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Chimeric dopamine D_2/ angiotensin AT_1 receptors: role of the length of third intracellular loop of D_2 receptors in conferring specificity of receptor binding and G-protein coupling[1]

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KEY WORDS dopamine D_2 receptors; angiotensin receptors; dopamine agonists; dopamine antagonists; chimeric proteins; polymerase chain reaction; phosphatidylinositols; G-proteins

AIM: To define roles of the third intracellular loop (IL_3) length of G-protein coupled receptors in conferring the specificity for receptor binding and G-protein coupling. **METHODS:** By polymerase chain reaction (PCR), the IL_3 of D_2 receptor was replaced with the counter part of AT_1 receptor which has the shortest loop among all G-protein coupled receptors. D_2/AT_1 receptor cDNA was then stably transfected into Chinese hamster ovary cells and a clone with high level expression was obtained for receptor binding and agonist-induced phosphatidylinositols (PI) turnover experiments. **RESULTS:** Comparing to the D_2 receptor, D_2/AT_1 chimeric receptor had lower affinities for all D_2 receptor antagonists tested (spiperone, haloperidol, (+)-butaclamol, chlopromazine, clozapine, trifluoperdazine) and different affinity profiles to agonists (apomorphine, dopamine, quinpirole, bromocriptine). But the chimeric receptor failed to couple to G-protein and subsequent stimulation of PI turnover. **CONCLUSION:** The length of IL_3 of D_2 receptor participates defining receptor binding sites conformation, and structure beyond IL_3 may affect receptor G-protein coupling.

G-protein-coupled receptors have 7 membrane-spanning domains with 3 extra- and 3 intra-cellular loops. Seven-membrane spanning regions form a binding sites inside membrane that bind ligands[1]. Of particular interest is the third intracellular loop (IL_3) which is important in determining the specificity of effector coupling. For example, by changing the IL_3 of muscarine M_3 receptor, which coupled to PI turnover, with the IL_3 of α_2-adrenergic receptor (α_2-AR) which inhibits adenyl cyclase, the created M_3/α_2-AR chimeric receptor displayed ligand binding properties of M_3 receptor, but an inhibition of adenyl cyclase[2]. The dopamine D_2/muscarine M_1 chimeric receptor bind dopaminergic ligands with affinities similar to the D_2 receptor, but elevate [Ca^{2+}] in response to dopamine[3]. Similar findings have been described for β_2/α_1 chimeric rceptor as well[4]. However, all these chimeric receptors have comparable lengths of IL_3, which may not affect receptor conformation if exchanged. What could be the consequence if we substitute IL_3 from one receptor into that of another when IL_3 are very different in lengths between two receptors? Do the chimeras still preserve the ability of conferring the specificity of receptor binding and G-protein coupling? Based on this speculation, we constructed a D_2/AT_1 chimeric receptor in which the IL_3 of D_2 receptor was replaced by the counter part of AT_1 receptor. AT_1 receptor has the shortest IL_3 among all receptors coupled to PI turnover. The D_2/AT_1 receptors were stably expressd in Chinese hamster cells (CHO) and studied for their ability to bind dopaminergic ligands and confer the specificity of G-protein coupling including sensitivity to guanine nucleotides and stimulation of phosphatidylinositol hydrolysis.

MATERIALS AND METHODS

Reagents Dopamine, apomorphine, quinpirole, bromocriptine, spiperone, haloperidol, (+)-butaclamol, chlopromazine, clozapine, and trifluoperdazine were purchased from RBI; [^{3}H]spiperone was purchased from Amersham.

Construction and transfection of chimeric receptor The IL_3 of D_2 receptor cDNA was replaced by the counter part of AT_1 receptor cDNA by PCR. In brief, two complimentary chimeric primers (5′-TAT ACC CTT ATT

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TGG AAA GCT CTA AAG GCT TAT GAA ATT CAA AAG AAC AAA CCA AGA AAC FAT GAC ATC GCC ATT GTT CTC GGC GTG TTC ATC ATC TGC-3′) containing the IL_3 of AT_1 receptor sequences were used in conjunction with primer A (5′-TCC AAG CTT CTG GAT CCA CTG AAT CTG TCC-3′) and primer B (5′-GCA TCT AGA TCA GCA GTG AAG GAT CTT CAG-3′) derived from published D_2 receptor sequences to amplify the N- and C-terminal portion of the chimeric receptor. Full length of chimeric receptor cDNA was amplified in a secondary PCR reaction using A and B primers and the overlapping chimeric N- and C-terminal fragments as templates. The amplified chimeric receptor cDNA was cloned into the TA vector, then re-inserted into the mammalian expression vector RC/CMV. CHO cell were propagated in F-12 medium with 10 % fetal bovine serum in 5 % CO_2. The chimeric receptor cDNA or D_2 receptor cDNA or AT_1 receptor cDNA was transfected into CHO (6×10^{10} cells · L^{-1}) by electroporation techniques. Cells grew for 48 h prior to the addition of G-418 sulfate 100 $\mu g \cdot L^{-1}$. The medium was replaced every 3 d and cells surviving the G-418 selection were grown and maintained in selective medium. A clone producing high level of the D_2/AT_1 receptors or D_2 receptors or AT_1 receptors was isolated and analyzed for receptor binding and agonist-induced PI turnover.

Measurement of agonist-induced PI hydrolysis PI hydrolysis was measured as the lithium-dependent accumulation of [^{3}H]inositol phosphates in cells prelabeled with [^{3}H] inositol[5]. Briefly, cells grown in 12-well plates were labeled with 93 kBq/dish of myo-[^{3}H]inositol for 24 h in the F-12 medium without serum. Labeled cells were then washed and preincubated at 37 ℃ for 20 min in physical buffer solution containing LiCl 5 mmol·L^{-1}. Dopamine 100 μmol·L^{-1} or agiotensin II 100 nmol·L^{-1} was added to stimulate the cells expressing either D_2 receptors, D_2/AT_1 receptors, or AT_1 receptors and the reaction was allowed to proceed at 37 ℃ for another 45 min. The reaction was terminated by addition of ice-cold trichloroacetic acid. Accumulation of [^{3}H] inositol phosphate was measured by anion exchange chromatography (AG-1 × 8, formate form) via eluting the column with ammonium formate 1 mol·L^{-1}/ formic acid 0.1 mol·L^{-1}.

Receptor binding assay For the assay of [^{3}H]spiperone binding to crude membrane, the cells grown in 150-mm dishes were washed twice with phosphate buffer solution, collected and homogenized with a polytron for 45 s followed by sonication for 1 min in Tris buffer 5 mmol·L^{-1} (pH 7.4) containing edetic acid 5 mmol·L^{-1}. The homogenates were spun at 40 000 × g for 20 min and the pellets were resuspended in buffer containing Tris-HCl 25 mmol·L^{-1}, $MgCl_2$ 8 mmol·L^{-1} and edetic acid 1 mmol·L^{-1} for binding assay. Binding studies were made with [^{3}H] spiperone 1 nmol·L^{-1} for at 25 ℃ 1 h, in 0.5 mL of the above Tris-Mg^{2+}-edetic acid buffer. Nonspecific binding was determined in the presence of (+)-butaclamol 10 μmol·L^{-1}. The binding reaction was terminated by rapid filtration on Whatman GF/G glass fiber filters monitored for ^{3}H radioactivity.

All reported values are $\bar{x} \pm s$. Comparisons between groups were made using t-test.

RESULTS

Ligand binding properties of D_2/AT_1 chimeric receptors expressed in CHO cells D_2 receptors and D_2/AT_1 chimeric receptors were stably expressed in CHO cells alone and tested for their ability to bind dopaminergic ligands. Equilibrium binding studies of the D_2 receptor antagonist [^{3}H] spiperone with both cell lines showed saturable binding. Scatchard transformation of the data showed that the cells expressing D_2 receptors and D_2/AT_1 chimeric receptors produced similar B_{max} (3.12 nmol/g protein *vs* 2.06 nmol/g protein). In contrast, the K_d value of AT_1 receptor for [^{3}H]spiperone was 28-fold higher than that of D_2 receptor (3.38 *vs* 0.12 nmol·L^{-1}). Untransfected CHO cells did not display any specific binding for [^{3}H]spiperone.

Competition binding experiments were carried out to determine the affinities with which different ligands bound to D_2 receptors or D_2/AT_1 chimeric receptors. The K_i values of all antagonists for displacing [^{3}H] spiperone were higher for D_2/AT_1 chimeric receptors than D_2 receptors. Haloperidol and chlopromazine showed 1/90 of the affinity for D_2/AT_1 chimeric receptors than for D_2 receptors, whereas D_2/AT_1 chimeric receptors displayed 1/10, 1/3, and 1/3 of the affinity for (+)-butaclamol, trifluoperdazine and clozapine, respectively, compared with D_2 receptors. In contrast, D_2/AT_1 chimeric receptors exhibited different affinity profiles for D_2 receptor agonists with increased affinity for dopamine and quinpirole (3-fold and 2-fold, respectively), but decreased affinity for apomorphine and bromocriptine (3-fold and 2-fold, respectively) (Tab 1).

Influence of guanosine triphosphate (GTP) on dopamine displacement of [^{3}H]spiperone binding In the D_2 receptor, dopamine competitively inhibited the specific [^{3}H]spiperone binding. The

Tab 1. K_i values ($nmol \cdot L^{-1}$) for agonists and antagonists in D_2/AT_1 chimeric receptor expressed in CHO cells. $n=8$, $\bar{x} \pm s$. $^cP<0.01$ vs D_2 receptor.

	D_2	D_2/AT_1
Agonist		
Apomorphine	24 ± 4	290 ± 24[c]
Dopamine	430 ± 174	129 ± 12[c]
Quinpirole	590 ± 240	290 ± 46[c]
Bromocriptine	23 ± 8	500 ± 139[c]
Antagonist		
Spiperone	0.19 ± 0.04	3.8 ± 0.8[c]
Haloperidol	0.23 ± 0.05	22 ± 6[c]
(+)-butaclamol	3.00 ± 0.55	30 ± 7[c]
Chlopromazine	0.47 ± 0.07	44 ± 5[c]
Clozapine	17.40 ± 0.40	51 ± 3[c]
Trifluoperdazine	1.20 ± 0.22	40.1 ± 0.9[c]

displacement curve was better fit by a two-site model than a one site model with a high affinity sites (IC_{50} = 9.0 $nmol \cdot L^{-1}$; 95 % confidence limits, 8.3 − 9.7 $nmol \cdot L^{-1}$) comprising 36 % of the total number of specific binding and low affinity sites (IC_{50} = 3.0 $\mu mol \cdot L^{-1}$; 95 % confidence, 2.8 − 3.3 $\mu mol \cdot L^{-1}$) comprising the remaining 64 % binding sites. In the presence of GTP 10 $\mu mol \cdot L^{-1}$, high affinity binding sites were decreased 16 %. However, in the D_2/AT_1 chimeric receptor, no significant effect of GTP 10 $\mu mol \cdot L^{-1}$ on dopamine displacement curve was seen (Fig 1).

Agonist-stimulated effects on PI hydrolysis

The ability of D_2/AT_1 receptor to mediate stimulation of PI hydrolysis was also examined. Angiotensin II (0.1 $\mu mol \cdot L^{-1}$) treatment of cells transfected with AT_1 receptors resulted in pronounced increase in inositol phosphates (InsPs) level. On the other hand, dopamine (100 $\mu mol \cdot L^{-1}$) stimulation of CHO cells transfected with either D_2 receptors or D_2/AT_1 chimeric receptors did not result in any significant increase in InsPs accumulation (Tab 2).

DISCUSSION

Results presented in the study showed that D_2/AT_1 chimeric receptors bound all the D_2 receptor antagonists with lower affinity than D_2 receptors, but displayed selective binding profile for D_2 receptor

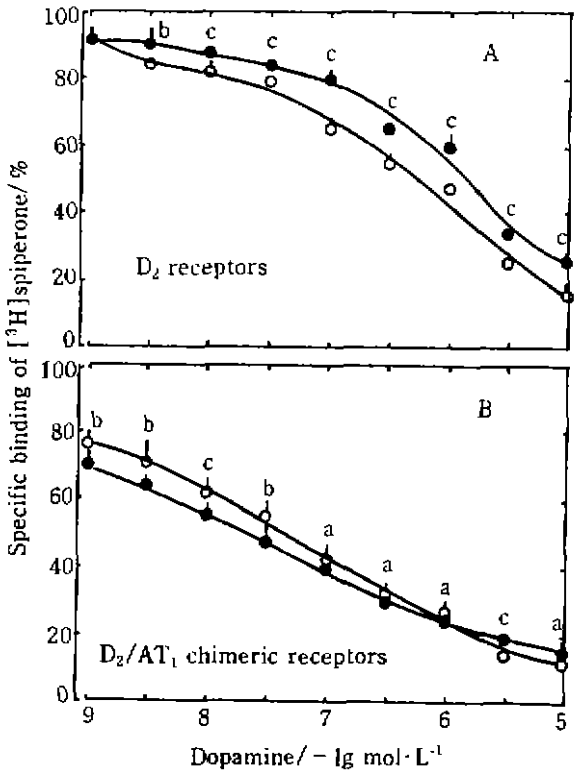

Fig 1. Effect of GTP on dopamine displacement of [^{3}H]spiperone binding. ○: −GTP, ●: +GTP. $n=6$, $\bar{x} \pm s$. $^aP>0.05$, $^cP<0.01$ vs D_2 receptor.

Tab 2. Effect of activation of D_2, AT_1, and D_2/AT_1 receptors on PI hydrolysis. $\bar{x} \pm s$ of triplicate determinations from 6 experiments. $^aP>0.05$, $^cP<0.01$ vs D_2 receptor, CHO cells expressed D_2 or D_2/AT_1 receptor were incubated with dopamine 100 $\mu mol \cdot L^{-1}$; CHO cells expressed AT_1 receptor were incubated with angiotensin II 100 $nmol \cdot L^{-1}$.

	$InsP_3$ accumulation (Bq)	
	Basal	Agonists
D_2	16.8 ± 1.3	18.5 ± 1.5[a]
AT_1	16.8 ± 1.2	32.0 ± 1.7[c]
D_2/AT_1	17.3 ± 1.6	16.7 ± 1.1[a]

agonists with affinity increase for dopamine and quinpirole, but affinity decrease for apomorphine and bromocriptine compared with D_2 receptors. These data suggest that substitution with short IL_3 from AT_1 receptor affects the ligand binding sites by introducing a conformational change of TM domains, which results in alteration of ligand

affinity at D_2/AT_1 receptor. Another two D_2 chimeric receptors have been constructed previously by other investigators. D_2/M_1 chimeric receptor containing IL_3 from M_1 receptor bound to dopaminergic ligands with affinities similar to D_2 receptor[3]. In contrast, D_2/D_3 chimeric receptor containing IL_3 from D_3 receptor had higher affinity for agonists than D_2 receptor, but antagonists binding was not significantly altered[6]. By combining data from D_2/M_1 chimeric receptor and D_2/D_3 chimeric receptor with our results from D_2/AT_1 receptor, the new insights have emerged that length and/or primary sequence of IL_3 could affect ligand binding selectivity by exerting conformational effects on ligand binding sites.

Receptors exist in 2 main conformational states, receptor alone and receptor coupled to the G-protein (HRG ternary complex). The former has a low affinity while the latter has a high affinity to agonists. GTP activates G-protein and at mean time, reduces HRG ternary complex. Therefore GTP can greatly decrease the high affinity sites for agonists[7] as seen in our experiment with D_2 receptor. However, in our experiment the displacement curve for dopamine in the presence of GTP was still better fit to a two-sites model than a single site model. The reasons might be: (1) HRG dissociation and association is always in a dynamic balance. Although in the presence of high concentration of GTP the balance is greatly inclined towards the dissociation, theoretically there should be still a small portion of HRG ternary complex, *ie*. high affinity sites left; (2) In our experiment we used GTP which was quite easy to be hydrolyzed during the 60 min incubation at 25 ℃. Our results have shown that in the D_2/AT_1 chimeric receptor GTP did not shift the dopamine displacement curve, and D_2 receptors agonist did not stimulate PI hydrolysis, indicating a perturbation of the interaction between the receptor and G protein. Studies with M_1/M_2, D_2/M_1 and β_2/α_{1B} chimeric receptors described above show that sequences beyond IL_3 exert little influence on receptor-G protein coupling. However, only when D_2/AT_1 was constructed in which AT_1 IL_3 was significantly shorter than that of D_2 receptor, the chimeras was found to lose its ability to couple to G protein. It suggested that there may exist minimal size of IL_3 necessary for chimeric receptor to couple with G protein and other regions of receptor in addition to IL_3 may play a role in determining the receptor-G protein interaction. Certainly, we can not exclude the possibility that the phenomenon is simply caused by the special amino acid sequences of the AT_1 receptor IL_3.

In conclusion, we have demonstrated that IL_3 of AT_1 receptor influences ligand binding selectivity by exerting conformational changes on receptor binding sites. Moreover, structures beyond IL_3 may also be involved in defining G-protein and receptor coupling properties, which may largely depend on the size of IL_3.

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多巴胺 D_2/血管紧张素 AT_1 嵌合受体：D_2 受体第三细胞内环影响受体与配基结合以及与 G 蛋白偶联的特性

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关键词 多巴胺 D_2 受体；血管紧张素受体；多巴胺激动剂；多巴胺拮抗剂；嵌合体蛋白类；聚合酶链反应；磷脂酰肌醇；G蛋白类

偶联

目的：研究受体第三细胞内环（IL_3）的长度对受体与配基结合及与 G 蛋白偶联特性的影响。方法：用目前已知的 G 蛋白偶联受体中 IL_3 最短的血管紧张素 II AT_1 受体的 IL_3 替换野生型 D_2 受体较长的 IL_3，组成 D_2/AT_1 嵌合受体。结果：与野生型 D_2 受体相比，D_2/AT_1 嵌合受体与拮抗剂的亲和性均降低，与激动剂的亲和性有的增高，有的降低。嵌合受体失去与 G 蛋白偶联的能力，也不能产生磷酸肌醇水解。结论：受体的 IL_3 对受体配基结合位点和空间构象有一定影响；受体与 G 蛋白的偶联不仅与 IL_3 有关，而且还受非 IL_3 区域的影响，而 IL_3 的长度是决定这两方面影响的因素之一。

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Effect of saponins of *Panax notoginseng* on synaptosomal ^{45}Ca uptake

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KEY WORDS ginseng; saponins; synaptosomes; calcium radioisotopes; nimodipine

AIM: To explore the calcium uptake antagonism of saponins of *Panax notoginseng* (PNS). **METHODS:** Synaptosomes were prepared from rat cerebral cortex by using differential Ficoll gradients. The effects of PNS on synaptosomal ^{45}Ca uptake were measured *in vitro* or after acute treatment. **RESULTS:** PNS 50 - 800 mg·L^{-1} produced a concentration-rated inhibition of Ca^{2+} uptake [IC_{50} = 111 (46 - 176) mg·L^{-1}]. Both initial and maximal uptake were inhibited. Similar effect was obtained after acute PNS treatment with 200 mg·kg^{-1} ip. The blocking effect of PNS was reversed by calcium in media. **CONCLUSION:** PNS is a calcium channel blocker in neurons.

Saponins of *Panax notoginseng* (PNS) protected brain or primary cultured myocytes from anoxic injuries[1-3]. Balance perturbation of calcium in the cell constitutes the final common pathway of cell damage during ischemia/reperfusion[4]. PNS blocked calcium into vascular smooth muscles and cultured myocardial cells[5,6]. In this study the effects of PNS on synaptosomal calcium uptake were tested.

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MATERIALS AND METHODS

Drugs and chemicals PNS was extracted and purified by Dept of Phytochemistry, Institute of Medicinal Plant Development, purity 94.5 %. $^{45}CaCl_2$ (148 GBq·L^{-1}) was purchased from Beijing Institute of Atomic Energy. Ficoll400 was obtained from Pharmacia and ATP-Na_2 was purchased from Sigma. All other chemicals were AR grade.

Preparation of synaptosomes Synaptosomes were obtained from rat cerebral cortex scraped free of as much white matter as possible before homogenization[7]. After centrifugation at 65 000 × g for 50 min, the material between the 7.5 % and 12 % Ficoll interface (synptosomes)