Role of a $G\alpha_{i2}$ protein splice variant in the formation of an intracellular dopamine D_2 receptor pool

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Summary

Treatment of D_2-receptor-expressing cells with specific drugs upregulates the receptor number at the cell surface independently of protein synthesis, leading to the concept of an intracellular receptor pool. However, how this pool is operating is still an enigma. Here, we report that a splice variant of the $G\alpha_{i2}$ protein, protein $sG\alpha_{i2}$, plays a crucial role in the maintenance of this D_2-receptor pool. Co-expression of sG_{i2} with D_2 receptor reduced receptor localization to cell surface by one-third. This effect is associated with specific intracellular protein-protein interaction and the formation of a sG_{i2}–D_2-receptor complex. It has been suggested that the formation of this complex serves to prevent D_2 receptors from reaching the cell membrane. Treatment of D_2-receptor-expressing cells with agonists increased the number of cell surface D_2 receptors and coincided with a reduction in these receptors from intracellular complexes, suggesting that agonist treatment released D_2 receptors from the complex allowing them to localize to the cell membrane. Thus, in addition to elucidating how the intracellular pool of D_2 receptor functions, our findings uncover a novel mechanism regulating the density of cell surface D_2 receptors.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/13/2171/DC1

Key words: Protein-protein interaction, Intracellular receptor pool, D_2 receptor, $G\alpha_{i2}$ protein, Receptor upregulation, Receptor density

Introduction

Drug-induced augmentation of D_2 receptor on plasma membrane has been reported in a cell line that expresses endogenous D_2 receptors (Ivins et al., 1991), as well as in HEK-293 cells (Boundy et al., 1995; Filtz et al., 1993), CHO cells (Itokawa et al., 1996; Zhang et al., 1994), C_6 glioma cells and Ltk^- cells (Starr et al., 1995), and Sf9 cells (Ng et al., 1997) when expressing recombinant D_2 receptors. This upregulation was not affected by the inhibition of protein synthesis (Filtz et al., 1993; Starr et al., 1995; Ng et al., 1997) and, therefore, it was proposed that this increase of receptors was owing to their recruitment from existing intracellular reservoir(s) (Ng et al., 1997). Increase in receptor numbers following drug treatments has also been observed in other transmitter-receptor systems (Creese and Sibley, 1981). The most recent work describing agonist exposure of cells that express D_2 receptors found translocation of receptor from cytoplasm to plasma membrane (Ng et al., 1997), providing direct evidence of functional receptor pool in the cytoplasm. It is well known that the interaction of plasma-membrane-bound dopamine D_2 receptor with $G\alpha_{i2}$ protein is fundamental for signal transmission. However, we and others have shown previously that, in contrast to $G\alpha_{i2}$ protein – which is localized at the cell surface, sG_{i2} is an intracellular protein and not found at plasma membrane (Khan and Gutierrez, 2004; Montmayeur and Borrelli, 1994). A Proline-rich motif at the C-terminus is thought to be crucial for the intracellular translocation of this protein (Picetti and Borrelli, 2000). The sG_{i2} transcript encodes a protein with a different C-terminus, in which a 24-amino acid (aa) strech is replaced by a 35-aa sequence. Like sG_{i2}, other G-proteins have also been identified on intracellular membranes (Audigier et al., 1988; Weiss et al., 2001), where they participate in different functions, such as protein transport (Bomsel and Mostov, 1992; Erkolani et al., 1990; Helms, 1995; Pimplikar and Simons, 1993). On the basis of the interaction between non-spliced $G\alpha_{i2}$ protein and dopamine D_2 receptor (Gazi et al., 2003; Senogles, 1994), and the localization of sG_{i2} protein in brain dopaminergic cells (Khan and Gutierrez, 2004) where prominent expression of D_2 receptors was also found (Khan et al., 1998a), we hypothesized that the sG_{i2} protein takes part in the translocation of dopamine D_2 receptors to cell surface. To our surprise, sGi2 protein not only regulated the density of D_2 receptor at cell surface but also participated in the formation of an intracellular reservoir of this receptor.

Results

In intact BHK cells, co-expression of the long isoform of the D_2 receptor (D_{2L}) and the sG_{i2} protein led to a 31% decrease in the number (B_{max}) of plasma-membrane-bound receptor (5.25±0.41 fmol per 10^6 cells of D_{2L} alone versus 3.65±0.35 fmol per 10^6 cells of D_{2L} with sG_{i2}). This loss in D_{2L} receptor was dependent on the amount of sG_{i2} protein co-expression (Fig. 1A). As the level of expression of sG_{i2} increased, more reduction in B_{max} of D_{2L} receptor was observed. However, no

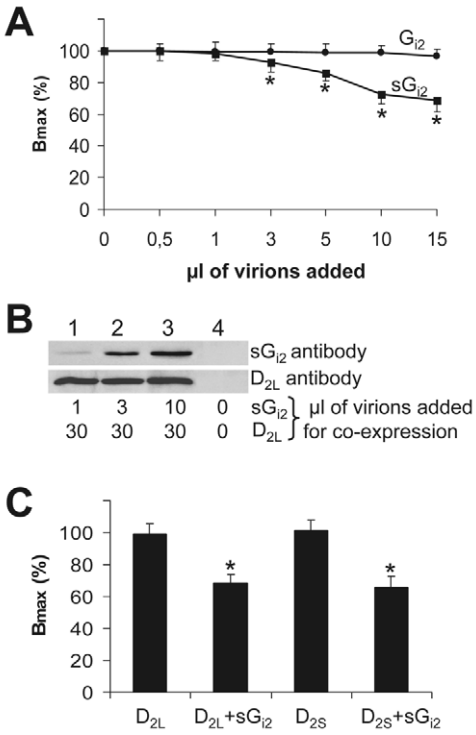

Fig. 1. Expression of sG_{i2} reduces the dopamine D_2 receptor density at the plasma membrane. (A) Infection of BHK cells with 30 µl pseudovirions of D_{2L} (long isoform of D_2 receptor) and 0-15 µl of sG_{i2} (●) or $G\alpha_{i2}$ (G_{i2}, ■) protein showed a gradual decrease in the number of plasma-membrane-bound D_2 receptors as the expression of sG_{i2} protein was increased. G_{i2} had no effect. (B) Immunoblots of cells from A show that, as the addition of sG_{i2} pseudovirion was increased, higher expression of this protein. D_{2L} expression remained the same. Non-transfected cells are shown in lane 4. (C) In JEG-3 cells that lack endogenous G_{i2} protein, co-expression of sG_{i2} with either D_{2L} or D_{2S} (short isoform of D_2 receptor) receptors also yielded a 32-35% reduction in cell surface D_2 receptors, similar to that as seen in BHK cells in A. *P<0.05, significant change from control. Values are representative of six to eight different experiments.

further reduction was observed at 30 µl of pseudovirions (not shown). The substitution of sG_{i2} with $G\alpha_{i2}$ had no effect (Fig. 1A). This finding suggests that the receptor loss seen is specifically associated to sG_{i2} protein. To exclude the possibility that sG_{i2} inhibits protein expression, we checked the expression levels of both D_{2L} and sG_{i2} proteins in these experiments by immunoblotting, by using affinity-purified antibodies [for data on specificity tests for D_{2L} and D_{2S} antibodies see Khan et al. (Khan et al., 1998a) and for sG_{i2} antibodies see supplementary material Fig. S1]. In fact, we found that the D_{2L} receptor concentration was unchanged, whereas expression of sG_{i2} protein was increased as expected (Fig. 1B), indicating that reduction of D_{2L} was not due to lower expression of this receptor. Next, we analyzed the binding affinity (K_d) of D_2 receptor that might have been compromised during co-expression of both proteins, but no discernable change was observed (K_d=80±15 pM without sG_{i2} and 73±16 pM with sG_{i2}). A similar effect of sG_{i2} protein on D_{2L} as well as D_{2S} receptors was also observed in other cell lines, including JEG-3 (Fig. 1C) and NG108-15 (Fig. 3B). These results not only confirm the observations made in BHK cells but also implicate that the regulation of the density of cell surface D_{2S} and D_{2L} receptors by sG_{i2} protein is a common characteristic among various cell types. The use of human carcinoma JEG-3 cells that lack D_2 receptor and $G\alpha_{i2}$ (Guiramand et al., 1995) also showed effects similar to other cell lines. It was observed that co-expression of either D_{2L} or D_{2S} receptors with sG_{i2} protein produced a loss of 32% or 35%, respectively (Fig. 1C). Furthermore, evidence from immunoblot analysis (Fig. 1B) suggests that, even though the total expression of D_{2L} receptors (cell-surface-bound plus intracellular) was unchanged, localization of D_{2L} receptor on the cell surface was reduced. Therefore, we further examined the localization of D_2 receptors in these cells by double immunofluorescence labeling (Fig. 2) and found that, when D_{2S} or D_{2L} receptors were expressed alone, they mainly localized at plasma membrane (Fig. 2A), co-expression with sG_{i2}, membrane localization of the receptor was noticeably reduced. This reduction in cell surface localization was probably due to accumulation of D_{2S}-sG_{i2} protein complex seen in the intracellular membranes (Fig. 2B,C,D).

To further demonstrate that the loss of D_2 receptor activity in intact cells was due to a reduction in functional cell surface receptor population, we performed in-vivo Ca^{2+}-transient studies in these cells by Ca^{2+} imaging. It is known that antagonist-mediated blockade of D_2 receptor augments intracellular Ca^{2+} flow via membrane-bound voltage-gated Ca^{2+} channels (Chronwall et al., 1995; Pauwels et al., 2001). Therefore, we used this paradigm expecting that a decrease in the cell-surface-associated D_2 receptor after co-expression of sG_{i2} proportionally reduces the D_2-antagonist-mediated Ca^{2+} rise. Because of downstream D_2-signaling pathways, NG108-15 – a neuroblastoma/glioma cell line of neural origin (Pilon et al., 1994) – was also included in this study. Indeed, application of 15 µM raclopride, a D_2 receptor antagonist, produced a transient increase in the intracellular Ca^{2+} concentration, which was significantly reduced (30-35%) in cells that co-expressed sG_{i2}, similar to the observation made in ligand-binding experiments (D_{2L} 2.1±0.1 versus D_{2L}+sG_{i2} 1.53±0.1 and D_{2S} 2.2±0.2 versus D_{2S}+sG_{i2} 1.44±0.1; values are F/F_o ratios of Ca^{2+} changes after drug application from the baseline) (Fig. 3A-C). In control experiments, the use of neither $G\alpha_{i2}$ protein in place of sG_{i2} nor D_1 in place of D_{2S} or D_{2L} receptor produced any such effect (Fig. 3C). In contrast to antagonist, D_2 receptor activation by agonist reduces Ca^{2+} flow (Lledo et al., 1990; Wolfe and Morris, 1999). Consistent with this, agonist treatment led to a decrease in Ca^{2+} levels in cells expressing D_2 receptor; however, co-expression of sG_{i2} reduced this decrease (see supplementary material Fig. S2). These results suggest that a reduced efficiency of Ca^{2+} flow is associated with a lower number of D_2 receptors at the plasma membrane. To rule out the participation of intracellular Ca^{2+} stores in our experiments, we used 2-aminoethoxydiphenyl borate (APB), an inhibitor of the IP3 receptor. Treatment of cells with APB did not influence the Ca^{2+} transients, whereas the thapsigargin-stimulated Ca^{2+} release from intracellular stores could still be observed. This observation suggested that intracellular Ca^{2+} stores were intact but did not participate in D_2-modulated Ca^{2+} increase. In addition, the use of Ca^{2+}-free

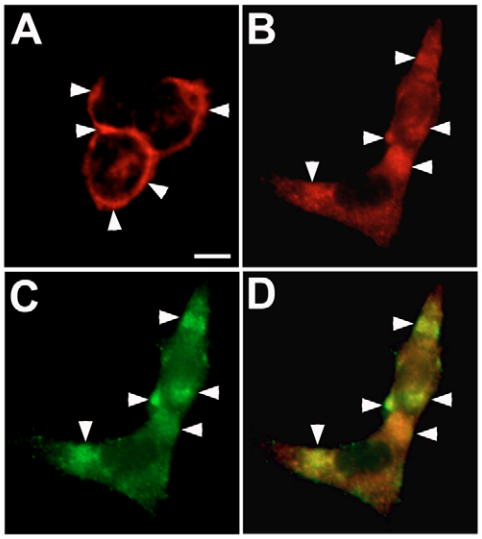

Fig. 2. (A-D) Co-accumulation of D_2 receptor and sG_{i2} protein complex in cytoplasmic space. (A) D_{2S} receptors were expressed alone in BHK cells and immunodetected by antibodies to D_{2S} receptor. In these cells, most of the D_{2S} proteins were localized at the cell surface (arrowheads). However, when D_{2S} receptors were co-expressed with sG_{i2} proteins (B and C) in these cells, cell surface localization of receptors was reduced markedly (arrowheads indicate protein immunolabeling). They co-accumulated in cytoplasm space with sG_{i2} protein (arrowheads in D). To detect the co-labeling of both proteins, specific antibodies against D_{2S} (B) and sG_{i2} (C) were used in double labeling immunofluorescence experiment. Bar, 12 μm.

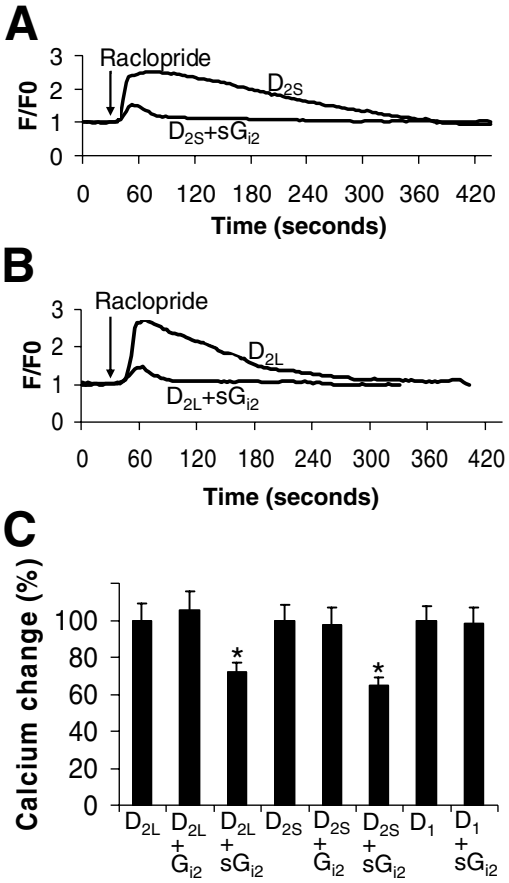

Fig. 3. Reduced D_2 receptor-mediated plasma membrane Ca^{2+} channel activity after co-expression with sG_{i2} demonstrates the reduced D_2 receptor density at cell surface. (A,B) Typical intracellular Ca^{2+} transient in a single BHK cell expressing (A) D2S and (B) D2L receptor, after application of 15 μM raclopride, a D_2 antagonist. This activity was significantly reduced in cells when sG_{i2} was co-expressed. (C) Summary of the results from experiments using NG108-15 cells. Co-expression of sG_{i2} with either D_{2L} or D_{2S} produced a 30-35% decrease in Ca^{2+} transients, similar to receptor binding results shown in Fig. 1. This activity was associated with D_2 and not to D_1 receptors. In addition, expression of G_{i2} instead of sG_{i2} did not show any change. F/F_0 represents the change in fluorescence intensity over baseline (see Materials and Methods). $^*P<0.05$, significant change from control. Values are representative of six different experiments.

medium or medium containing EGTA and $CoCl_2$ (blockers of membrane Ca^{2+} channels) and APB yielded the same results, further supporting this notion (see supplementary material Fig. S3).

Direct evidence of interaction between D_2 receptor and sG_{i2} protein came from the co-elution of a D_2-sG_{i2} complex using affinity-columns (Fig. 4A). The columns were prepared with affinity-purified specific antibodies against D_{2L}, D_{2S} (Khan et al., 1998a; Khan et al., 2001) or sG_{i2} protein (see supplementary material Fig. S1 for evidence on antibody specificity). Both affinity-columns that were immobilized with antibodies against D_{2L} and D_{2S} co-eluted sG_{i2} protein (Fig. 4A). Using the sG_{i2} antibody affinity-column, we observed co-elution of D_{2S} and D_{2L} receptors but not of D_1 receptor (Fig. 4A). To further demonstrate the functional interaction between sG_{i2} protein and active D_2 receptors, solubilized proteins from cells were incubated with antiserum against sG_{i2} and presence of co-imunoprecipitated D_2 receptor was determined. We observed 24.6±4.9% co-precipitation of D_2 receptor with sG_{i2} antibodies (Fig. 4B). The fact that sG_{i2} antibody did not bind D_1 receptor suggests again that the D_2-sG_{i2} interaction is specific.

To find out whether the complex of D_2-sG_{i2} also exists in brain tissues, we used extracts from substantia nigra, a region where most neurons express high number of dopamine D_{2S} receptors (Khan et al., 1998a) and where we have also observed a strong immunolabeling of sG_{i2} protein (Khan and Gutierrez, 2004). Immunoaffinity co-elution experiments similar to those described above confirmed the existence of a D_{2S}-sG_{i2} protein complex in this tissue (Fig. 5).

Furthermore, we performed deletion experiments with cDNA of sG_{i2} to dissect the site involved in their intracellular interaction. As indicated in Fig. 6A, deletion constructs of sG_{i2} were co-expressed with D_{2S} receptor in BHK cells and D_2 receptor density at cell surface was determined by whole-cell binding assays. Truncated protein constructs lacking 108 bp of the extreme 3′-terminal end (sG_{i2}-N1, sG_{i2}-N2 and sG_{i2}-C2) lost the capability to interact (Fig. 6B); in contrast to sG_{i2} and its deletion construct (sG_{i2}-C1) that both contain this extreme

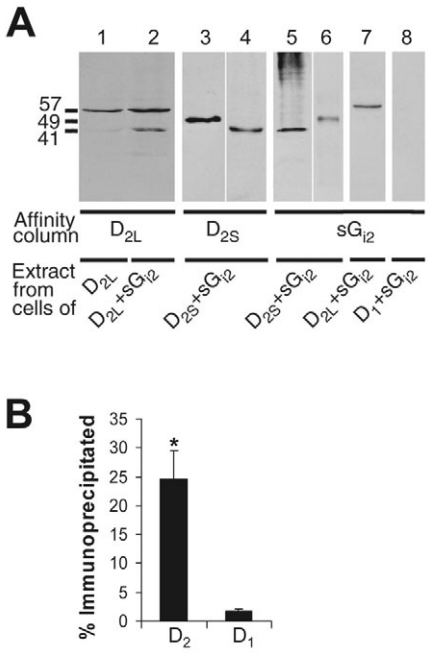

Fig. 4. Co-immunoelution of sG_{i2} protein with D_{2S} and D_{2L} receptors. (A) BHK cell extracts were passed through immunoaffinity columns and resultant proteins were identified on immunoblots with a mixture of antibodies against D_{2L} and sG_{i2} (lanes 1, 2) and antibodies to D_{2S} (lanes 3, 6), sG_{i2} (lanes 4, 5), D_{2L} (lane 7) and D_1 (lane 8). sG_{i2} protein (41 kDa) co-eluted with both D_{2L} (57 kDa) and D_{2S} (49 kDa) antibodies and vice-versa. D_1 receptor was not co-eluted with sG_{i2}. (B) sG_{i2} antibodies co-immunoprecipitated D_2 (^{3}H-raclopride) but not D_1 (^{3}H-SCH 23390) receptor binding sites from extracts of BHK cells expressing sG_{i2} and D_{2S} or D_1. Values are representative of four different experiments.

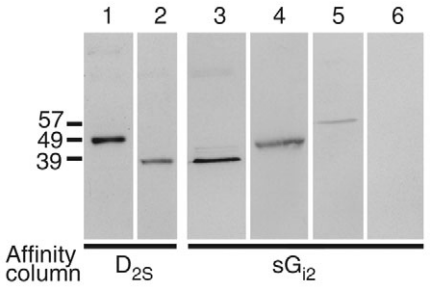

Fig. 5. Co-immunoelution of sG_{i2} protein with D_{2S} and D_{2L} receptor from the extract of monkey brain. Similar to Fig. 4, extract from the substantia nigra was passed through immunoaffinity columns as indicated. Eluted proteins were then identified in immunoblots with antibodies raised against D_{2S} (lanes 1, 4), sG_{i2} (lanes 2, 3), D_{2L} (lane 5), D_1 (lane 6). sGi2 antibody co-eluted D_{2S} and D_{2L} receptors but not D_1 receptor.

3′-terminal, which retained this activity. These results suggest that the 36 amino acids C-terminal of sG_{i2} are essential for the binding with D_2 receptor and necessary to invoke the effect of complex formation.

To further test the effect of D_2 drugs on cell surface receptor density, we treated cells that expressed both D_{2S} and sG_{i2} protein with D_2-agonists for 30 minutes. Exposure with 10 μM of dopamine or 5 μM quinpirole led to an increase in the density of cell-surface-bound D_{2S} receptors (Fig. 7A). These results are in agreement with earlier reports, in which an increase in the cell-surface-bound D_2 receptor was observed after exposure to dopamine D_2 drugs (Filtz et al., 1993; Starr et al., 1995; Ng et al., 1997). Treatment of the same cells with 5 μM raclopride, a D_2 antagonist, had no effect (Fig. 7A). Next, we used the cells from the experiment described in Fig. 7A to isolate the sG_{i2}-D_2 complex with sG_{i2}-immunoaffinity columns and to determine the concentration of D_{2S} receptor bound to sG_{i2}. The combination of immunoblots and optical-density measurements showed that dopamine and quinpirole treatment reduced the amount of D_2 bound to sG_{i2}, whereas levels of D_2 in both raclopride-treated and untreated control cells was unchanged (Fig. 7B,C). The levels of sG_{i2} under these conditions were unchanged (Fig. 7B). Our findings suggest that D_2-agonist-mediated activity has freed D_2 receptors from sG_{i2}-containing intracellular complexes, and that these D_2 receptors then localized to the plasma membrane. Values obtained in the experiments using D2L or D2S were not significantly different from each other.

Discussion

Here, we have presented evidence that the sG_{i2} protein participates in the formation of an intracellular D_2 receptor pool by specific protein-protein interaction and in the regulation of the density of these receptors at the cell membrane. To our knowledge, this constitutes the first description of how the D_2 receptor pool system functions within the cell. Our results of intracellular D_{2S}- and D_{2L}-receptor sequestration by sG_{i2} protein, and their translocation to cell surface after D_2-agonist treatment explain how an increase in numbers of D_2 receptor is possible while protein synthesis is blocked. An increase in the number of D_{2L} receptors on the cell surface and a proportional decrease in the number of the same receptors in the cytoplasm were observed when D_{2L}-expressing cells were exposed to agonist (Ng et al., 1997). In rats treated with haloperidol, dopamine challenge led to upregulation of the dopamine D_2 receptor (Creese et al., 1976; Severson et al., 1984). However, the prevalence of steady-state D_2 receptor RNA was unaffected (Goss et al., 1991). It was therefore, suggested that an intracellular D_2 receptor pool is needed to upregulate D_2 during dopamine challenge. It is likely that this pool supplies receptor to plasma membrane in conditions such as those described by ‘the law of denervation’ and when normal protein synthesis is not capable to fulfill the requirement. Furthermore, the maintenance of the D_2 receptor reservoir while protein synthesis is active (Starr et al., 1995), suggests that the machinery synthesizing dopamine D_2 receptor is not a substitute for the D_2 receptor pool. However, it remains to be explored whether, after synthesis, dopamine D_2 receptors are first localized to the intracellular reservoir before being translocated to the cell surface, or whether they are transported directly to the plasma membrane when the reservoir is saturated. It is also reasonable to argue that this sG_{i2}-driven reservoir might not only function as a stock room for D_2 receptor but might also control the amount of D_2-mediated signal to be transmitted inside the cell by regulating the cell surface density of this receptor.

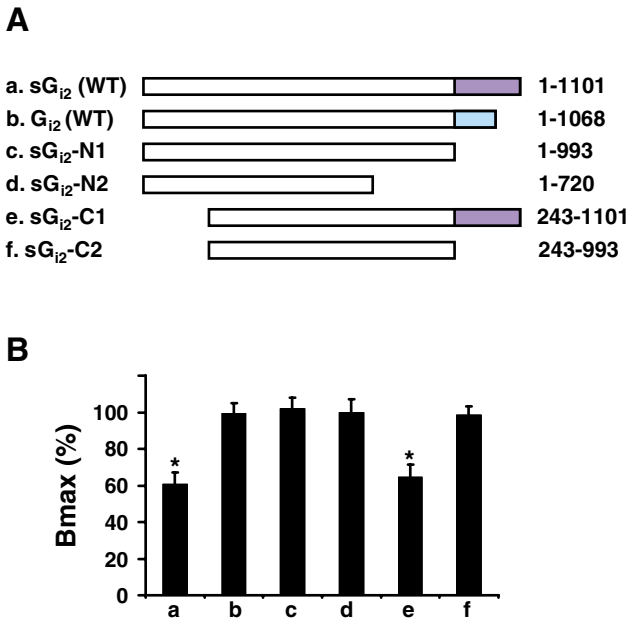

Fig. 6. The extreme C-terminal end of sG_{i2} is essential for the intracellular interaction. (A) For deletion experiments, sG_{i2} constructs were prepared by eliminating the part of the complete cDNA sequence with the use of specific PCR primers and then cloned. Each bar reflects the approximate size and location of each construct included in the study. Numbers on the right indicate start and end of the cDNA sequence. sG_{i2} (WT) and $G\alpha_{i2}$ (WT) are complete genes and rest (c-f) are the constructs derived from sG_{i2} (WT). Shaded areas show the sequences that differ between sG_{i2} and $G\alpha_{i2}$. $G\alpha_{i2}$ was included in the study as control. (B) Each of the constructs or WT (a-f) from (A) was co-expressed with D_{2S} receptor in BHK cells and the receptor density at cell surface was assayed in intact cells. The protein expression of these constructs was confirmed by western blots (see supplementary material Fig. S5).The 108 base pairs on the extreme 3′-end were necessary for the intracellular interaction and retention of the receptor. Values are representative of five different experiments.

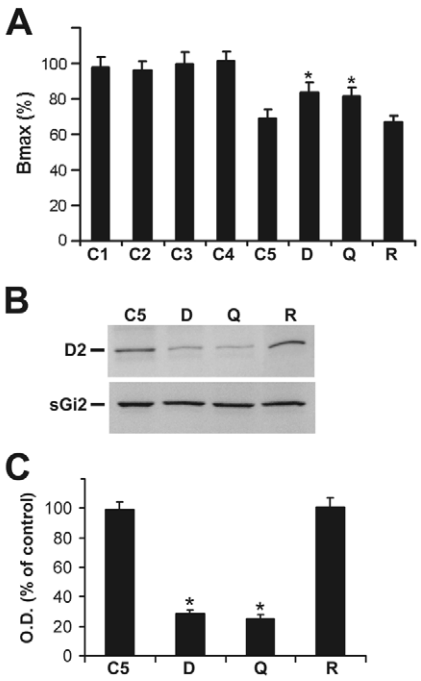

Fig. 7. Treatment with dopamine agonists increases the D_2 receptor density at plasma membrane and decreases the D_2 level from sG_{i2}-D_2 complex. (A) Incubation of BHK cells expressing D_{2S} and sG_{i2} protein with 10 μM dopamine (D) or 5 μM quinpirole (Q) for 30 minutes showed a significant increase (18%) in D_2 receptor localization at plasma membrane. By contrast, however, treatment with antagonist [raclopride (R), 5 μM] produced no effect. Controls are from C1 to C5 (C1, cells expressing D_{2S} receptors alone and treated with dopamine; C2, cells treated with quinpirole; C3, cells expressing $G\alpha_{i2}$ as well as D_{2S} receptor and treated with dopamine; C4, cells treated with quinpirole; C5, cells expressing sG_{i2} and D_{2S} receptor but left untreated). (B) Extracts of same cells as used in A were passed through sG_{i2} immunoaffinity columns and eluted to determine sG_{i2}-bound D_{2S} receptor in above conditions. Immunoblot assays show that agonist treatment resulted in loss of ~75% (equals to 22% of the binding results) D_{2S} component (49 kDa) from sG_{i2}-D_{2S} complex, whereas the level of sG_{i2} was the same in all conditions. (C) Average OD change in immunoblot experiments from B. Results show that only 28.6±2.48% and 25±2.78% of the D_{2S} receptor remained bound in the sG_{i2}-protein complex eluted from dopamine (D)-treated and quinpirole (Q)-treated cells, respectively. Values are representative of five different experiments.

The presence of sG_{i2}-D_2 receptor complex in brain tissues suggests their physiological importance in brain function. Apart from expression in neuronal intracellular compartments (Khan and Gutierrez, 2004), sG_{i2} proteins were also found in abundance in axons and spines, where the most nota observation was its frequent localization to or near to the neck of spines (70% of 41 labeled spines observed) (see supplementary material Fig. S4). In addition, these proteins are often localized not at, but in close proximity to, the synapse. Given the binding capability of sG_{i2} with D_2 receptor, this strategic extra-synaptic presence of sG_{i2} proteins suggests that these proteins bind D_2 receptors and prevent their localization at the synapse and, as a result, this binding can interrupt the full participation of D_2 receptors in synaptic neurotransmission events. Therefore, this mechanism might fine-tune the D_2-mediated synaptic transmission, depending on the requirement of local circuits. Although we have confirmed the non-binding to other dopamine, glycine and $GABA_A$ receptors, it remains to be determined whether sG_{i2} protein binds to other receptors and synaptic proteins to participate in similar processes.

The C-terminal of the $G\alpha_{i2}$ protein is known to interact with the dopamine D_2 receptor in order to transmit signals at the plasma membrane (Boundy et al., 1993; Damaj et al., 1996; Senogles et al., 2004); the third cytoplasmic loop of dopamine D_2 receptor was found to be crucial for this G_i protein interaction (Malek et al., 1993). Though, in sG_{i2}, this terminal end is replaced, our results using deletion constructs demonstrate that 36 amino acids of the C-terminal end are essential for the intracellular interaction with the D_2 receptor. In contrast to sG_{i2}, proteins that interact with the third intracellular loop of the dopamine D_2 receptor, such as spinophilin (Smith et al., 1999), filamin A (Li et al., 2000; Lin et al., 2001) and heart fatty-acid-binding protein (Takeuchi and Fukunaga, 2003), have also been identified. In addition, the

dimerization of D_2 receptors through interaction has also been shown (Lee et al., 2003). Thus, these evidences point to the ability of dopamine D_2 receptor to participate in protein-protein interaction with various cellular proteins and not only with sG_{i2} protein, as reported here.

In the central nervous system, cell surface dopamine D_2 receptors are the major target of all effective antipsychotic drugs. Their interaction is considered to be the key event associated with improvements in patients (Kapur and Remington, 2001; Seeman and Kapur, 2000) and also in the generation of extrapyramidal side effects (Strange, 2001). Usually, antipsychotic drugs block dopamine D_2 receptor signalling; therefore, intracellular sequestration of D_2 receptor may offer an alternative in reducing the D_2-mediated signaling without blocking its function. The advantage of this approach is that intracellular sequestration of D_2 receptor might reduce inhibitory components of D_2 signaling, one of the main causes for side effects in patients using antipsychotics.

In conclusion, our results demonstrate that the sGi_2 protein and the dopamine D_2 receptor form intracellular complexes that serve as of D_2 receptor reservoir. Treatment with D_2-specific drugs break down this protein complex and free D2 receptor can translocate to cell surface. We postulate that, in contrast to a long-term strategy where protein synthesis is essential, this mechanism is a short-term cellular strategy to cope with the demand for D2 receptor while the protein synthesis machinery is unable to respond.

Materials and Methods

cDNA

cDNA clone of $G\alpha_{i2}$ (GenBank accession number, M17528) and $G\alpha_{i3}$ (GenBank accession number M20713) was provided by Randall R. Reed (Johns Hopkins University, Baltimore, MD). Human D_1, D_{2S} and D_{2L} cDNA clones were from Olivier Civelli (University of California, Irvine, CA). The full-length cDNA of sG_{i2} was obtained from human brain poly A^+ RNA (Clontech) and was submitted to GenBank at the accession number AY677118.

Cloning

Genes were amplified by PCR using primers containing *Mlu*I and *Apa*I or *Mlu*I and *Xba*I restriction sites at their 5′ and 3′ ends, respectively. For the deletion constructs, PCR primers were designed to amplify the sG_{i2} DNA sequences containing restriction sites like those described above. The digestions were performed by incubating 3 μg of DNA with 40 units of *Mlu*I and *Apa*I or *Mlu*I and *Xba*I at 37° C for 4 hours. Digested DNA was separated on 1.4% agarose gels and recovered with Gel Extraction Kit (Qiagen). Digestion of pSinRep5 plasmid vector (Sindbis Expression System from Invitrogen) was also performed as above and was used for ligation. Gene DNA (0.5 μg) was added to 0.5 μg of digested plasmid DNA, ligated in the presence of 2 Weiss units of bacteriophage T4 DNA ligase and incubated for 1 hour at room temperature. The ligation mixture was directly transformed into a competent TOP10 One Shot cells (Invitrogen). Colonies were selected on LB agar plates containing 100 μg/ml ampicillin. After isolation of plasmid DNA with Wizard Plus Minipreps DNA Purification System (Promega) from several colonies, they were analyzed for the presence of gene by restriction digestion with *Mlu*I and *Apa*I and then by PCR using a combination of primers from both plasmid and gene. Usually, two to three colonies that showed correct size gene insert were then processed for large-quantity DNA isolation using Wizard Maxi-Plasmid Preparation System (Promega). The isolated DNA was quantified, aliquoted and stored at –20° C. These recombinant samples, including deletion constructs, were sequenced to confirm the DNA sequence.

In vitro transcription, transfection and preparation of pseudovirions

The above described recombinant genes were used as template to produce recombinant RNA with the InvitroScript Cap SP6 in vitro Transcription Kit (Invitrogen). Briefly, 5 μg of recombinant DNA was linearized with 50 units of *Not*I. The digest was extracted once with phenol-chloroform and 0.1 volumes of 5 M ammonium acetate and 2 volumes of ethanol were added and the mix was incubated at –20°C for 1 hour. After centrifugation, DNA pellet was suspended in RNase-free water to 0.5 μg/μl. The in vitro transcription reaction was set up at room temperature by mixing 1 μg of the linearized recombinant DNA with the SP6 transcription reagents as indicated by the Invitrogen protocol. The reaction was mixed gently and incubated for 2 hours at 37°C. A typical reaction yielded 10-20 μg of RNA. The RNA product was purified with phenol-chloroform extraction, quantified with spectrophotometer, aliquoted into 10 μl samples and stored at –80°C.

2×10^5 baby hamster kidney (BHK) cells were seeded into six-well culture plates in 2 ml of growth medium and incubated at 37° C in 5% CO_2 for 12-18 hours until 80% confluency. Cells of each well were then washed with 2 ml OPTI-MEM I reduced-serum medium at room temperature. For liposome-mediated transfection, DMRIE-C reagent from Gibco was used. RNA-lipid complexes were prepared by adding 9 μl of liposome reagent (DMRIE-C), 9 μg of recombinant RNA and 9 μg of helper RNA to 1 ml of OPTI-MEM I in polystyrene tubes and were mixed briefly by vortexing. The lipid-RNA complexes were immediately added to the washed BHK cells and incubated for 4 hours at 37°C. Following the incubation, transfection medium was replaced with complete growth medium containing αMEM medium supplemented with 2 mM L-glutamine and 5% fetal bovine serum and the cells were incubated for an additional 36 hours. During this period, recombinant RNA are packaged into pseudovirion particles and then released into the medium. The medium from the cells was collected, aliquoted into 1 ml samples and stored at –80°C.

Infection of cells with recombinant pseudovirions

Cell lines used in this study were obtained from the American Type Culture Collection and they were cultured at 37°C in a 5% CO_2 atmosphere. BHK cells were grown in αMEM medium supplemented with 2 mM L-glutamine and 5% fetal bovine serum. JEG-3 human carcinoma cells were cultured in Eagle's MEM with 2 mM L-glutamine and Earle's BSS containing 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. NG108-15 neuroblastoma/glioma cells were grown in DMEM with 4 mM L-glutamine without sodium pyruvate and modified to contain 4.5 g/l glucose, 1.5 g/l sodium bicarbonate, 0.005 mM pyridoxin-HCl, HAT supplement, and 10% fetal bovine serum. Cells for infection were grown to approximately 70-80% confluency in 60-mm tissue culture plates, and pseudovirions (0-30 μl) diluted to 450 μl was added to each well. After incubation at room temperature for 1 hour, 4 ml of medium were added and cells were incubated for 30-34 hours for expression of functional protein. The optimal amount of pseudovirions needed for maximal protein expression was determined by making serial dilutions of the stock.

Whole-cell binding assays

After infection with recombinant pseudovirions and protein expression, intact cells were harvested, counted and processed for the binding assays. As described previously (Khan et al., 1998b; Khan et al., 2001), the binding of [^{3}H]sulpiride (NEN-PerkinElmer) to 10^5 cells was done by incubation with 0-1 nM of the radioligand for 1 hour at 24°C in a total volume of 0.5 ml. The reaction was terminated by rapid filtration through glass filters and counted for the retained radioactivity. This value was considered as total binding. Non-specific binding was determined with 1 μM of (+)-butaclamol-HCl or fluphenazine (RBI-Sigma). Specific binding was calculated by subtracting non-specific binding from total binding. B_{max} and K_d values were calculated with Prism program (GraphPad Software). Data are presented as the mean ± s.d. from six to eight independent experiments.

To control the amount of receptor expression, cells were homogenized in parallel experiments and used for receptor binding, similar as described for whole-cells receptor binding, to determine the total number of receptors in each condition. Variations in the total number of receptor within the same experiment and also between experiments were below 3%. Furthermore, the amount of pseudovirions and number of cells used in experiments were kept constant throughout the study.

Antibodies

A peptide corresponding to sG_{i2} protein residues 343-354, LSGPDQHPHPSP (GenBank accession number AY677118), was synthesized and coupled to keyhole limpet hemocyanin (KLH). Peptide conjugation and rabbit immunizations were performed as described previously (Khan and Gutierrez, 2004; Khan et al., 1998a; Khan et al., 1998b). Development of immune response was monitored by ELISA using immobilized synthetic peptides. Antibodies against sG_{i2} were affinity-purified on the corresponding immobilized peptide as described in detail elsewhere (Khan et al., 1993). Briefly, peptide (5 mg) was coupled to 1 g of activated thiopropyl-Sepharose 6B (Pharmacia LKB). One milliliter of antiserum diluted fivefold in 10 mM phosphate-buffered saline (PBS) (10 mM Na_2PO_4, 0.14 M NaCl, 0.01 M KCl, pH 7.4) was circulated through the peptide column. After washing, the antibody was eluted with 50 mM glycine-HCl pH 2.3, and collected in 1-ml fractions. OD_{280} was determined for each fraction and fractions containing antibodies were pooled and dialyzed in PBS. Antibodies were stored as 50-μl aliquots at –20°C. Specificity of affinity-purified antibody was then determined (Khan and Gutierrez, 2004) (supplementary material Fig. S1). The isoform-specific D_{2S} and D_{2L} antibodies had been prepared earlier by us and their specificity have already been demonstrated (Khan et al., 1998a; Khan et al., 2001).

Immunoblots

Immunoblots were done as described previously (Khan et al., 1998a; Khan et al., 1993). Solubilization of proteins from harvested intact cells was done with solubilization buffer provided in Seize X Mammalian Immunoprecipitation kit (Pierce). These solubilized proteins were separated by 10% SDS-PAGE and transblotted to nitrocellulose membranes. Membranes containing proteins were incubated with 5 μg/ml antibodies to sG_{i2}, D_{2S} or D_{2L}, followed by incubation with anti-rabbit IgG-HRP (1:2000; Amersham). Bands were visualized using an ECL kit (Amersham).

Calculation of percentile in immunoblots

The concentration of bands in blot experiment (Fig. 7B) was obtained by OD measurements (Fig. 7C). These results suggest that approximately 75% of D_2 receptor was uncoupled from the D2-sGi2 complex after drug treatment. This calculation is based on the assumption that D_2 receptor population bound to sGi2-D2 complex is 100% (Fig. 7B, C5) under normal conditions when D_2 receptor and sGi2 are co-expressed. However, when comparing data of blots with binding experiments, this 100% of D_2 receptor population of the D2-sGi2 complex represent approximately 30% in binding experiments (see Fig. 1). Therefore, normalizing the results of both blots and binding experiments to the same level (30%), the 75% value of blots comes down to 22%. A slightly lower value in the binding experiments might reflect the population of unbound receptor still in transit.

Fluorescence immunocytochemistry

After infection with recombinant pseudovirions and after protein expression, cells grown on Flask-style glass slides (Nunc) were fixed with 4% paraformaldehyde and 0.2% glutaraldehyde for 10 minutes and permeabilized with 0.3% Triton X-100. Immunofluorescence staining of cells was performed as described earlier (Khan et al., 1998a; Khan et al., 1998b; Khan et al., 2001; Khan and Gutierrez, 2004; Lopez-Aranda et al., 2006). Briefly, after incubation with sG_{i2} antibody (1:500), cells were incubated with FITC (green) coupled to anti-rabbit Fab2 fragment (1:100; Jackson), followed by incubation with D_{2S} antibody (1:200) and Cy3-conjugated secondary antibody(red) (1:200; Jackson). Images were taken with Zeiss confocal microscope.

Ca^{2+} imaging

Cells infected with recombinant pseudovirions were grown on glass coverslips and incubated with 2 μM fluo-4-AM (fluo-4 acetoxymethyl ester from Molecular Probes) for 15-30 minutes. The fluorescence change in cells after application of 15 μM raclopride was measured with a Zeiss LSM 410 confocal laser scanning microscope system as described previously (Koulen et al., 1999). Images were acquired every 500 mseconds. Changes in fluorescence intensity were calculated by dividing the fluorescence intensity during drug application (F) by the average baseline fluorescence intensity (F_0). Non-stimulus-related spontaneous changes in fluorescence were 1-3%. Data are presented as the mean ± s.d. of four independent experiments.

Immunoaffinity elution

Protein solubilization, antibody immobilized affinity column preparation and protein elution was performed as described in Seize X Mammalian Immunoprecipitation Kit (Pierce). In brief, 0.8 ml of gel-immobilized protein G was washed with PBS buffer (10 mM Na_2PO_4, 0.14 M NaCl, 0.01 M KCl, pH 7.4) by centrifugation and incubated with 2 mg of affinity-purified antibodies for 1 hour. The mixture was then transferred to spin cups and centrifuged. Flow-through was collected to determine the amount of antibody bound to resin. Approximately 80-90% of antibodies were bound; 1.3 mg of DSS crosslinker in DMSO was added to the resin and gently mixed by inversion for 1 hour. Resin was washed with Tris buffer (25 mM Tris, 0.15 M NaCl, pH 7.2) in spin cups and stored in 1 ml of PBS buffer containing 0.01% sodium azide.

After infection with recombinant pseudovirions, whole cells (2×10^6) were harvested and lysed with 2 ml of M-PER Mammalian Protein Extraction reagent (Pierce) for 10 minutes. After removing cell debris by centrifugation, clear supernatant was diluted 1:1 with PBS buffer and added to spin columns with resin bound to antibody. The samples were incubated for 2 hour at 4°C and eluted with 400 μl of elution buffer (pH 2.8). Immunoaffinity eluted proteins were then analyzed by immunoblots.

For the brain tissues, membrane was prepared as described earlier (Khan et al., 1998a; Khan et al., 1998b; Khan et al., 2001; Khan et al., 1993). The prepared membrane was then used for the protein solubilization, binding with affinity-column and elution as explained above.

Co-immunoprecipitation

Cells were infected with recombinant pseudovirions, harvested and their proteins were solubilized with 1% digitonin (Khan et al., 1998a; Khan et al., 1998b). After centrifugation, the supernatant was used for incubation with 20 μl of affinity-purified sG_{i2} antibody. The protein-antibody complexes were separated by incubation with 80 μl of proteinA-agarose (Sigma) followed by centrifugation. The non-immunoprecipitated supernatant was used for the binding assay using D_2-specific ([^{3}H]raclopride) and D_1-specific ([^{3}H]SCH 23390) radioligands as described elsewhere (Khan et al., 1993; Khan et al., 1998a; Khan et al., 1998b; Khan et al., 2001). For the binding assay, supernatant was incubated with 1 nM radioligand in total of 0.5 ml. Reaction was terminated by rapid filtration and retained radioactivity was counted as described above in detail in whole-cell binding assays. The amount of co-immunoprecipitated receptors was calculated by subtracting the binding values of supernatant from the total (100%) binding of radioligands. Incubation without addition of antiserum represented 100% binding.

Calculation of immunoprecipitation values

For calculation and deduction of immunoprecipitation values as in Fig. 4, proteins extract of cells was incubated with pre-immune serum or antiserum against sGi2 protein after termination of the experiment. The immunocomplex (sGi2-D2 complex) was precipitated using proteinA-agarose. The supernatant portion of this reaction was used for binding assays and cpm values were obtained after counting in scintillation counter. A value of 100% was assigned to the cpm values obtained from the extract treated with preimmune serum. The immunoprecipitation values were calculated by subtracting the values of supernatant originated from extract treated with antiserum to the 100% value, meaning amount of supernatant derived from extract treated with preimmune serum minus amount of supernatant derived from extract treated with antiserum equal the amount of immunoprecipitated receptor.

Drugs treatment

Following infection and protein expression, harvested intact cells were incubated with agonists (10 μM dopamine and 5 μM quinpirole, both from Sigma/RBI) and antagonist (5 μM raclopride from Sigma/RBI) for 30 minutes. After washing, cells were processed for whole-cell binding assays and immunoblots as described above.

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