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Dopamine D2 Receptor Dimers and Receptor-Blocking Peptides

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Dopamine D2 receptors exist as dimers in whole cell lysate, crude membranes prepared from human caudate, and following solubilization and immunoprecipitation of the receptor from these tissues. Photoaffinity labelling experiments confirmed that D2 receptors exist either as monomers that are selective targets for spiperone or as dimers that are targets for nemonapride. Incubation of D2 dimers with peptides derived from the putative transmembrane (TM) domains of the D2 receptor, or incubation under high temperatures or low pH resulted in the dissociation of the dimer to monomer. D2-TM peptides were unable to dissociate dopamine D1 and serotonin 5-HT_{1B} receptor dimers, suggesting that receptor dimers are formed by specific intermolecular noncovalent interactions involving TM regions. This opens a path to new selective therapeutic receptor-blocking compounds based on this principle of mimicking transmembrane portions of neurotransmitter receptors.

MATERIALS AND METHODS

Cross-linking of intact living D2 receptor expressing Sf9 cells. Human dopamine $D2_{long}$ receptors were expressed in Sf9 cells (D2/Sf9 cells) [7]. Glutaraldehyde (50 % w/v) was added to D2/Sf9 cells at 48 h post-infection (~95% viable as assessed by trypan blue staining) in PBS to give a final concentration of 1 % w/v. After incubation of cells at room temperature for 1 min, cells were pelleted by rapid centrifugation, and cells washed in ice-cold (5 vol) PBS (twice), and cell lysate prepared (cells were disrupted in cold buffer (5 mM Tris-HCl, 2 mM EDTA, 5 μg/ml leupeptin, 10 μg/ml benzamidine and 5 μg/ml soybean trypsin inhibitor), and the lysate recovered by centrifugation at 100 $\times$ g).

Immunoblot analysis. In brief, tissues were solubilized in SDS buffer consisting of 50 mM Tris-HCl pH 6.5, 10% SDS, 10% glycerol, 0.003% bromophenol blue, and 10% 2-mercaptoethanol (20% DMSO was added for cross-linked tissues). Samples were subjected to SDS-PAGE, and electroblotted on to nitrocellulose. Immunoblot analysis of the D2 receptor was performed as described [7] with the anti-D2 antibody (AL-26), a polyclonal raised in rabbits against a 120-amino acid sequence in the third intracellular loop of the human dopamine D2 receptor [10] (a.a. 661-1020). The specificity of this antibody for D2 but not for D1, D3, D4 or D5 receptors has been reported elsewhere [10]. Immunoblot analysis of c-myc epitope-tagged D1 and 5-HT_{1B} receptors was performed as described [8,9] with the monoclonal 9E10 antibody raised against a 32-amino acid sequence of c-myc (a.a. 408-439) (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were developed with BCIP/NBT substrate (BioRad, ON).

Binding of radiospiperone and radionemonapride to D2 receptors. The binding of [^{3}H]spiperone and [^{3}H]-nemonapride to P2 membranes prepared from D2/Sf9 cells has been described previously [7]. The photoincorporation of 2 nM [^{125}I]azido-phenethylspiperone by the D2 receptor monomer, and the photoincorporation of 1.5 nM [^{125}I]4-azido-5-iodo-nemonapride into both monomers and dimers was done under previously described conditions [8]. The photoincorporation of both radioligands into the tissue was prevented by the presence of 1 μM (+)-butaclamol.

Dissociation of dopamine D2 dimers. Increasing concentrations of a peptide corresponding to a particular TM region of the D2 receptor were used to dissociate D2 dimer into monomers. Three pmol of solubilized and immunoprecipitated D2 receptors in buffer A (0.5% digitonin, 100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 5 μg/ml leupeptin, 10 μg/ml benzamidine, and 5 μg/ml soybean trypsin inhibitor) were incubated in the absence or presence

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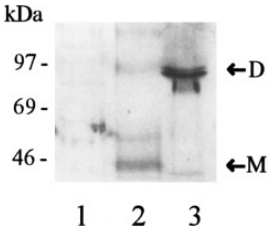

FIG. 1. Immunoblot of human dopamine D2 receptors using the AL-26 antibody. Lane 1: Wild-type Sf9 cell lysate. Lane 2: Lysate from D2/Sf9 cells. Lane 3: Lysate from D2/Sf9 cells which had been previously exposed to 1% glutaraldehyde. 20 μg membrane protein in each lane. This blot is representative of 2 experiments. D indicates the position of the dimer, M, the position of the monomer.

of peptide. Peptides were freshly prepared in buffer A with 10% DMSO. All samples were made up to a final volume of 20 μL with buffer A containing 10% DMSO. An aliquot of 10 μL of SDS buffer was added to all samples which were then incubated at 37 °C for 30 min prior to SDS-PAGE and immunoblotting. Solubilization and immunoprecipitation of receptors from P2 membranes from receptor expressing Sf9 cells have been reported in detail elsewhere [7-9].

Temperature and acid stability of D2 dimers. 1.5 pmol of solubilized and immunoprecipitated D2 receptors in buffer A were incubated at 23, 37, 65, and 90 °C for 30 min with SDS buffer at a final volume of 30 μl. For acid stability experiments, 1.5 pmol of solubilized and immunoprecipitated D2 receptors in buffer A was treated with H_2O, or 0.1 N tartaric acid (final concentration), or 0.1 N HCL (final concentration), or 0.1% glacial acetic acid (final concentration) (approximate pH 3 for acid solutions). Treated samples were incubated at 37 °C for 30 min with SDS buffer at a final volume of 30 μl. Incubations were followed immediately by SDS-PAGE and immunoblot analysis.

RESULTS AND DISCUSSION

Human dopamine D2 receptors, expressed in insect *Spodoptera frugiperda* cells, were immunoblotted with a D2-selective antibody. Fig. 1 (lane 2) shows a major band at 44 kDa, consistent with the predicted size of the cloned D2 receptor [10], and another band at 90 kDa, compatible with a dimer of the D2 receptor. An immunoreactive band at ~180 kDa may represent higher size oligomers of D2 receptors, but this form of D2 was less abundant than the monomer and dimer species.

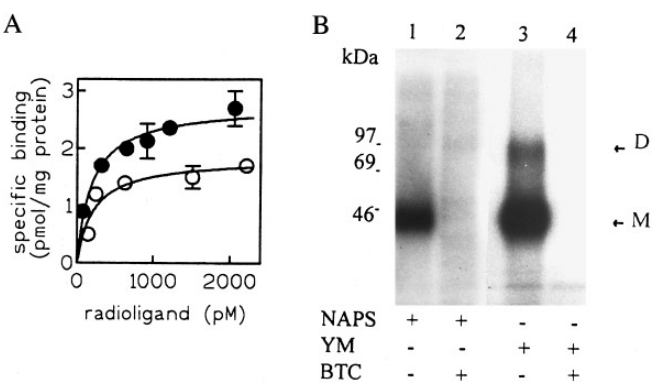

FIG. 2. Radioligand detection of human dopamine D2 receptors. (A) A representative experiment (n=6) showing the saturation isotherms for [^{3}H]spiperone and [^{3}H]nemonapride specific binding to an identical P2 membrane preparation from D2/Sf9 cells. In this experiment, B_{max} (pmol/mg of protein) and K_D (pM) are as follows: [^{3}H]spiperone (○), B_{max} = 1.8, K_D = 215; for [^{3}H]YM-09151-2 (●), B_{max} = 2.8, K_D = 150. These B_{max} and K_D values differed < 10% from the mean of 6 independent experiments. (B) A representative autoradiogram (n=4) showing the photoincorporation of [^{125}I]azidophen- ethylspiperone (NAPS) by the D2 monomer, and the photoincorporation of [^{125}I]4-azido-5-iodo-nemonapride (YM) into both monomers and dimers. The photoincorporation of both radioligands into the tissue was prevented by the presence (+) of 1 μM (+)-butaclamol (BTC).

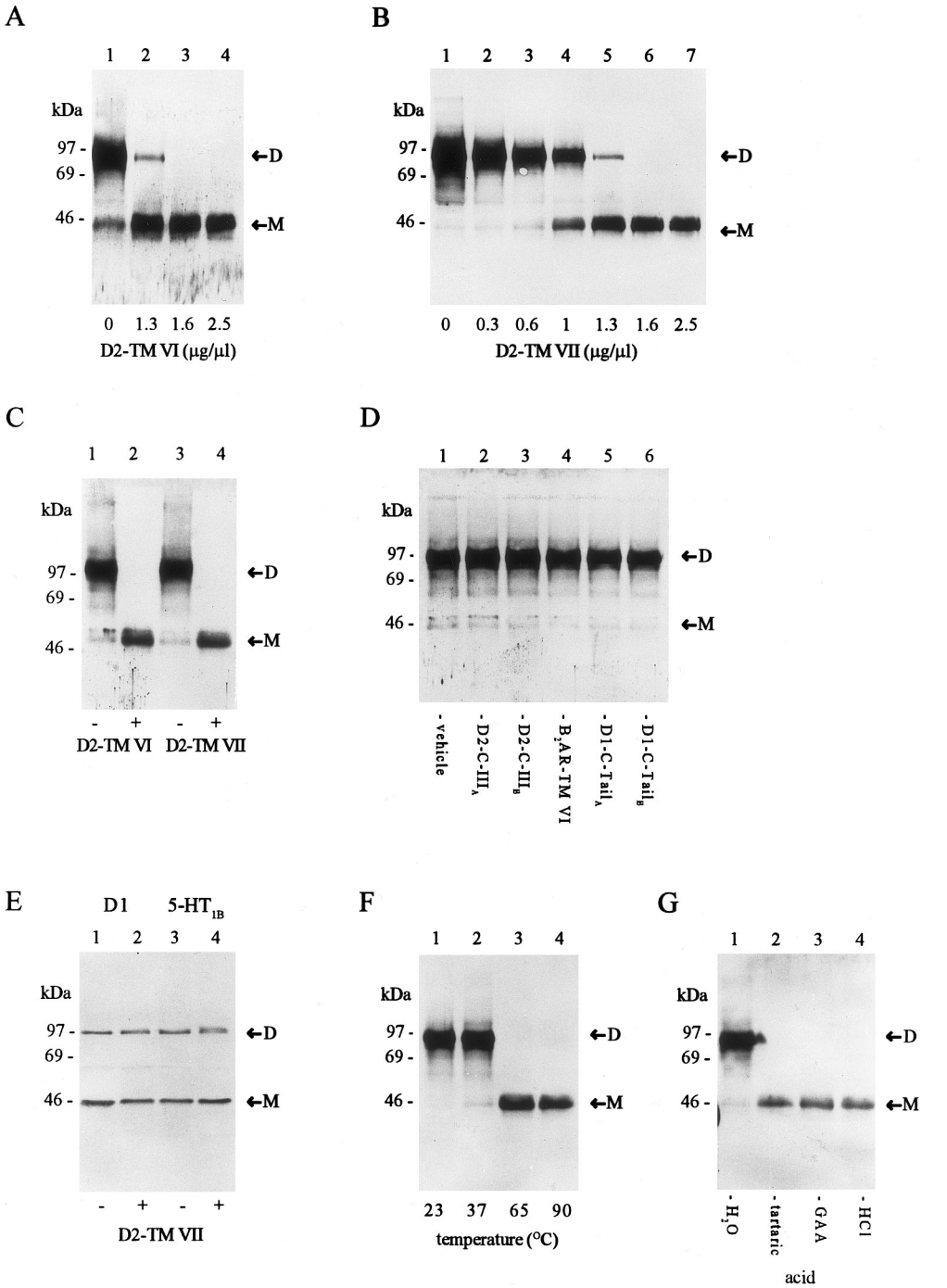

FIG. 3. Dissociation of human dopamine D2 receptor dimers into monomers. (A) Increasing concentrations of D2-TM VI peptide (a.a. 375 to 394). (B) Increasing concentrations of D2-TM VII peptide (a.a. 407 to 426). (C) D2-TM VI and D2-TM VII peptides dissociated D2 dimers from human caudate nucleus (case 1271) into monomers. (D) Lack of effect of control peptides on D2 dimers. Lane 1: Buffer. Lane 2: Peptide corresponding to intracellular third loop of the D2 receptor (a.a. 244-262). Lane 3: Peptide corresponding to intracellular third loop of the D2 receptor (a.a. 284-303). Lane 4: Peptide corresponding to TM VI domain (a.a. 276-296) of the β_2-adrenoceptor. Lane 5: Peptide corresponding to the carboxyl tail (a.a. 369-383) of the dopamine D1 receptor. Lane 6: Peptide corresponding to the

Exposure of living D2/cells to chemical cross-linking with glutaraldehyde resulted in an increase of D2 dimers with a disappearance of D2 monomers presumably due to the irreversible cross-linking of receptor monomers, confirming that D2 dimers are present before cell fractionation and are not an artifact of membrane preparation or solubilization (Fig. 1, lane 3). GTPγS and endoglycosidase F studies ruled out that the 90 kDa species could be a receptor-G-protein complex or that it was a glycosylated monomer (data not shown). Altogether, therefore, these findings suggested that the 90 kDa species was a D2 dimer.

The presence of multiple D2 receptor species was confirmed by radioligand binding. In the same membrane preparation from D2/Sf9 cells, the benzamide D2 antagonist [^{3}H]nemonapride detected $\sim$1.5 fold the receptor density estimated by the butyrophenone D2 antagonist [^{3}H]-spiperone (Fig. 2A); however, an explanation for this difference in the receptor density estimated by these ligands has been lacking. The receptor species-specificity of these ligands was clarified using two photoaffinity radioligands, [^{125}I]azidophenethylspiperone and [^{125}I]4-azido-5-iodo-nemonapride. As shown in Fig. 2B, [^{125}I]azidophenethylspiperone labelled the monomer, while [^{125}I]4-azido-5-iodo-nemonapride labelled both the monomer and the dimer. These data indicate that the spiperone and benzamide congeners labelled different populations of the D2 receptor, resolving the density discrepancies found with these two radioligands in positron tomography [1-6]. In other words, because the benzamide attaches to both monomers and dimers, the density of [^{11}C]raclopride sites would be expected to exceed the density of D2 monomer sites labelled by [^{11}C]methylspiperone.

Because the TM regions of receptors may interact to result in dimers [11], we tested whether peptides with the same amino acid sequence as the receptor TM regions could affect the dimer. Such peptides, corresponding to the D2-TM regions VI and VII, effectively dissociated the dimer, as illustrated in Fig. 3 (A-C). A small increase in the molecular mass of the receptor monomer after peptide treatment suggested the formation of a peptide-D2 receptor heterodimer (Fig. 3, A-C).

Such peptide actions on the dimer were receptor-specific and region-specific. That is, the D2 dimer was not dissociated by a peptide with an amino acid sequence outside the D2-TM region (a.a. 244-263, or 284-303) or by peptides corresponding to the TM VI region of the β_2-adrenoceptor (a.a. 369-383) or the carboxyl tail of the D1 receptor (a.a. 416-431) (Fig. 3D). Indicating further specificity for this effect, the D1 and serotonin 5-HT$_{1B}$ receptor dimers [8,9] were not dissociated by a peptide corresponding to the TM VII region of the D2 receptor (Fig. 3E). However, peptides matching the TM regions of the 5HT$_{1B}$ receptor were effective in dissociating the 5-HT$_{1B}$ dimer (data not shown). These data confirm that TM interactions are receptor specific, and mediate specific intermolecular interactions for dimerization. The most likely explanation for TM peptide actions might be that the TM peptide binds to a TM domain thus preventing its intramolecular interactions with other TM regions that are critical for the 3-dimensional conformation of the receptor monomer. Such a peptide-monomer heterodimer may also result in the disruption of intermolecular TM interactions mediating dimers and/or inhibition of dimer formation. In fact, the peptide-dissociated D2 receptors did not bind ligand (data not shown). D2 dimers also dissociated as a function of increasing temperature (Fig. 3F) and in the presence of acid (pH $\sim$3) (Fig. 3G). A slightly reduced recovery of the monomer in these experiments may be attributed to some proteolysis (in spite of protease inhibitors)

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carboxyl tail (a.a. 416-431) of the dopamine D1 receptor. (E) Lack of effect of D2-TM VII peptides on the D1 dimer or on the serotonin 5-HT$_{1B}$ dimer. Lanes 1 and 3: Buffer. Lanes 2 and 4: D2-TM VII peptide. (F) Dissociation of D2 dimers by high temperature. Lane 1: 23 °C. Lane 2: 37 °C. Lane 3: 65°C. Lane 4: 90°C. (G) Dissociation of D2 dimers by acidification. Lane 1: pH 7.4. Lane 2: 0.1 N Tartaric acid (pH 3). Lane 3: 0.1% Glacial acetic acid (pH 3). Lane 4: 0.1 N HCl (pH 3). Blots A-C, F and G are representative of 3 or more independent experiments. Blots D and E are representative of 2 replicates. Receptor monomers (M) and dimers (D) are indicated.

and/or that such heat and acid treatments render the receptors less immunoreactive to the antibody. We conclude that D2 dimerization is mediated at the protein level involving specific intermolecular, noncovalent, electrostatic interactions of residues within TM α-helices.

In summary, therefore, D2 receptors can exist as dimers targeted by benzamide drugs and which can be dissociated by specific transmembrane-like peptides. The selectivity of the transmembrane peptides for dissociating the dimer may have far-reaching implications in medicinal chemistry and clinical therapeutics insofar as this principle may be used to design many different types of new drugs which target membrane-embedded receptors.

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