC1 and AC8. AC isoforms 2, 3, 6, 7, and 9 are expressed in rat renal proximal tubules (9). These AC isoforms plus AC4 and AC5 are also expressed in the mouse and rat renal collecting duct (10, 11).

The human D_1R (hD_1R), as with other GPCRs and ACs, are regulated by lipid rafts and caveolae (12-24). Lipid rafts are dynamic structures, rich in cholesterol and sphingolipids that are important in organizing signal transduction cascades. Caveolae are a subset of lipid rafts characterized by their invaginated morphology, formed by the crosslinking of their characteristic marker protein, caveolin-1 (Cav-1) (3, 13, 14). Ca^{2+}-sensitive ACs (AC1, AC3, AC5, AC6, and AC8) are found in lipid rafts, while Ca^{2+}-insensitive ACs (AC2, AC4, and AC7) are found in non-lipid rafts (4, 14). The association of GPCRs with specific AC isoforms has been reported. AC5 and AC6 are associated with: β_2 adrenergic receptors (β_2AR) in lipid rafts in cardiac, vascular, and bronchial smooth muscle cells (16-18); nicotinic acetylcholine receptor (nAChR) in lipid rafts in pheochromocytoma cells (PC12 cells) (19, 20); and μ- but not δ-opioid receptor in lipid rafts in human embryonic (HEK) cells (23). Toll-like receptor 4 is associated with AC6 in lipid rafts in a murine macrophage cell line (24), while stomatin-related olfactory protein and AC3 are found in lipid rafts in olfactory cilia (22). By contrast, AC2 and E prostanoid type 2 receptors are found in non-lipid rafts in mouse bronchial smooth muscle cells (21).

The association of D_1R and AC5/6 in several cells, including HEK-293 cells, has been reported (12, 25). However, the AC isoform associated with D_5R has not been established. In the current study, we investigated whether or not specific AC isoforms are differentially regulated by hD_1R and hD_5R in lipid rafts in HEK-293 cells heterologously expressing either hD1R (HEK- hD_1R) or hD_5R (HEK- hD_5R) and in human renal proximal tubule (hRPT) cells endogenously expressing D_1R and D_5R. Our data indicated that AC 5/6 was differentially distributed in lipid rafts in these renal epithelial cells and their integration in membrane microdomains is important in maintaining basal AC activity and dopamine receptor-mediated signaling.

2. Methods

2.1 Cell treatment

Well-characterized HEK-hD_1R, HEK-hD_5R, and hRPT cells were used (12, 26-30). The cells, pre-starved in serum-free αMEM medium (SFM) for 1hr, were grown in 100 cm dishes to 90% confluence. The cells were treated for 15 min at 37°C with vehicle, D_1-like receptor agonist fenoldopam (1.0 μmol/L), D_1-like receptor antagonist SCH23390 (5.0 μmol/L) alone, or combination of fenoldopam and SCH23390 (SCH23390 added to the cells 5 min prior to the addition of fenoldopam). To disrupt the lipid rafts, the cholesterol depleting reagent methyl-β-cyclodextrin (βCD) was used. The cells were washed once with SFM and then incubated with vehicle or βCD (2%/ 1 h) at 37°C in SFM (27). Cholesterol repletion was performed by incubating the cells in a pre-mixed solution containing cholesterol (stock solution in 100% ethanol at 50 mg/ml), 100 μg/ml, and βCD (2%). The cells were also treated with drug combinations: βCD+fenoldopam (βCD added to the cells 1 h prior to the addition of fenoldopam); βCD+cholesterol (mixture added to cells 1 h prior to addition of fenoldopam); and βCD+cholesterol+fenoldopam (βCD+cholesterol mixture added 1 h prior to addition of fenoldopam).

2.2 Cell transfection

HEK-hD_1R, HEK-hD_5R, and hRPT cells were transfected with vehiclethat contained only the transfection reagent and served as control (Con), non-silencing*mock-siRNA* that consisted of a scrambled sequence and served as another negativecontrol, or siRNA that was specific for a particular AC isoform *(AC isoform-siRNA)*, as described previously (27). The cells were seeded in 6-wellplates at a density of 5×10^5/well at day 1 and then transfected with *mock-siRNA*, or *AC isoform-specific-siRNA* at day 2. AC protein and AC activity were determined at day 4. To measure AC activity, the cells were seeded in 12-well plates at a density of 2×10^5/well at day 1, followed by transfection with *AC isoform*-specific-siRNA at day 2; cAMP concentration was measured at day 4 using a cAMP direct immunoassay kit.

2.3 Subcellular fractionation

To prepare lipid and non-lipid rafts, the cells, pre-treated with vehicle (control), fenoldopam (1 μmol/L, 15 min), or βCD 2% at 37°C, were subjected to sucrose density gradient centrifugation, using a detergent-free protocol, as described previously (12, 27).

2.4 Measurement of cAMP accumulation

To measure cAMP accumulation the samples were prepared as described previously (12, 28). HEK-hD_1R, HEK-hD_5R, or hRPT cells were seeded into 12-well plates (2×10^5 cells/well) in complete culture medium. When the cells had grown to 90% confluence, they were incubated for 20 min at 37°C with (0.2 ml/well) MHI medium [αMEM serum-free medium, HEPES (10 mmol/L), isobutylmethylxanthine (IBMX, 1mmol/L), and ascorbic acid (100 μmol/L)]. The cells were then treated with vehicle (MHI medium) or fenoldopam (1 μmol/L) and other drugs diluted in MHI medium (vehicle or drug volume=50μl/well) for 10 min at 37°C. The reactions were stopped by adding 0.25 ml of 0.2N HCl to each well. The cells, collected by scrapping, were transferred into labeled microcentrifuge tubes and centrifuged at 2000 x *g* for 5 min. The supernatants were obtained and cAMP was measured using a cAMP immunoassay kit, and expressed as pmol/mg protein/min which was later converted to % change of control.

2.5 Co-immunoprecipitation and immunoblotting

To determine the association of D_1R or D_5R with specific AC isoforms, co-immunoprecipitation experiments were performed, as described previously (12). HEK-hD_1R and HEK-hD_5R cells were treated with vehicle, fenoldopam (1 μmol/L), SCH23990 (5 μmol/L, added 5 min prior to the addition of fenoldopam), or the combination of fenoldopam and SCH23390 for 10 min. The cells were lysed in MBST buffer and equal amounts of cell lysate proteins (500 μg) were mixed with a polyclonal anti-AC5/6 antibody and incubated at 4 °C overnight. Protein A/G beads (30 μl) were added to each sample with rocking for 2 h at 4°C on the next day. The immune complexes were washed 3X with cold PBS. The bound proteins were eluted by the addition of Laemmli buffer (20 μl) and boiled for 5 min. The samples were subjected to immunoblotting with mouse monoclonal anti-Myc (for hD1R) or anti-V5 (for hD_5R) antibodies, as indicated. Normal rabbit IgG was used as a negative control. The immunoreactive bands were semi-quantified by densitometry (12, 27).

2.6 Statistical analysis

Data are expressed as Mean ± standard error (SEM). Significant differences between two groups were determined by Student's *t-test* Significant differences among more than 2 groups were determined by one-way factorial ANOVA, followed by Tukey post-hoc test; $P<0.05$ was considered significant.

3. Results

3.1 AC isoform mRNA profile in human kidney cells

The first aim of our studies was to determine the endogenous mRNA expression of AC isoforms in untransfected HEK-293 and hRPT cells using the primers listed in Table S1. We found that *AC3*, *AC5*, *AC6*, *AC7* and *AC9* mRNA were expressed in HEK-293 and hRPT cells (Figure 1). The endogenous expression of AC2, AC4, AC5/6, and AC9 has been reported in some but not all HEK-293 cell lines (31, 32). Sequencing of the mRNA products confirmed the identity of these AC isoforms (Table S2), except for AC2 and AC4. Band B under *AC2* mRNA (Figure 1) was chromodomain helicase DNA binding protein 8. The apparent expression of *AC4* mRNA in HEK-293 cells was not confirmed by sequencing

because it was not expressed in hRPT cells. *AC8* mRNAs was not found in either human kidney cell line (HEK293 and hRPT). The *AC* mRNA profile in hRPT cells differed from rat RPT cells, which expressed AC2 in addition to AC3, AC6, AC7, and AC9; AC5 was found in hRPT but not rat RPT cells (9, 33), indicating species specificity; *AC8* mRNA was not found in either human or rat RPT cells but may be minimally expressed in HEK cells. *AC1* gene expression was not studied in human renal epithelial cells because this gene is expressed mainly in brain (4).

3.2 Distribution of AC isoforms in lipid and non-lipid raft microdomains

HEK-hD_1R, HEK-hD_5R, and hRPT cells have been characterized in ourprevious reports (12, 26-30). We now report the distribution of AC isoform proteins in lipid and non-lipid rafts, using sucrose gradient fractionation samples from these cells. Among the AC isoforms only AC5/6 protein (using an antibody that recognizes both AC5 and AC6) was expressed mainly in lipid rafts in these kidney cells, in agreement with previous reports (13, 14, 16, 17, 23) (Figures 2A and 2B, **upper panels**). In the basal state, more AC5/6 was distributed in lipid rafts than in non-lipid rafts and to a greater extent in HEK-hD_1R cells (84.5±2.3 % of total density units, DU) than HEK-hD_5R cells (69±3.1 % total DU) ($P<0.05$, n=4, ANOVA) (Figure 2A). In HEK-hD1R cells, AC5/6 was concentrated in lipid raft fraction 4 but, in HEK-hD_5R cells, AC5/6 was distributed in fractions 3-6 in lipid rafts and fractions 7 and 8 in non-lipid rafts, indicating that the concomitant expression of a specific receptor may alter AC localization in membrane microdomains. In hRPT cells, basal AC5/6 protein profile was similar to that observed in HEK-hD1R cells, in which AC5/6 was mainly concentrated in lipid raft fraction 4 (66.6±2.2 % total DU) (Figure 2B). In HEK-hD_1R cells, fenoldopam increased AC5/6 in lipid raft fractions 3 and 4 (+17.2±3.9 % change from control) and decreased AC5/6 in non-lipid rafts (-47.3±5.3 % change from control) ($P<0.05$, n=4, ANOVA) (Figure 2A). By contrast, in HEK-hD_5R cells, fenoldopam had little effect on AC5/6 in lipid rafts (-6.3±1.7 % change from control) but increased the expression of AC5/6 in non-lipid raft fractions 8 and 9 (+67.1±20.5 % from control, $P<0.05$, n=4) (Figure 2A). In hRPT cells, fenoldopam increased AC5/6 protein in lipid rafts in fractions 4 and 5 (*$P<0.05$, n=5, ANOVA) but had little effect on AC5/6 expression in non-lipid rafts (+14.9±13.5 % of control) (Figure 2B), which seems to be the algebraic sum of that found in HEK-hD1R and HEK-hD_5R cells.

AC3 protein was also present in lipid and non-lipid rafts in HEK-hD_1R, in agreement with previous reports (13, 14, 23), and hRPT cells, but present only non-lipid rafts in HEK-hD_5R cells, supporting the aforementioned notion that the concomitant expression of a specific receptor may alter AC localization in membrane microdomains. Fenoldopam decreased AC3 protein in lipid rafts in HEK-hD_1R and hRPT cells but had no effect in HEK-hD_5R cells (Figure 2C).

AC7 protein was expressed mainly in non-lipid rafts and minimally expressed in lipid rafts in both HEK- hD1R (12.1± 0.8 %) and HEK-hD_5R cells (13.3±4.1% of total DU) but was diffusely distributed in lipid rafts (fractions 3-6) (29.4±5.4 % total DU) and non-lipid rafts (fractions 7-12) in hRPT cells (Figure 2D). Fenoldopam had little or no effect on AC7

protein in HEK-hD$_1$R and HEK- hD$_5$R cells but shifted all of AC7 in lipid rafts to non-lipid rafts in hRPT cells.

AC9 was found mainly in non-lipid rafts and its membrane distribution was not affected by fenoldopam in HEK-hD1R, HEKhD$_5$R, and hRPT cells (Figure 2E). The finding that the AC7 and AC9 distributions in lipid and non-lipid rafts and response to fenoldopam are similar in both HEK-hD$_1$R and HEK-hD$_5$R cells, unlike that found for AC3 and AC5/6, indicate that the concomitant expression and response to agonist stimulation of a specific GPCR, D1R and D$_5$R in this instance, is isoform-specific.

3.3 Effect of βCD on AC5/6 distribution

βCD, a cholesterol-depleting reagent, has been used to determine the effect of disruption of lipid rafts on the distribution of proteins in cell membranes (16-20, 23). The cells were treated with βCD (2%) at 37°C for 1hr, and the lysates were subjected to sucrose gradient centrifugation as in previous experiments (12, 27) (Figure 3A). Similar to those shown in Figure 2B, in the basal state, AC5/6 was distributed to a greater extent in lipid than non-lipid rafts in HEK-hD$_1$R and hRPT cells, while in HEK-hD$_5$R cells AC5/6 was distributed in fractions 3-6 in lipid rafts and fractions 7-9 in non-lipid rafts (Figure 3A). In HEK-hD$_1$R and HEK-hD$_5$R cells, βCD did not alter the membrane distribution of AC5/6 but decreased the molecular size of AC5/6 in lipid rafts in HEK-hD$_1$R cells, indicating that the presence of a particular protein in the more buoyant sucrose gradient fractions does not necessarily indicate presence in lipid rafts. By contrast, βCD shifted all AC5/6 to non-lipid rafts in hRPT cells.

To determine if the change in molecular size of AC5/6 in HEK-hD$_1$R cells caused by βCD was related to deglycosylation, the cell lysates were incubated with N (PNG-F)-glycanase, O-glycanase, or sialidase and then probed with specific polyclonal anti-AC5/6 antibodies. The molecular size of AC5/6 in vehicle-treated cells was 160 kDa and was not affected by PNG-F alone but decreased to 120 kDa following treatment with the combination of PNG-F, O-glycanase, and sialidase A (Figure S1). Therefore, disruption of lipid rafts with βCD may cause AC5/6 deglycosylation in HEK-hD$_1$R cells.

We also studied the association of Cav-1, a lipid raft marker protein, with AC5/6 in hRPT cells; similar studies in HEK cells were not performed because Cav-1 is not expressed in some HEK-293 cells, including ours (12, 16, 34, 35), although it may be present in other HEK-293 cell lines (33), albeit minimally (36). In hRPT cells, the D1-like receptor agonist fenoldopam increased the association of AC5/6 protein with Cav-1 (147.5±17.2 DU vs. control 70.3±12.6 DU) ($P<0.05$, n=4, ANOVA) (Figure 3B), supporting the finding that fenoldopam increased AC5/6 protein in lipid rafts (Figure 2A). Cav-1 has been reported in hRPT cells (27, 29) which can form caveolar membranes (14, 17). The stimulatory effect of fenoldopam was blocked by the D$_1$-like receptor antagonist Sch23390, indicating specific effect on D1-like receptors.

3.4 Association of AC isoforms with specific dopamine receptor subtypes

The GPCR coupling to specific AC isoforms has been reported (16-24). We, therefore, determined if D$_1$R and D$_5$R colocalize with AC5/6 (Figure 4A). Fenoldopam promoted the

co-localization of hD_1R with AC5/6 and stimulated cAMP accumulation in HEK-hD_1R cells at 15 min that became markedly attenuated at 60 min. Fenoldopam also promoted the co-localization of D_5R with AC5/6 and cAMP production in HEK-hD_5R cells but to a lesser degree than that observed for hD_1R in HEK-hD_1R cells.

The D_1-like receptor agonist fenoldopam promoted the interaction of75-kDa-D_1R with AC5/6 (41.9 ± 4.1 vs. control 15.1 ± 3.5 DU)($P<0.006$, n=4, ANOVA) in HEK-hD_1R cells (Figure 4B, **left graph**), while the 45-kDa- but not the 60-kDa-D_5R interacted with AC5/6 (76.2 ± 9.7 DU vs. control 12.0±2.1 DU) ($P<0.005$, n=4, ANOVA) in HEK-hD_5R cells (Figure 4B, **right graph**). In addition, D_1R interacted with AC3 (Figure 4C); D_5R did not interact with AC3 in HEK-D_5R cells (Figure S2, supplementary file), in agreement with the inability of fenoldopam to alter the non-lipid raft distribution of AC3 in HEK-hD_5R cells. The interaction of hD_1R and hD_5R with AC5/6 and interaction of D_1R with AC3 were prevented by the D_1-like receptor antagonist SCH23990, indicating that the interaction was specific to D_1R and D_5R (Figures 4B and 4C). There was interaction between AC4 and D_1R and AC4 and D_5R (Figure S3, supplementary file). However, the association of D_5R but not D_1R with AC4 was reversed by the D_1-like receptor antagonist SCH23990. We also found that D_1R interacted with AC9 (Figure S4, supplementary file), but the interaction was not specific to D_1R because the D_1R-AC9 interaction was not prevented by the D_1-like receptor antagonist SCH23990 (data not shown). Neither D_1R nor D_5R interacted with AC7 (Figure S4, supplementary file), in agreement with the inability of fenoldopam to alter the lipid and non-lipid raft distribution of AC7.

hRPT cells endogenously express D_1R and D_5R (27-30). Therefore, co-immunoprecipitation studies were also performed in hRPT cells to determine whether or not AC5/6 protein could associate with endogenously expressed D_1R and D_5R. In agreement with studies in heterologously expressed D_1R (HEK-hD1R) and D_5R (HEK-hD_5R), fenoldopam promoted the co-immunoprecipitation of endogenous AC5/6 with endogenous D1R (110.1±9.9 DU vs. control 77.2±4.2 DU) and D_5R (182.4±31.4 DU vs. control 96.7±3.6 DU) ($P<0.05$, n=5-6, one-way ANOVA) (Figure 4D); control D_5R was designated as the control for all the experiments in Figure 4.

3.5 Differential effects of βCD on AC activity in kidney cells

cAMP accumulation was measured in whole cell lysates following drug treatment. Basal cAMP accumulation was greater in HEK-hD_5R cells (120±11 pmol/mg protein/min) than HEK-hD1R cells (71±4 pmol/mg protein/min) ($P<0.003$, n=4, ANOVA). Fenoldopam markedly increased cAMP accumulation in both cell lines (Figure 5A and Table 1). However, the percent (not absolute) response to fenoldopam was greater in D1R (183±22 % of control=100±4.0) than D_5R (140±4.8 % of control=100±3.0). Disruption of lipid rafts with βCD decreased basal and fenoldopam-stimulated AC activity by 90 % in both cell lines (4±1.6 pmol/mg protein/min in HEK-hD_1R cells and to 15±4.6 pmol/mg protein/min in HEK-hD_5R cells), indicating that lipid rafts keep the AC enzyme in an active state in HEK-293 cells. Cholesterol repletion partially restored AC activity but at levels that were still below control levels (32.4±7.0 pmol/mg protein/min, HEK-hD1R and 39.1±7.7 pmol/mg protein/min, HEK-hD_5R cells, vs. βCD treatment and control) ($P<0.05$, ANOVA).

AC accumulation was also measured in lipid and, non-lipid rafts following drug treatment and sucrose gradient centrifugation. Fenoldopam markedly increased AC activity in lipid rafts in HEK-hD1R (Figure 5B) and HEK-hD_5R (Figure 5C). The increase in AC activity occurred mainly in lipid rafts in HEK-hD1R cells similar to our previous report (12). AC activity in response to fenoldopam was greater in HEK-hD_1R (512±47 pmol/mg/min) than HEK-hD_5R cells (132±9.1 pmol/mg/min). However, AC activity was greater in non-lipid rafts in HEK-hD_5R than HEK-hD_1R cells.

In hRPT cells, basal cAMP accumulation was very low (0.04±0.003 pmol/mg protein/min), relative to HEK-hD_1R and HEK-hD_5R cells. βCD treatment increased cAMP accumulation 6.8±0.5-fold (0.29±0.05 pmol/mg protein/min) ($P<0.001$, n=4, ANOVA) (Figure 5D), the opposite of the response observed in HEK-hD1R and HEK-hD_5R cells. Cholesterol repletion restored the cAMP accumulation close to basal levels. Fenoldopam markedly increased cAMP accumulation (120±0.6-fold) that was decreased by βCD treatment and partially restored by the addition of cholesterol. Therefore, caution is needed in translating studies in embryonic kidney cells (HEK) to adult RPT cells.

3.6 Effect of siRNA-mediated silencing of specific AC isoforms on AC activity

siRNAs against specific AC isoforms were used to determine which AC isoform contributed to hD_1R or hD_5R signaling. Silencing the gene expression of *AC3*, *AC5/6*, *AC7*, and *AC9* decreased the AC isoform protein expression: 70% for AC3, 50% for AC5/6, 80% for AC7, and 80% for AC9 in HEK-hD1R cells (Figure 6A). Silencing of a single *AC* isoform gene with an AC isoform-specific siRNA *(AC3, AC5/6, AC7*, and *AC9*) partially decreased the fenoldopam-induced increase in AC activity to similar degrees in HEK-hD1R cells and HEK-hD_5R cells (Figure 6B). These studies show that the failure of fenoldopam to alter the lipid and non-lipid raft distribution of AC7 and AC9 or their co-immunoprecipitation with D_1R or D_5R did not preclude their involvement in cell signaling. Indeed, silencing multiple *AC* isoform genes with *AC3*- *AC5/6*-, and/or *AC7*-specific siRNA augmented their ability to decrease the fenoldopam-mediated increase in AC activity (60±2.8 % of mock-siRNA +Fen) relative to the silencing *C5/6* gene (73.4±2.1 % of mock-siRNA) in HEK-hD_1R cells ($P<0.001$, n=4, ANOVA) (Figure 6C).

To further confirm the effect of *AC5*-specific siRNA and *AC6*-specific siRNA on AC activity, the effects of **only** *AC5*-specific siRNA, **only** *AC6*-specific siRNA, or their combination were studied. Silencing AC5, AC6, or both had no effect on basal AC activity compared with mock-siRNA in either HEK-hD_1R or HEK-hD_5R cells, but the basal level of AC activity was greater in HEK-hD_5R than HEK-hD_1R cells (Figure S5, supplementary file), in agreement with previous reports (37, 38). Silencing the *AC5* gene reduced the fenoldopam-stimulated AC activity to almost the same extent in HEK-hD_1R and HEK-hD_5R cells (Figure 6D), in agreement with previous reports (37, 38). In contrast, silencing the *AC6* gene impaired the ability of fenoldopam to increase AC activity to a greater extent in HEK-hD_1R than HEK-hD_5R cells ($P<0.05$, ANOVA). Silencing both *AC5* and *AC6* genes did not decrease further the effect of either AC5 or AC6 depletion on fenoldopam-mediated increase in AC activity in HEK-hD_1R cells; however, silencing both *AC5* and *AC6* caused a lesser impairment in the ability of fenoldopam to increase AC activity, similar to that caused by

AC6-specific siRNA alone, in HEK-hD_5R cells ($P<0.05$, vs. siAC5+6-D_1R, ANOVA) (Figure 6D). These results could be taken to indicate that AC5 is important in the agonist response of both D_1R and D_5R while AC6, independent of AC5, is also involved in the positive agonist response of D_1R. AC6 may be needed with AC5 to abet the positive agonist response of D_5R, in agreement with the colocalization of these AC isoforms in lipid rafts.

4. Discussion

Several lines of evidence have shown that some AC isoforms are distributed in lipid rafts and coupled to different GPCRs and downstream effectors endogenously expressed in tissues and cells (16-22, 24, 37) or in over-expression systems (23, 24, 37). However, the regulation of endogenous AC enzymes in human kidney cells has not been reported. We now report the endogenous *AC* gene expression profiles in HEK-293 and hRPT cells. There are differences among HEK-293, hRPT, and rat RPT cells in the expression of AC isoforms: 1) *AC8* is not expressed in rat RPT and hRPT cells, but minimally expressed in HEK-293 cells; 2) *AC5* is expressed in HEK-293 and hRPT cells but not in rat RPT cells; and 3) *AC2* is expressed in rat RPT cells but not in HEK-293 or hRPT cells (9, 33).

There are other novel findings in the current studies. First, the distribution of endogenous AC5/6 in lipid and non-lipid rafts in HEK-293 cells is affected by the heterologous expression of D_1R and D_5R. Second, the D_1-like receptor agonist fenoldopam increases AC5/6 protein in lipid rafts and decreases it in non-lipid rafts in HEK-hD_1R cells, increases AC5/6 in non-lipid rafts only in HEK-hD_5R, and increases AC5/6 in lipid rafts only and its association with Cav-1 in hRPT cells. Third, endogenous AC5/6 and AC3 are linked to 75-kDa-D_1R in HEK-hD_1R cells, while AC5/6 is linked to 45-kDa-D_5R in HEK-hD_5R cells. Fourth, gene silencing experiments demonstrated that of all AC isoforms endogenously expressed in HEK-293 cells, only AC3, AC5/6, AC7, and AC9 can mediate D_1R and D_5R signaling. AC5 is important in the agonist response of both D_1R and D_5R, while AC6, independent of AC5, is also involved in the positive agonist response of D_1R. AC6 may be needed by AC5 for the agonist response of D_5R.

Lipid rafts are dynamic cholesterol- and sphingolipid-enriched structures that play an important role in organizing signal transduction cascades (13, 14, 38). The extent by which a given GPCR regulates an AC isoform may depend on its plasma membrane localization (14, 37). For example, the β_2 adrenergic receptor and AC6 are localized with caveolae in cardiac myocytes, which allow the preferential stimulation of AC6, whereas the prostanoid EP2 receptor, which is distributed outside the caveolae, is coupled to AC6 with far less efficiency (21, 38). The μ-opioid receptor (μ-OPR), but not δ-OPR, is localized in lipid rafts and is more sensitive than δ-OPR to cholesterol depletion (23). In the current studies, we found that endogenous AC5/6 protein is concentrated in lipid raft fraction 4 in HEK-hD1R while AC5/6 protein is diffusely expressed in lipid raft fractions 3-6 in in HEK-hD_5R cells. The localization of AC5/6 in lipid rafts may have contributed to differences in basal activity (D_5R>D1R) and percent responses to agonist stimulation (D_1R>D_5R). Disruption of lipid rafts with βCD promoted the de-glycosylation of AC5/6 in HEK-hD1R cells, and shifted all AC5/6 protein to non-lipid rafts in hRPT cells. The decrease in basal AC activity in HEK-293 cells with disrupted lipid rafts suggests that the complex composed of the GPCR,

G protein, and AC is in an active state in HEK-293 cells. It is possible that the diffuse localization of AC5/6 in the lipid raft fractions may be responsible for the greater basal AC activity in D_5R than D_1R (39-42). Although the potency of agonist stimulation of cAMP production is greater for D_5R than D_1R (37-42), the percent response to maximum AC stimulation with fenoldopam is greater in D_1R than D_5R in the current study. It is possible that the increased expression of AC5/6 in lipid raft fraction 4 in HEK-D_1R cells is responsible for the greater percent maximum effect of agonist stimulation in HEK-D1R than HEK-D_5R cells. There may be greater coupling of D_1R in HEK-D_1R cells because signaling molecules such as Gαs, Gβγ, PKA subunits are also concentrated in the same lipid raft fraction in HEK-hD_1R cells (12). Indeed, the maximum fenoldopam-stimulated AC activity is greater in HEK-hD_1R than HEK-hD_5R, in agreement with a previous report (42).

βCD is widely used to disrupt lipid rafts (15-22). Disruption of lipid rafts with βCD in HEK-293 cells did not alter the lipid raft fraction 4 localization of AC5/6 but shifted its expression to a lower molecular size from 160kDa to 120kDa in HEK-hD1R cells, indicating that AC5/6 protein is not sensitive to cholesterol extraction in HEK-293 cells but was sensitive to cholesterol extraction in hRPT cells, as indicated above. The decreased molecular size of AC5/6 may be due to deglycosylation, in agreement with the report that AC6 is a glycosylated protein (32). However, we do not know why βCD treatment would have caused AC5/6 de-glycosylation in HEK-hD1R cells but not in HEK-hD_5R and hRPT cells.

Both D_1R and D_5R are coupled to Gαs while D_5R but not D_1R is also coupled to $G\alpha_{12/13}$ (7, 44). The complex of D1R-Gαs-AC5/6 may be less stable than the complexes of D_5R-Gsα/Gα $_1$2/13-AC5/6 in HEK-hD1R and HEK-hD_5R, and D_1R/D_5R/G-proteins/Cav-1 in hRPT cells. Although AC5/6 distribution pattern is similar in HEK-hD_1R and hRPT cells, there is a major difference in that AC5/6 protein completely moved out of lipid rafts in hRPT cells but not in HEK-hD_1R and HEK-hD_5R cells, following disruption of lipid rafts, indicating that AC5/6 is sensitive to cholesterol extraction in hRPT cells, in agreement with previous reports (16, 17). The difference in AC5/6 properties between embryonic (HEK-293) and terminally differentiated (hRPT) cells is another example of cell specificity. It may be because of the presence of Cav-1, which is responsible for the formation of caveolae (10, 17), in hRPT cells but minimally present or even absent in HEK-293 cells (12, 16, 34, 35). These data could be taken to suggest that different signaling systems are localized in a specialized subdomain of the plasma membrane to optimize the efficiency of signal transduction (10, 45, 46).

βCD treatment has been reported to stimulate or inhibit AC activity (15-22). Cholesterol depletion with βCD augmented isoproterenol-, zinterol-, and forskolin-stimulated accumulation of cAMP in rat ventricular cardiomyocytes (16). βCD treatment or Cav-1 knockdown in C6 glioma cells also increased isoproterenol- or thyrotropin-stimulated cAMP accumulation (15). However, the inhibition by nicotine of the forskolin-stimulated AC activity in a pheochromocytoma cell line (19), and basal and α7 nicotinic receptor-stimulated AC activity in airway epithelial cells were prevented by βCD (20). βCD treatment increased basal forskolin-stimulated cAMP accumulation in HEK-293 cells transfected with δ-and μ-opioid receptors but blocked μ- but not δ-opioid agonist-induced sensitization of AC

activity (23). In the current studies, we found that disruption of lipid rafts decreases the basal and agonist-stimulated AC activity in HEK-293 cells but increases it in hRPT cells, indicating cell specificity or cell differentiation, as indicated earlier. Thus, lipid rafts keep AC in a less active state in hRPT cells and in a more active state in HEK-293 cells.

Knockdown of a single *AC* isoform gene with siRNA partially reduces the fenoldopam-stimulated AC activity in HEK-D_1R and HEK-D_5R cells, while the knockdown of combination of *ACs* genes with several AC-siRNAs (AC3, AC5/6, and AC7) further reduces the fenoldopam-stimulated AC activity in HEK-D_1R cells. This may suggest that maximal activation of AC requires the formation of multi-AC isoform complex for full agonist stimulation (35, 47). The silencing experiments with siRNA can determine the specific AC isoform effect. We found that AC5 is important in the agonist response of both D_1R and D_5R but AC6, independent of AC5, is also involved in the positive agonist response of D_1R. In contrast, in the case of D_5R, AC6 may be needed with AC5 to abet the positive agonist response, in agreement with the colocalization of these AC isoforms in lipid rafts. AC3 may also be associated with hD_1R.

5. Conclusions

Our results emphasize the key role of lipid rafts in the regulation of D_1R and D_5R-GαsAC signaling in human RPT cells. The integrated membrane microdomain localization of GPCRs and AC isoforms is important in maintaining basal and dopamine receptor-mediated signaling transduction. These findings also suggest that AC isoform-selective signaling complexes likely contribute to various functional consequences of cAMP elevation in human kidney cells.

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Abbreviations

AC	Adenylyl cyclase
DA	dopamine receptor
LRs	lipid rafts
hRPT	human renal proximal tubule
βCD	methyl- β-cyclodextrin

Highlights

1. The distribution of endogenous AC5/6 in lipid and non-lipid rafts in HEK-293 cells is affected by the heterologous expression of hD_1R and hD_5R.
2. The D_1-like receptor agonist fenoldopam differentially affects AC5/6 protein: a) increased in lipid rafts and decreased in non-lipid rafts in HEK-hD_1R cells; b) increased in non-lipid rafts but no effect in lipid rafts in HEK-hD_5R; and c) increased in lipid rafts but no effect in non-lipid rafts in hRPT cells.
3. Endogenous AC5/6 and AC3 interact with 75-kDa-D_1R in HEK-hD_1R cells, while AC5/6 interacts with 45-kDa-D_5R in HEK-hD_5R cells.
4. Gene silencing experiments demonstrated that in HEK-293 cells, AC3, AC5/6, AC7, and AC9 could mediate D_1R and D_5R function. However, AC5 is important in the agonist response of both D_1R and D_5R, while AC6, independent of AC5, is also involved in the positive agonist response of D_5R.

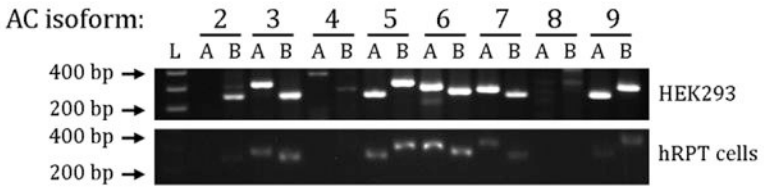

Figure 1. mRNA profiles of adenylyl cyclase (AC) isoforms in human kidney cells

mRNA was extracted from untransfected HEK-293 and hRPT cells from normotensive subjects using the RNeasy RNA Extraction Kit (Qiagen). RT-PCR was performed, as described previously (9). The sets of primers (**A** and **B**) used for human *AC* isoform mRNA detection are shown in Table S1, supplementary file.

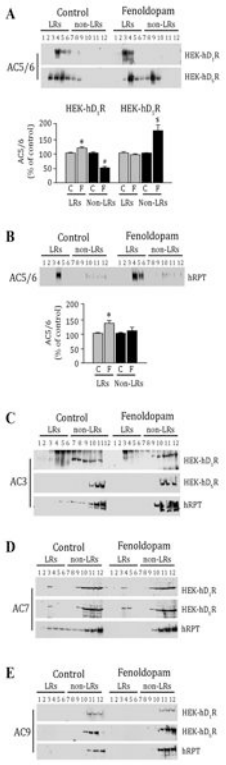

Figure 2. Distribution of adenylyl cyclase (AC) isoforms in lipid (LRs) and non-lipid rafts (non-LRs)

A-B. HEK-hD$_1$R, HEK-hD$_5$R, and hRPT cells were treated with vehicle (Control, C) or fenoldopam (F, 1 μmol/L) for 15 min. The cell lysates were then subjected to sucrose gradient centrifugation. Proteins (20 μl/lane) from sucrose gradient fractions (from 2 to 12 fractions) were immunoblotted with the antibodies against AC5/6. The immunoblot from one of 4-5 separate experiments is shown (upper panel). The immunoreactive bands for AC5/6 were semi-quantified as described previously (12, 27). The results are expressed as % of control for HEK-D$_1$R and HEK-D$_5$R cells in Figure 2A (lower panel) and for hRPT cells in Figure 2B (lower panel). Values are Mean ± SEM (n=4-5/group).

A: *P<0.01 and $^{\#}$P<0.001 vs. others in HEK-hD$_1$R cells, $^{\$}$P<0.006 vs. others in HEK-hD$_5$R cells, n4=/group, one-way factorial ANOVA, Tukey post-hoc test.

B: *P<0.05, vs others, n=5, one-way factorial ANOVA, Tukey post-hoc test.

C-E. Proteins (20 μl/lane) from sucrose gradient fractions (from 2 to 12 fractions) were immunoblotted with the antibodies against AC3 (Figure 2C), AC7 (Figure 2D), and AC9 (Figure 2E)

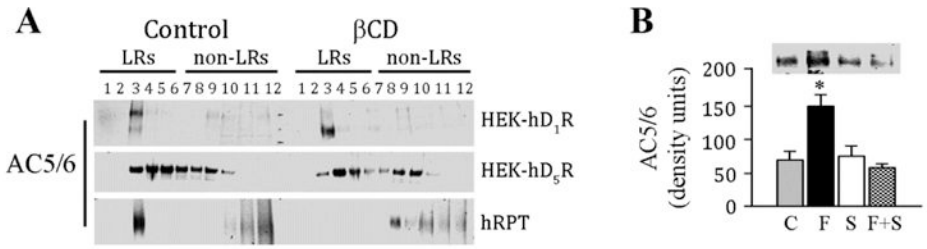

Figure 3. Effect of methyl-β-cyclodextrin (βCD) on the distribution of AC5/6

A. Cells from HEK-hD$_1$R, HEK-hD$_5$R, and hRPT were treated with vehicle (Control) or βCD (27%) for 1hr at 37°C. The cell lysates were then subjected to sucrose gradient centrifugation and the proteins (20μl/lane) from the sucrose gradient fractions (form 2 to 12 fractions) were immunoblotted with AC5/6 antibody. The distribution of AC5/6 in lipid rafts (LRs) and nonlipid rafts (non-LRs) is shown. n=3/group

B. The lipid raft marker caveolin 1 (Cav-1) co-immunoprecipitates with AC5/6 in hRPT cells

hRPT cells were grown to 90% confluence and then treated with vehicle (Control, C), fenoldopam (F, 1μmol/L), or SCH23390 (S) (5 μmol/L), or a combination of fenoldopam and SCH23390 (F+S). The cell lysates were immunoprecipitated with a polyclonal anti-Cav-1 antibody. Proteins from immunocomplexes were probed with a polyclonal anti-AC5/6 antibody. The immunoreactive bands were semi-quantified. Values are Mean ± SEM (n=4/group).

*P<0.05, vs. all others, one-way factorial ANOVA, Tukey post-hoc test. One immunoblot is shown.

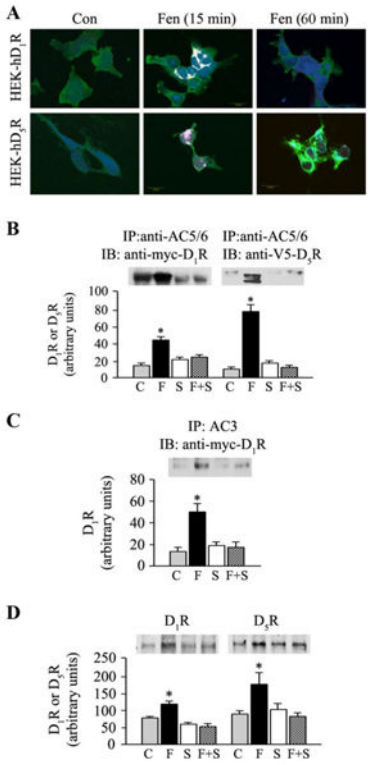

Figure 4. D_1R and D_5R association with AC isoforms

A. Co-localization of hD_1R or hD_5R with AC5/6 and cAMP in HEK-hD_1R and HEK-D_5R cells. HEK-hD_1R and HEK-hD_5R cells, grown on coverslips, were treated with vehicle (Control) or fenoldopam (Fen, 1μmol/L) for 15 or 60 min. The cells were then washed, fixed, and triple immunofluorescence staining was performed. Fluorescence images were obtained using laser confocal scanning microscopy (Olympus Fluoview FV600) at excitation and emission wavelengths of 495/519, 579/603 and 632/648 nm. Myc-tagged D_1R or V5-tagged D_5R, green; AC5/6, blue; cAMP, red; Scale bar=20 μm.

B-C. The interaction of D_1R and D_5R with AC5/6 or with AC3

HEK-hD_1R and HEK-hD_5R cells were treated for 15 min at 37°C with vehicle (Control, C), fenoldopam (Fen, 1μmol/L), SCH23390 (S) (5 μmol/L), or combination of Fen and SCH23390 (F+S). Cell lysates were immunoprecipitated with specific polyclonal anti-AC5/6 antibody (Figure 4B) or anti-AC3 antibody (Figure 4C). Proteins were eluted from the immunocomplexes and probed with anti-Myc- (D_1R) (Figures 4B and 4C) or anti-V5- (D_5R) (Figure 4B) tagged antibodies. The immunoreactive bands were semi-quantified. Values are Mean ± SEM (n=4-6/group: $^*P<0.006$ vs. all others, one-way factorial ANOVA, Tukey post-hoc test. One immunoblot is shown.

D. The interaction of D_1R or D_5R with AC5/6 in hRPT cells. hRPT cells were treated for 15 min at 37°C with vehicle (Control, C), fenoldopam (F, 1μmol/L), SCH23390 (S) (5umol/L), or combination (F+S) as in Figures 4B and 4C. Cell lysates were immunoprecipitated with polyclonal anti-AC5/6 antibody and proteins from immunocomplexes were probed with polyclonal rabbit anti-D_1R and -D_5R antibodies. The immunoreactive bands were semi-quantified. Values are Mean+SEM (n=6/group). $^*P<0.05$,

vs. all others, in AC5/6-D1R or AC5/6-D_5R, one-way factorial ANOVA, Tukey post-hoc test. One immunoblot for D_1R or D_5R is shown.

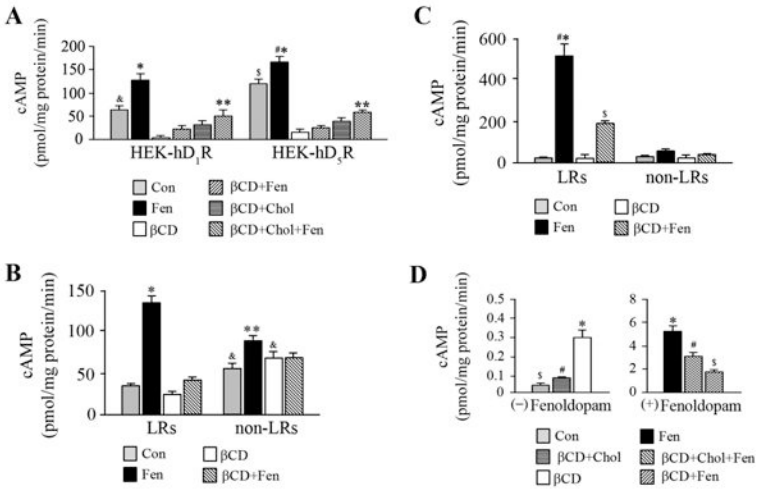

Figure 5. Effect of fenoldopam and methyl-β-cyclodextrin (βCD) on cAMP accumulation

A. Effect of fenoldopam and βCD on cAMP accumulation in whole cells

HEK-hD_1R and HEK-hD_5R cells were seeded in 12-well plates at5×10^5cells/well. The cells, pretreated for 1 h at 37°C with vehicle (control, Con), βCD (2%), or cholesterol (Chol, 100 μg/ml) plus βCD (βCD+Chol), were treated for 10 min at 37°C with vehicle (Con) or fenoldopam (Fen, 1μmol/L) or in combination (βCD+Fen, βCD+Chol+Fen). The cells were lysed in 0.1N HCL as described in "Methods". cAMP accumulation was measured in each well using a cAMP assay kit and expressed as pmol/mg protein/min. Values are Mean ± SEM (n = 4/group). *P<0.001 vs. all others in HEK-hD_1R or HEK-hD_5R, respectively, $^{\#}$P<0.05 vs. Fen-D_1R, $^{\&}$P<0.05 vs. βCD, βCD+Fen, and βCD+Chol in HEK-hD_1R cells, $^{\$}$P<0.003 vs. Con-HEK-h$D_5$R and βCD, βCD+Fen, βCD+Chol, and βCD+Chol +Fen in HEK-hD_5R cells, **P<0.05, vs. βCD in HEK-hD_1R or HEK-hD_5R, n=4/group, one-way factorial ANOVA, Tukey post-hoc test.

B-C. Effect of fenoldopam and methyl- β-cyclodextrin (βCD) on cAMP accumulation in LRs and non-LRs

HEK-hD_1R (**B**) and HEK-hD_5R (**C**) cells, grown in 150-mm dishes, pre-treated with vehicle (Control) or βCD (2%) for 45 min at 37°C, were then treated with vehicle or fenoldopam (Fen, 1 μmol/L), or βCD +Fen for 15 min (total 1h). The cell lysates were then subjected to sucrose gradient centrifugation as described in "Methods" (12, 27). Cell fractions 2-6 and 7-12 were combined as LR (fractions 2-6) and non-LR (fractions 7-12) fractions and cAMP accumulation was measured using a cAMP assay kit and expressed as pmol/mg protein/min. Values are Mean ± SEM (n = 4/group). *P< 0.001, vs. others in HEK-hD_1R or HEK-hD_5R cells, $^{\#}$P<0.001, vs. Fen, HEK-hD_1R (**B**), $^{\$}$P<0.001, vs. Con, βCD, non-LRs-HEK-hD_1R and -HEK-hD_5R,**P<0.05, vs. Con, , βCD-LRs,, non-LRs-HEK-hD_5R, one-way factorial ANOVA, Tukey post-hoc test, $^{\&}$P<0.006**,** vs. Fen-non-LRs HEK-hD_1R, *t*-test

C. Effect of fenoldopam and βCD on cAMP accumulation in hRPT cells. hRPT cells were treated with βCD and fenoldopam as described in Figure 5A. cAMP accumulation was measured in each well using a cAMP assay kit and expressed as pmol/mg protein/min. Values are Mean±SEM (n=4/group). *,$^{\#}$P<0.001 vs. all others, untreated and fenoldopam-treated cells, $^{\$}$P<0.002, vs. all others, untreated and fenoldopam-treated cells, one-way factorial ANOVA, Tukey post-hoc test.

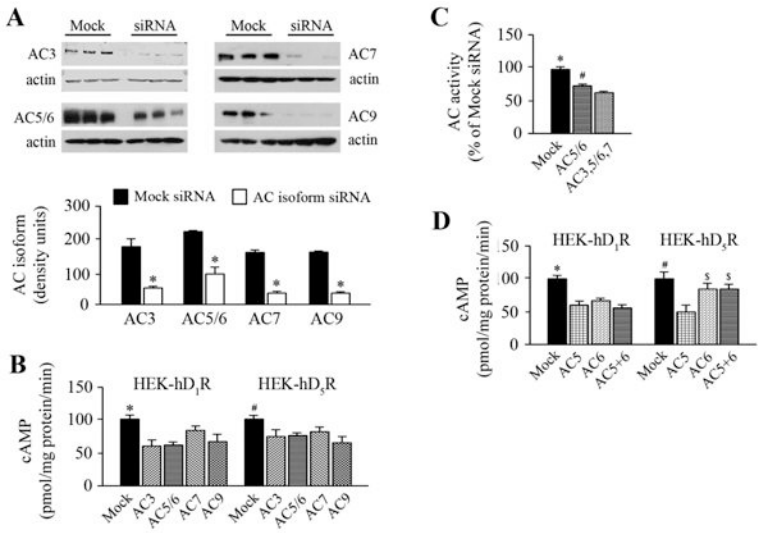

Figure 6. Effect of silencing *AC* isoform genes on AC isoform protein and cAMP accumulation

A. Effect of silencing AC isoforms genes on AC isoform protein

HEK-hD_1R and HEKhD_5R cells were seeded in 6-well plates at the cell density 5×10^5/well. The cells were transfected with scrambled sequence (mock-siRNA) that served as a negative control, or AC isoformspecific siRNA (AC-siRNA) for 48 h. The cell lysates were immunoblotted with polyclonal antibodies against specific AC isoforms (**upper panels**). The immunoreactive bands were semiquantified and corrected for actin (**lower panel**). Values are Mean ± SEM (n=3/group). *,#P<0.001 vs. mock-siRNA, *t*-test.

B. Effect of silencing a specific *AC* isoform gene on cAMP accumulation

HEK-hD_1R and HEK-hD_5R cells were transfected with mock-siRNA or siRNA against the*AC3*, *AC5/6*, *AC7*, and *AC9* genes. The cells were then treated with vehicle (Con) or fenoldopam (1 μmol/L) for 10 min. The cells were lysed in HCl solution at a final concentration of 0.1N. cAMP accumulation was measured in each well using a cAMP assay kit and expressed as pmol/mg protein/min. Values are Mean ± SEM (n=7/group). *P<0.05 vs. others in HEK-hD_1R; #P<0.05 vs. others, in HEK-hD_5R, one-way factorial ANOVA, Tukey post-hoc test.

C. Effect of silencing multiple *AC* genes on cAMP accumulation in HEK-hD_1R cells.

HEK-hD_1R cells were transfected with mock-siRNA, *AC5/6*-specific siRNA, or a combination of siRNAs against *AC3*, *AC5/6*, and *AC7* for 48 h. The cAMP accumulation in the cell lysate was measured as above. Values are Mean ± SEM (n=6/group). *,#P<0.001 vs. others, one-way factorial ANOVA, Tukey post-hoc test.

D. Effect of silencing *AC5*, *AC6*, or both on fenoldopam-stimulated cAMP accumulation

HEK- hD_1R and HEK-hD_5R cells were transfected with mock-siRNA or siRNA against *AC5*, *AC6*, or combination of *AC5+AC6* for 48 h as described above. cAMP accumulation was measured in the cell lysates as above. Values are Mean ± SEM (n=3-6/group). *P<0.001, vs. all others in HEK-D_1R (Figure 6D, **left graph**) and #P<0.003 vs. all others in HEK-hD_5R and $P<0.05 vs. AC5-siRNA in HEK-hD_5R (Figure 6D, **right graph**), one-way factorial ANOVA, Tukey post-hoc test.

Table 1
Effect of methyl- β-cyclodextrin (βCD) on cAMP accumulation

Drugs treatment	cAMP accumulation (pmol/mg protein/min)	
	HEK-hDjR	HEK-hD$_5$R
Control	70.9±4.3	119.8±11.3$^{\$}$
Fenodolpam	128.8±13.3*	166.6±14.2*
βCD	4.0±1.6	15.4±4.6
βCD+Fen	23.0±7.2$^{\#}$	27.8±3.2$^{\$}$
βCD+Cho	32.4±7.0	39.1±7.7
βCD+Cho+Fen	49.5±13.6$^{\#}$	59.3±8.5$^{\$}$

HEK-hD$_1$R or HEK-hD$_5$R cells were seeded in 12-well plate at 5×10^{5}cells/well. Cells, pretreated for 1hr at 37°C with vehicle or βCD (2%) or βCD plus cholesterol (βCD+Cho), were treated for 10min at 37°C with vehicle (Control, Con) or fenoldopam (Fen, 1μmol/L) or in combination (βCD+Fen, βCD+Cho+Fen). Cells were lyzed in 0.1N HCL as described in "Methods". The amount of cAMP in each well was measured using cAMP assay kit. cAMP accumulation was calculated and corrected for total protein (mg/ml) and expressed as pmol/mg protein/min. Values are Mean ± SEM (n = 4/group).

*P<0.001 vs. others,

$^{\#}$P<0.05, vs. Fen-HEK-hD$_1$R,

$^{\$}$P<0.003, vs. Con-D$_1$R, and βCD, βCD+Fen, βCD+Cho and βCD+Cho+Fen in HEK-hD$_5$R, n=4, one-way factorial ANOVA, Tukey post-hoc test.

Table 2
Effect of fenoldopam and βCD on cAMP accumulation in LR and non-LR fractions

Treatment	cAMP acculmulation (pmol/mg protein/min)	
	HEK-hDjR	HEK-hD$_5$R
Con-LRs	30.5±2.1	35.3 ±2.9
Fen-LRs	512.5 ±47.4$^{*\#}$	131.9±9.1*
βCD-LRs	19.7±1.0	23.4 ±3.4
βCD+Fen-LRs	185.3 ±9.8$^{\$}$	40.3 ±3.6
Con-non-LRs	29.6 ±3.3	54.9 ±5.0$^{\&}$
Fen-non-LRs	50.0±5.1	88.7 ± 5.8**
βCD-non-LRs	25.4 ±2.0	67.1 ±8.3$^{\&}$
βCD+Fen-non-LRs	48.2 ±3.4	68.9 ± 5.3

Cell sucrose gradient fractions 2-6 and 7-12 were combined as LR (2-6) and non-LR (7-12) fractions. The amount of cAMP in each well was measured using cAMP assay kit. cAMP accumulation was calculated and corrected for total protein (mg/ml) and expressed as pmol/mg protein/ min. Values are Mean ± SEM (n = 4/group).

*P<0.001, vs. all others in HEK-hD$_1$R and HEK-hD$_5$R cells, respectively,

$^{\#}$P<0.001, vs. HEK-hD$_5$R,

$^{\$}$P<0.001, vs. others HEK-hD$_1$R, and βCD+Fen-LRs HEK-hD$_5$R cells,

**P<0.05, vs. all others except βCD+Fen-LRs HEK-hD$_5$R cells, ANOVA, Tukey test,

$^{\&}$P<0.006, vs. Con-non-LRs and βCD-non-LRs, HEK-hD$_1$R, *t*-test.