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Biochemical Pharmacology 63 (2002) 859–863

Biochemical Pharmacology

Association of dopamine D_3 receptors with actin-binding protein 280 (ABP-280)

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Received 18 January 2001; accepted 31 July 2001

Abstract

Proteins that bind to G protein-coupled receptors have been identified as regulators of receptor localization and signaling. In our previous studies, a cytoskeletal protein, actin-binding protein 280 (ABP-280), was found to associate with the third cytoplasmic loop of dopamine D_2 receptors. In this study, we demonstrate that ABP-280 also interacts with dopamine D_3 receptors, but not with D_4 receptors. Similar to the dopamine D_2 receptor, the D_3/ABP-280 association is of signaling importance. In human melanoma M2 cells lacking ABP-280, D_3 receptors were unable to inhibit forskolin-stimulated cyclic AMP (cAMP) production significantly. D_4 receptors, however, exhibited a similar degree of inhibition of forskolin-stimulated cAMP production in ABP-280-deficient M2 cells and ABP-280-replent M2 subclones (A7 cells). Further experiments revealed that the D_3/ABP-280 interaction was critically dependent upon a 36 amino acid carboxyl domain of the D_3 receptor third loop, which is conserved in the D_2 receptor but not in the D_4 receptor. Our results demonstrate a subtype-specific regulation of dopamine D_2-family receptor signaling by the cytoskeletal protein ABP-280.

Keywords: Dopamine; G protein-coupled receptor; Protein interaction; Actin-binding protein 280; Receptor subtypes; Signaling

1. Introduction

The dopamine D_2 receptor regulates a variety of physiological processes including locomotion, feeding, endocrine functions, reinforcement, emotion, and cognition. Molecular cloning has identified three genes, D_2, D_3, and D_4, that encode distinct D_2-family receptors [1–6]. However, relatively little is known about functional differences among these three D_2 receptor subtypes. A number of studies have suggested that these different D_2-family receptors may have preferential coupling to various G proteins [7]. Many other studies, on the other hand, have emphasized the functional significance of receptor subtype-specific protein–protein interactions. For example, the β_2-adrenergic receptor has been shown to bind to Na^+/H^+-exchanger regulatory factor (NaH ERF) through a four amino acid motif at the receptor C-terminus. As the binding motif is present only in β_2- and not in β_1- or β_3-adrenergic receptors, this interaction is subtype-specific [8]. Similarly, a subtype of somatostatin receptors has been shown to associate with the cortactin-binding protein [9]. Recently, proteins that bind to specific dopamine receptor subtypes have been identified. A 24 kDa single transmembrane protein, calcyon, has been shown to interact with the D_1 dopamine receptor. As a result of this interaction, D_1 receptors can shift effector coupling from adenylate cyclase stimulation to a robust release of intracellular calcium [10]. The γ-aminobutyric acid A ($GABA_A$)-ligand-gated channel complex has been shown recently to selectively bind to D_5 receptors, but not D_1 receptors [11]. This physical association of D_5 and $GABA_A$ receptors enables a mutually inhibitory functional interaction between these receptor systems.

In our previous work, we found that the D_2 and the D_3, but not the D_4, receptor specifically binds to the cytoskeletal protein ABP-280 through its third cytoplasmic loop [12]. We have also shown that the absence of this association dramatically reduces the coupling efficiency of D_2

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Abbreviations: ABP-280, actin-binding protein 280; cAMP, cyclic AMP.

0006-2952/02/$ – see front matter
PII: S0006-2952(01)00932-7

receptor activation to the inhibition of adenylate cyclase. In the current study, we establish that the physical interaction of the D_3 receptor with ABP-280 is essential for the coupling of D_3 receptor activation to adenylate cyclase inhibition.

2. Materials and methods

2.1. Yeast two-hybrid assay

A yeast two-hybrid assay was performed as described previously [12]. Briefly, the third cytoplasmic loop of the rat D_3 receptor [1] was divided into four segments at residues 241, 273, and 338. Each segment was then subcloned into the GAL4 binding domain. The third cytoplasmic loop of the human $D_{4,4}$ receptor [13] (residues 214–346) was also amplified by polymerase chain reaction (PCR) and subcloned in-frame into the GAL4 DNA-binding domain vector pGBT9 to generate pGBT9-$D_{4,4}$. ABP-280 (residues 1779–2134) was subcloned into a GAL4 activation domain. For interactions in a yeast two-hybrid system, colony growth on histidine-deficient agar (HIS3) and β-galactosidase activity (β-Gal) were analyzed as detailed in [12].

2.2. Cell culture and transfection

A human melanoma cell line (M2) that does not express ABP-280 endogenously, and a subclone of M2 (A7) that has been stably transfected with ABP-280 cDNA [14], were used. M2 and A7 cells were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum and penicillin. M2 and A7 cells were transfected with dopamine D_3 and D_4 receptor constructs and were selected with Zeocin (100 μg/mL) (Invitrogene). Clones surviving the selections were expanded and analyzed by receptor radioligand binding and cAMP assay.

2.3. Receptor radioligand binding

Stably transfected cells were grown to confluency on 150 mm tissue culture plates. Cell pellets were resuspended in ice-cold TEM buffer (25 mM Tris–HCl, pH 7.4, 1 mM EDTA, and 6 mM $MgCl_2$) at a concentration of 200–400 μg total protein/mL and homogenized using a polytron (Brinkmann Instruments) at a setting of 5 for 10 s on ice. Radioligand binding was performed in a volume of 1 mL using approximately 200 μg total protein per tube. Competition binding assays were performed using 0.1–0.3 nM [^{3}H] spiperone (99 Ci/mmol, Amersham) and various concentrations of dopamine or 6,7-ADTN [(±)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene HBr; RBI]. Reaction mixtures were then incubated for 1 hr at room temperature and terminated by rapid vacuum filtration through GF/B filters pre-soaked in 0.5% polyethylenimine (Sigma) using a 24-port harvester (Brandel). Filters were washed rapidly with 5 mL of TEM buffer and air-dried, and individual filter discs were placed in counting vials with 5 mL of scintillation fluid for counting in a Beckman LS-6800 liquid scintillation counter. Data were analyzed using GraphPad software (GraphPad Software Inc.).

2.4. Receptor functional analyses

Cells were seeded 24 hr before the assay at a density of 1×10^5 per well. They were washed with warm HBBS buffer (20 mM HEPES, pH 7.2, 118 mM NaCl, 4.6 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose) and incubated for 10 min at 37° in the same buffer with 2 μM Ro 20-1724 to inhibit cAMP phosphodiesterase. All cells were stimulated with 10 μM forskolin and increasing concentrations of dopamine. Drug incubation was carried out at 37° for 30 min and was terminated by the addition of ice-cold 70% ethanol. cAMP samples were collected in Eppendorf tubes. After drying down with a Savant Speed-Vac, cAMP levels in the samples were determined using a sensitive succinylation method as described [12].

2.5. Statistical analysis

Statistical analysis was carried out by using the unpaired Student's *t*-test.

3. Results

In a previous study, we found that the C-terminal region of the D_2 dopamine receptor third cytoplasmic loop is critical for binding to ABP-280 [12]. As the sequence of this region of the D_2 receptor is conserved in the D_3 receptor, but not in the D_4 receptor, we sought to analyze whether the interaction with ABP-280 is subtype-specific. Using a yeast two-hybrid analysis, we demonstrated (Fig. 1) that the D_3 receptor third cytoplasmic loop also interacted with ABP-280. However, there was no interaction between ABP-280 and the D_4 receptor third loop (data not shown). Fig. 1 further shows that the C-terminal 36 amino acid residues of the third cytoplasmic loop of the D_3 receptor are necessary and sufficient for interaction with ABP-280. The association between D_3 and ABP-280 was verified by *in vitro* fusion protein binding, which indicated that GST-D_3, but not GST-D_4, bound specifically to ABP-280 [12].

To investigate the physiological significance of the dopamine D_3 receptor and ABP-280 association, D_3 receptors were stably expressed in the melanoma cell lines M2 and A7. The M2 line does not express ABP-280, and the A7 line is an M2 subclone that has been stably transfected with ABP-280 cDNA [14]. Clones with similar receptor expression levels (around 1.5 and 0.5 pmol/mg protein)

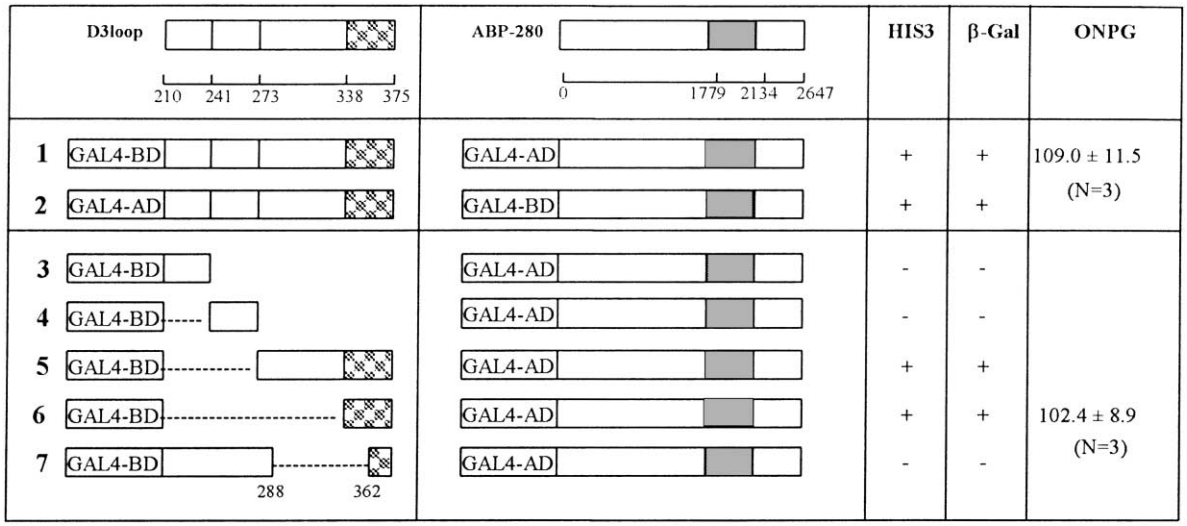

Fig. 1. Yeast two-hybrid assay of dopamine D_3 receptors with ABP-280. The third cytoplasmic loop of the D_3 receptor and ABP-280 (residues 1779–2134) were linked to GAL4 activation and binding domains (specified in each row) and tested in yeast for their interaction. Growth on histidine-deficient agar (HIS3) and β-galactosidase activity (β-Gal) were analyzed. The intensity of the protein–protein interaction was quantified using ONPG (*o*-nitrophenyl-β-D-galactopyranoside) as a substrate. The D_3 third loop was also divided into four segments at the residues indicated in the figure, and tested for interaction with ABP-280, respectively. Row 7 is the truncated D_3^* receptor third loop. Only the extreme C-terminus 36 amino acids of the D_3 third loop are involved in the binding.

were selected and paired for comparison in receptor binding and functional assays. D_4 receptors were similarly expressed in both M2 and A7 cells. In addition, we also generated a truncated D_3 receptor (D_3^*), with the C-terminal region (residues 288–362) of the third cytoplasmic loop removed. The mutant D_3^* receptor completely lost the ability to bind ABP-280 in the yeast two-hybrid assay (Fig. 1). This D_3^* receptor was also stably expressed in A7 cells. Stable clones with similar receptor expression levels were chosen for receptor binding and activation studies.

Radioligand binding experiments revealed that there was no significant difference in D_3 receptor binding of

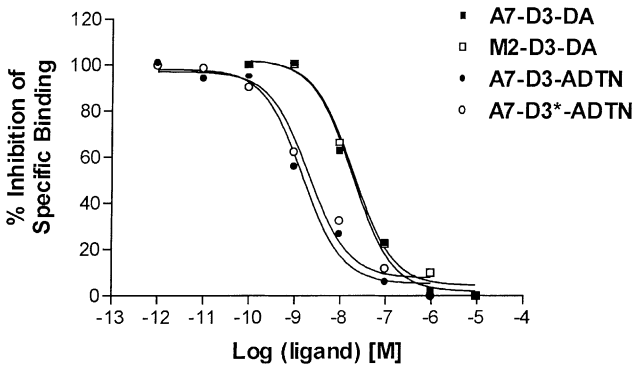

Fig. 2. Dopamine D_3 and mutant D_3^* receptor binding in M2 and A7 cells. Competition binding assays were performed using [^{3}H] spiperone (0.1–0.3 nM) and various concentrations of dopamine and 6,7-ADTN on D_3 stably expressed M2 (ABP-280-deficient), A7 (genetically reconstituted ABP-280), and mutant D_3^* stably expressed A7 cells. Curves were fitted as one-site competition binding. Representative curves from three independent experiments are shown. The wild-type D_3 receptor expression levels were 1.2 pmol/mg protein (M2) vs. 1.5 pmol/mg protein (A7). The mutant D_3^* receptor expression levels were 1.6 pmol/mg protein in A7 cells.

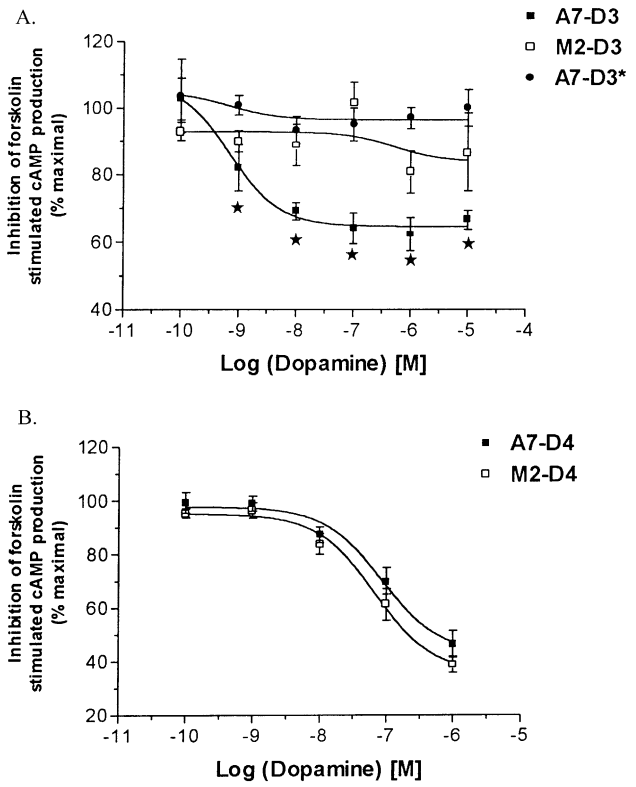

Fig. 3. Dopamine D_3 and D_4 receptor-mediated inhibition of cAMP accumulation in M2 and A7 cells. Concentration–response curves for (A) wild-type and mutant dopamine D_3 receptor and (B) dopamine D_4 receptor-mediated inhibition of cAMP accumulation were determined on cells stimulated with 10 μM forskolin and increasing concentrations of dopamine. Results are expressed as percentage of inhibition of the forskolin-stimulated cAMP level. Curves are plotted as the average ± SEM from three independent experiments. The D_4 receptor expression levels were 1.0 and 1.1 pmol/mg protein in both M2 and A7 cells, respectively, (★) $P < 0.05$ (unpaired Student's *t*-test of M2-D_3 and A7-D_3^* cells).

M2 and A7 cells (Fig. 2). The mutant D_3^* receptor showed an affinity to the dopamine agonist 6,7-ADTN similar to that of the wild-type D_3 receptor (Fig. 2). In A7 cells, dopamine-stimulated wild-type D_3 receptors were able to mediate a significant inhibition of forskolin-stimulated cAMP production (EC_{50} was 0.75 ± 0.51 nM; maximum inhibition level was $37.8 \pm 4.9\%$, $N = 5$, Fig. 3A). However, in D_3 receptor-expressing M2 cells (lacking ABP-280), dopamine did not inhibit adenylate cyclase (Fig. 3A). Consistent with the essential interaction of D_3 with ABP-280 for signaling, activation of the mutant D_3^* receptor in A7 cells did not induce any significant inhibition of forskolin-stimulated cAMP production (Fig. 3A). In contrast to the D_3 receptor, the signaling of the D_4 receptor, which does not bind to ABP-280 *in vitro*, was similar in M2 and A7 cells: the EC_{50} was 33.7 ± 6.35 nM and the maximum inhibition level was $59.3 \pm 3.05\%$ in M2 cells, while in A7 cells, the EC_{50} was 26.5 ± 5.74 nM and the maximum inhibition level was $53.6 \pm 5.05\%$ (Fig. 3B). These findings indicate that as with the D_2 receptor, the D_3 receptor was less efficient in coupling to adenylate cyclase in the absence of ABP-280 binding. We conclude that cytoskeletal protein ABP-280 regulates the signaling of D_2-family receptors in a subtype-specific manner.

4. Discussion

In this study, we have shown that dopamine D_2-family receptors interact with ABP-280 in a subtype-specific manner. We found that ABP-280 binds to a region of approximately 36 amino acids in the third cytoplasmic loop, which is conserved in D_2 and D_3 receptors. We have further shown that the association of dopamine D_2 [12] and D_3 receptors with ABP-280 plays an important role in coupling efficiency of both receptors with the inhibition of adenylate cyclase. In the absence of ABP-280 association, D_3 receptors were unable to inhibit forskolin-stimulated cAMP. Similarly, coupling of D_2 receptors to the inhibition of adenylate cyclase was attenuated significantly in both maximum effect and agonist potency [12]. However, the D_4 third cytoplasmic loop does not possess this conserved sequence and does not interact with ABP-280; thus, the absence of ABP-280 binding has no effect on D_4-mediated adenylate cyclase inhibition.

The exact mechanism by which ABP-280 modulates the signaling of D_2 and D_3 receptors is unclear. It is likely that ABP-280 may anchor D_2 and D_3 receptors to membrane subdomains that are enriched with G proteins or adenylate cyclases, and/or are deficient in negative regulators of signaling such as G protein-coupled receptor kinases. Studies have demonstrated that the distribution of G proteins in the plasma membrane is not random [15] and that G proteins or adenylate cyclases could also be attached to components of the cytoskeleton [16,17]. Thus, it is possible that by anchoring receptors and signaling molecules, the cytoskeleton may ensure more efficient signal transduction.

Other subtype-specific protein–protein interactions have been reported. The third cytoplasmic loop of D_2 receptors was shown to interact with spinophilin which binds protein phosphatase-1 [18,19]. While the exact functional significance of this association is still unknown, it has been suggested that spinophilin may play a role in organizing the D_2-receptor signaling complex. The dopamine D_4 receptor has been shown recently to interact with the SH2-SH3 adapter proteins Grb2 and Nck with a putative SH3 binding motif in its third cytoplasmic loop [20]. Such an SH3 binding domain, which is required to bind to SH2-SH3 adapter protein, is apparently absent in D_2 and D_3 receptors.

In summary, we have demonstrated a subtype-specific interaction of dopamine D_2-family receptors with the cytoskeleton protein ABP-280. Understanding the specific interactions between such intracellular proteins and dopamine receptors may be the key to understanding the functional differences between receptor subtypes. The existence of subtype-specific protein–protein interactions may also provide novel strategies of manipulating D_2-family receptor signaling.

Acknowledgments

We thank Yu-Tian Wang for efforts in the early phase of the project. This work was supported by grants from NIH (MH57889) and the Sloan foundation (Q.-Y. Zhou).

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