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A chimeric D_2 dopamine/m1 muscarinic receptor with D_2 binding specificity mobilizes intracellular calcium in response to dopamine

Bruce P. England, Martha S. Ackerman and Ronald W. Barrett

Affymax Research Institute, 3180 Porter Drive, Palo Alto, CA 94304, USA

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Using PCR methodology, a chimeric receptor cDNA was constructed in which the entire third cytoplasmic loop of the human D_2 dopamine receptor was replaced by the analogous portion of the human m1 muscarinic receptor. When expressed in CHO cells, the chimeric D_2/m1 receptor bound dopaminergic ligands with affinities similar to the native $D_{2(414)}$ receptor. Intracellular calcium levels (measured with Fura-2) were not altered when CHO cells expressing the $D_{2(414)}$ receptor were exposed to dopamine. In contrast, dopamine elevated intracellular calcium levels in cells expressing the D_2/m1 chimeric receptor in a dose-dependent manner which was blocked by the D_2 antagonist, fluphenazine. The ability to construct G-protein-linked receptor chimeras which mobilize calcium with nearly unaltered pharmacologic specificity raises the possibility of a generic strategy for creating non-radioisotopic reporter systems for use in drug discovery.

Dopamine receptor; Muscarinic receptor; Chimeric receptor; Effector coupling; Calcium mobilization

1. INTRODUCTION

G-protein-linked receptors are a large family of cell surface receptors characterized by their putative common topology of seven membrane-spanning helixes and by their ability to interact with heterotrimeric G-proteins. For several of these receptors, mutagenesis and chimeric receptor studies have identified functions associated with specific regions of the protein [1]. Of particular interest is the mechanism by which receptors specify their interaction with the family of G-proteins which transduce signals through different effector pathways. Previous studies have implicated the third cytoplasmic loop (i3 loop) as being important in specifying G-protein interactions [2–6]. The present investigation was designed to further address the role of the i3 loop and whether the signal transduction pathway of a G-protein-linked receptor can be altered without affecting its pharmacologic specificity. For this purpose, we have studied the binding and calcium mobilizing properties of a chimeric receptor in which the i3 loop of the human $D_{2(414)}$ dopamine receptor (which does not mobilize calcium when expressed in CHO cells) was replaced with the analogous portion of a calcium mobilizing receptor, the human m1 muscarinic receptor.

Correspondence address: R.W. Barrett, Affymax Research Institute, 3180 Porter Drive, Palo Alto, CA 94304, USA

Abbreviations: QNB, quinuclidinyl benzilate; $[Ca^{+2}]_i$, intracellular free calcium concentration; PCR, polymerase chain reaction; CHO, Chinese hamster ovary

2. MATERIALS AND METHODS

2.1. *Receptor clones*

The protein coding portion of the human m1 muscarinic acetylcholine receptor gene was cloned from human genomic DNA by PCR [7] using primers derived from the published DNA sequences [8,9] (5′-CGGAATTCCC AGCCCCACCT AGCCACCA-3′ and 5′-GGGGATCCGA GGGATGCAGG AGAGGGGAC-3′). Amplification was carried out in a 30 cycle, 25 μl reaction containing 0.5 μg of human placental DNA (Sigma) using the GeneAmp kit (Perkin-Elmer Cetus) according to the manufacturer's recommendations. The amplified m1 receptor DNA was cloned into the mammalian expression vector pBJ1-Neo [10] to produce the m1 receptor expression plasmid pSRm1-9. The m1 receptor insert of pSRm1-9 was sequenced [11] and found to code for the same peptide sequence as one of the published clones [8].

The coding portion of the cDNA for the short form of the human D_2 dopamine receptor ($D_{2(414)}$) was cloned from a λgt10 human retina cDNA library by PCR, using primers derived from the published sequences [12–14] (5′-GCGAATTCAT GGATCCACTG AATCTGTCC-3′ and 5′-GATAAGCTTC AGCAGTGGAG GATCTTCAG-3′). Two μg of phage DNA was used as template in a 30 cycle, 100 μl PCR reaction and the amplified $D_{2(414)}$ receptor fragment was cloned into pBJ1-Neo. The DNA sequences of the inserts of two recombinant plasmids were determined and each was found to contain a different point mutation introduced by the PCR amplification. Therefore, non-mutant restriction fragments from each clone were ligated together and re-cloned into pBJ1-Neo to produce the $D_{2(414)}$ receptor expression plasmid pSRD2(414)-C.

The D_2/m1 receptor chimera was made by the recombinant PCR technique [15]. Primers with sequences 5′-CTTCATTGTC ACCCTGCTGG TCTACTGGCG CATCTACCGG GAGA-3′ and 5′-GAACACGCCG AGAACAATGG CGAGGGTCCG AGCCGCCTTC TTCTC-3′ were used in a 10 cycle, 100 μl PCR reaction containing 400 ng of linearized pSRm1-9 to amplify a fragment coding for the third cytoplasmic loop of the m1 receptor fused at each end to sequences coding for the homologous portions of the fifth and sixth transmembrane segments of the D_2 receptor. 35 ng of

this chimeric DNA fragment was then used to prime a 10 cycle, 100 μl PCR reaction containing 2 μg of the λ phage library DNA described previously. Next, the D_2 receptor primers described previously were added to the reaction and 30 additional PCR cycles were performed and the PCR reaction products were cloned into pBJ1-Neo. As with the $D_{2(414)}$ receptor clone, the sequences of two D_2/m1 chimeric clones were analyzed and found to contain non-identical, PCR-induced errors, so restriction fragments from each clone were combined to make the D_2/m1 expression clone pSRD2m1-C.

2.2. *Cell culture and transfection*

CHO-K1 cells (American Type Culture Collection CCL 61) were grown in DMEM/F-12 1:1 (J R Scientific) containing 5% fetal bovine serum (HyClone), and 2 mM glutamine (J R Scientific). 40 μg of each receptor expression plasmid was linearized with *Sea*I and used to transfect 2×10^6 cells by electroporation [16]. Cells were grown for 48 h prior to the addition of 1 mg/ml G-418 sulfate (Geneticin; Gibco). The medium was replaced every three days and cells surviving the G-418 selection were grown and maintained in the selective medium. Experiments were done with cells harvested from 4 to 8 weeks after the initial transfection.

2.3. *Radioligand binding*

Cells were detached from culture flasks with EDTA, pelleted at 1000 × *g* and resuspended in 1 × binding buffer (100 mM Tris; 120 mM NaCl; 5 mM KCl; 1 mM $MgCl_2$; 2 mM $CaCl_2$; 1.1 mM ascorbic acid; pH 7.4) at $\sim 1 \times 10^6$ cells/ml. Cells were homogenized with a Polytron (Brinkman) and the crude membrane fraction was pelleted at 17 000 × *g* for 10 min. Membranes were resuspended in 1 × binding buffer and ~10 μg of membrane protein from CHO-$D_{2(414)}$ cells or ~20 μg of protein from CHO-D_2/m1 cells was used per 1 ml binding reaction. Triplicate binding reactions were done in 1 × binding buffer for 30 min at 37°C, then stopped by filtration through glass-fiber filters (Skatron) that were pre-soaked in 0.1% polyethyleneimine, followed by a 5 s wash with ice-cold 0.1 × binding buffer. Saturation binding experiments were done with 8 concentrations of [^{3}H]spiperone (Amersham) in the range between 5 pM and 1 nM. Non-specific binding was defined with 10 μM fluphenazine. Competition binding experiments were performed with 500 pM [^{3}H]spiperone and varying amounts of competing ligand. Competing ligands were all from Research Biochemicals Inc.

2.4. *Measurement of* $[Ca^{+2}]_i$

Cells from a single sub-confluent 150 cm^2 T-flask were detached with EDTA, resuspended in Dulbecco's phosphate buffered saline (D-PBS) containing 0.1% glucose and 1 μM Fura2-AM (Molecular Probes Inc.), and incubated for 30 min at 37°C. The cells were then centrifuged at 750 × *g* for 5 min and resuspended in 40 ml of D-PBS containing 0.1% glucose. The cells were counted and then washed by centrifugation and resuspension in the same buffer at a concentration of $\sim 1 \times 10^6$ cells/ml. A 2 ml aliquot of the cell suspension was centrifuged and the cells were resuspended in fresh buffer immediately prior to use. Fluorescence was monitored with a DMX-1000 spectrofluorimeter (SLM-Aminco) equipped with a cuvette stirrer. The excitation wavelengths were 340 and 380 nm and the emission wavelength was 510 nm. The ratio of the intensities of the fluorescence at the two excitation wavelengths was used to calculate $[Ca^{+2}]_i$ as described previously [17]. For each experiment and each group of cells, maximum and minimum fluorescence values were determined by the addition of 10 mM digitonin followed by 10 mM EGTA.

3. RESULTS AND DISCUSSION

Fig. 1 shows a schematic of the D_2/m1 receptor that was constructed. In contrast to previously published G-protein-linked receptor chimeras [2–6,18], the D_2/m1 chimera was synthesized entirely by PCR. Since it does not require restriction sites within the receptor sequence, the PCR approach is much more flexible in its ability to create DNA sequences with desired inserts. We were able to insert the entire i3 loop of the m1 receptor at exactly the putative ends of transmembrane helixes V and VI of the $D_{2(414)}$ receptor without introducing any amino acid changes. However, the PCR amplification produced sequence errors that were repaired by fusing the non-mutated portions of two D_2/m1 clones. The use of thermostable polymerases with higher fidelity than Taq polymerase may improve the recovery of chimeras with correct sequences.

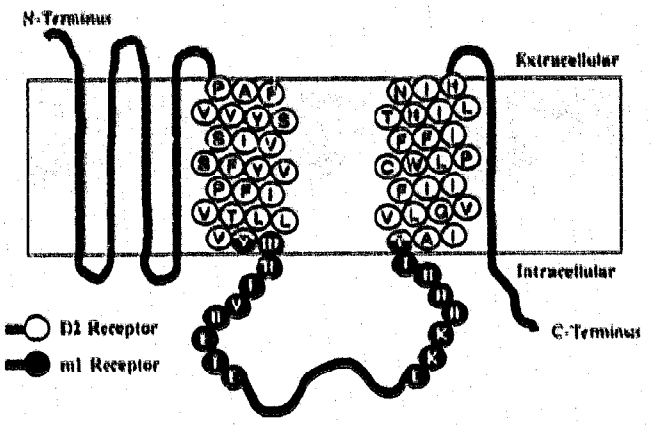

Fig. 1. Structure of the D_2/m1 chimeric receptor. The portion of the receptor derived from the $D_{2(414)}$ receptor is indicated by the grey line and open circles. The portion derived from the m1 receptor is indicated by the black line and filled circles. The amino acid residues at the chimera junctions (half-filled circles) were common to both sequences.

Preliminary studies with CHO cells showed no detectable specific binding for the muscarinic ligand [^{3}H]-QNB or the dopaminergic ligand [^{3}H]spiperone (data not shown). We therefore created cells expressing m1, $D_{2(414)}$ or D_2/m1 receptors by transfecting CHO-K1 cells with an expression vector containing the receptor DNA and a *neo* resistance gene. Transfected cells were then selected for G418 resistance. We compared the pharmacological profiles of the $D_{2(414)}$ and D_2/m1 receptors to see what effect, if any, the substitution of the i3 loop from the m1 receptor had upon the chimeric receptor's affinity for dopaminergic ligands. Shown in Fig. 2 is a representative Scatchard plot of the binding of [^{3}H]spiperone to crude membranes prepared from cells expressing either the $D_{2(414)}$ or the D_2/m1 receptor. The K_d of 65 ± 12 pM (mean ± SE ($n = 3$); B_{max} = 4.0 ± 1.1 pmol/mg protein) determined for the $D_{2(414)}$ receptor agrees well with a previous determination of the K_d for [^{3}H]spiperone for the rat $D_{2(415)}$ receptor [19]. The D_2/m1 receptor shows a modest, but statistically significant ($P < 0.05$; *t*-test), decrease in [^{3}H]spiperone affinity (K_d = 250 ± 10 pM ($n = 3$); B_{max} = 3.8 ± 0.6 pmol/mg protein). Next, we tested the binding affinities of six dopaminergic ligands in a com-

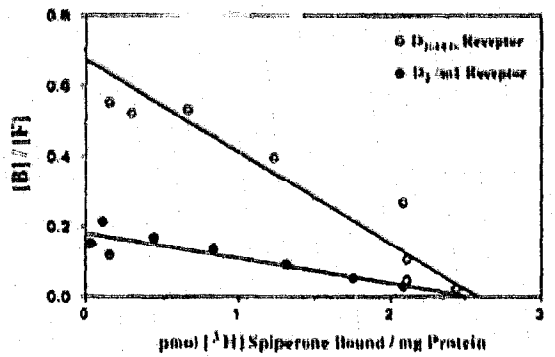

Fig. 2. Scatchard plot of [³H]spiperone binding to $D_{2(414)}$ and D_2/m1 receptors. Specific binding of various concentrations of [³H]spiperone was determined in triplicate. The figure is a representative experiment. Mean K_d and B_{max} values from three experiments (calculated with the program LIGAND [28]) are given in the text.

petition binding assay with [³H]spiperone. The results of 3 independent competition assays are summarized in Table I. There was no difference in K_i's of N-0434, (−)-sulpiride and SKF-38393 for the $D_{2(414)}$ and D_2/m1 receptor and although statistically significant ($P<0.05$; t-test), the differences in the K_i values for dopamine, fluphenazine and SCH-23390 were small.

Preliminary studies with non-transfected CHO cells indicated that $[Ca^{+2}]_i$ was unaltered by carbachol (100 μM) or dopamine (1 mM) (data not shown). Activation of the human m1 receptor has been previously shown to cause phosphatidylinositol hydrolysis and elevation of $[Ca^{+2}]_i$ [20]. When we expressed the human m1 receptor in CHO cells, it bound [³H]QNB with a K_d of 81 pM and elevated $[Ca^{+2}]_i$ in a dose-dependent manner in response to carbachol (data not shown). D_2 receptors, on the other hand, are classically associated with inhibition of adenylate cyclase [21] and thus thought to couple to a G_i-type G-protein. Both forms of the D_2 receptor have been shown to inhibit cAMP accumulation when expressed in the appropriate cell lines [13,22,25]. However, there have been some reports of dopamine-induced alteration of $[Ca^{+2}]_i$ [23,24], and studies with the rat $D_{2(415)}$ receptor have shown either dopamine-induced decreases or increases in $[Ca^{+2}]_i$ depending on the host cell line [25]. When we expressed the $D_{2(414)}$ receptor in CHO cells, dopamine (100 μM) failed to alter $[Ca^{+2}]_i$ (Fig. 3A). Shown in Fig. 3B are the results of calcium studies with the D_2/m1 chimeric receptor. $[Ca^{+2}]_i$ was elevated in a dose-dependent manner in response to dopamine. This effect of dopamine was blocked by the dopamine antagonist fluphenazine (Fig. 3C).

Our result, that the D_2/m1 chimeric receptor retains the pharmacologic specificity of a D_2 receptor and elevates $[Ca^{2+}]_i$ in response to dopamine, is significant in two respects. First, it provides additional support for the idea that the i3 loop of G-protein-linked receptors is important in determining the specificity of G-protein interaction. Second, it suggests the possibility of a generic strategy for directing members of the G-protein-linked receptor family to elevate $[Ca^{+2}]_i$ when this is not their native or preferred signal transduction pathway. Such an approach may prove useful for several purposes. For example, 'orphan' receptors, i.e. those receptors identified by homology to known receptors but for which the natural agonist is unknown, could be engineered to elevate $[Ca^{+2}]_i$ by the approach illustrated here. The calcium assay could then be used to identify agonists for the 'orphan' receptor. Chimeric receptors could also be important tools for drug discovery, where the identification of families of receptor subtypes has increased the need for inexpensive and facile assays for testing large numbers of potential drug candidates. Typically, radioisotopic ligand binding and second messenger assays are employed in screening drug candidates for their interaction with G-protein-linked receptors. But if the nucleotide sequence of a receptor is known, a chimeric receptor similar to the D_2/m1 receptor can be created quite easily using PCR. The $[Ca^{+2}]_i$ response is rapid, robust and likely to be automatable. Thus, it may be possible to screen large

Table I

K_i values (μM) for dopaminergic ligands

Competitor	$D_{2(414)}$ K_i (μM)	Receptor 95% C.L.	D_2/m1 K_i (μM)	Receptor 95% C.L.
Fluphenazine*	0.0031	(0.0016, 0.0057)	0.0072	(0.0053, 0.0098)
N-0434	0.021	(0.010, 0.042)	0.027	(0.012, 0.058)
(−)Sulpiride	0.028	(0.015, 0.054)	0.045	(0.011, 0.180)
Dopamine*	6.0	(4.9, 7.4)	2.1	(0.93, 4.63)
SCH-23390*	2.1	(1.5, 3.0)	3.6	(2.4, 5.3)
SKF-38393	21	(8.8, 51)	36	(19, 70)

* Difference between D_2 and D_2/m1 receptor is statistically significant ($P<0.05$; t-test)

Specific binding of [³H]spiperone (0.5 nM) at twelve different concentrations of competing ligand was determined in triplicate. IC_{50}'s were determined by analysis with the program ALLFIT [26] and K_i's were computed using the Cheng-Prusoff equation [27]. K_d values for [³H]spiperone, which differed between the two receptors, are given in the text. Values are the anti-log of the mean log K_i from three separate experiments and 95% confidence intervals calculated from the standard deviation of the log K_i

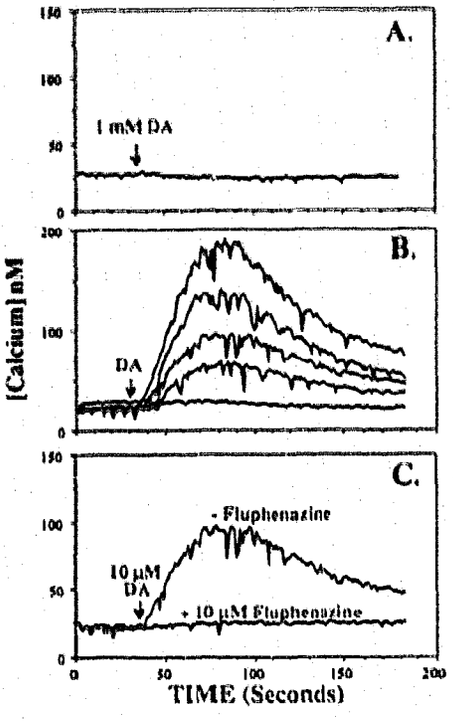

Fig. 3. Effect of dopamine on $[Ca^{+2}]_i$ in Fura-2 loaded CHO cells. (A) Dopamine (1 mM final concentration) was added to CHO cells expressing $D_{2(414)}$ receptors at the indicated time. (B) Various concentrations of dopamine were added at the time indicated to CHO cells expressing D2/m1 receptors. The final concentrations of dopamine from the bottom to the top curve were 0.1, 1, 10, 100, 1000 μM. (C) CHO cells expressing D2/m1 receptors were pre-incubated with or without 10 μM fluphenazine for 2 min followed by addition of 10 μM dopamine at the indicated time.

numbers of compounds as agonists or antagonists of any previously cloned member of the G-protein-linked receptor family and avoid the hazards and costs associated with the use of radioisotopes.

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