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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 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 for the regulation of D_1R- and D_5R-mediated AC signaling.

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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

Abbreviations: AC, Adenylyl cyclase; DA, dopamine receptor; LRs, lipid rafts; hRPT, human renal proximal tubule; βCD, methyl-β-cyclodextrin.

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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 hD_1R (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 1 h, 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 vehicle that contained only the transfection reagent and served as control (Con), non-silencing *mock*-siRNA that consisted of a scrambled sequence and served as another negative control, or siRNA that was specific for a particular AC isoform (*AC isoform*-siRNA), as described previously [27]. The cells were seeded in 6-well plates 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, 1 mmol/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 = 50 μL/well) for 10 min at 37 °C. The reactions were stopped by adding 0.25 mL of 0.2 N HCl to each well. The cells, collected by scrapping, were transferred into labeled microcentrifuge tubes and centrifuged at 2000 ×*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 3× 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 hD_1R) 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's 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 (Fig. 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 (Fig. 1) was a chromodomain helicase DNA binding protein 8. The apparent expression of *AC4* mRNA in HEK-293 cells was not confirmed by

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