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ORIGINAL ARTICLE

Role of $G\alpha_{12}$- and $G\alpha_{13}$-protein subunit linkage of D_3 dopamine receptors in the natriuretic effect of D_3 dopamine receptor in kidney

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The D_3 dopamine receptor is the major D_2-like receptor that regulates sodium transport in the renal proximal tubule (RPT) and helps maintain blood pressure in the normal range. In Wistar–Kyoto (WKY) rats chronically fed high-salt diet, the intrarenal arterial infusion of a D_3 receptor agonist, PD128907, increased absolute and fractional sodium excretion. We have reported that $G\alpha_{12}$ and $G\alpha_{13}$, which participate in the signal transduction of the D_5 receptor, are expressed in RPTs. As the D_3 receptor is also expressed in RPTs, we hypothesized that it may also interact with $G\alpha_{12}/G\alpha_{13}$ in RPTs from WKY rats. There were co-localization and co-immunoprecipitation of D_3 receptor and $G\alpha_{12}/G\alpha_{13}$ in renal brush border membranes (BBMs) and RPT cells. The intrarenal infusion of PD128907 ($1\,\mu g\,kg^{-1}\,min^{-1}$) that increased sodium excretion also increased the co-immunoprecipitations of $D_3/G\alpha_{12}$ and $D_3/G\alpha_{13}$ in renal BBMs; their co-immunoprecipitation was confirmed in RPT cells. As $G\alpha_{12}$ and $G\alpha_{13}$ increase sodium pump and transporter activity (for example, Na^+–K^+–ATPase, NHE3), an increased association of D_3 receptors with $G\alpha_{12}/G\alpha_{13}$ receptors after D_3 receptor activation may be a mechanism to prevent $G\alpha_{12}/G\alpha_{13}$-mediated stimulation of sodium transport (and thus enhance natriuresis). We conclude that a D_3 receptor interaction with $G\alpha_{12}/G\alpha_{13}$ that increases sodium excretion may have a role in the regulation of blood pressure.
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Keywords: dopamine; G proteins; kidney; natriuresis; receptors

INTRODUCTION

Dopamine produced in neural and non-neural tissues is now recognized to serve an important role in the regulation of blood pressure and sodium balance by direct actions on renal and intestinal epithelial ion transport, by interaction with other receptors, by modulation of the secretion of hormonal/humoral agents, such as aldosterone, catecholamines, renin and vasopressin, and by actions on brain appetite centers.[1–3] Dopamine receptors are classified into D_1- (D_1, D_5) and D_2-like (D_2, D_3 and D_4) subtypes based on their structure and pharmacology. Under euvolemic conditions or volume expansion, dopamine, via D_1-like and D_3 receptors, acts to increase sodium excretion and decrease blood pressure.[1–3]

The effects of dopamine are exerted by cell surface receptors that belong to the rhodopsin-like or class A family of membrane receptors. These receptors, characterized by seven membrane-spanning domains, are called G protein-coupled receptors because of their interaction with heterotrimeric G proteins, composed of α, β and γ subunits.[1–5] There are more than 20 Gα-subunits, grouped into four subfamilies ($G\alpha_S$, Gαi, Gαq and Gα12). In mammals, the two D_1-like dopamine receptors, D_1 and D_5, are coupled to the stimulatory Gα subunit ($G\alpha_S$) and Gαq,[6] whereas the three D_2-like receptors, D_2, D_3 and D_4, are coupled to the inhibitory Gα subunit, $G\alpha_i$. $G\alpha_S$ is stimulatory, whereas Gαi is inhibitory of adenylyl cyclase activity.[1–3] However, D_3 receptor linkage to $G\alpha_i$ is not robust, in contrast to that observed for the D_2 and D_4 receptors.[7] In some instances, the D_3 can be linked to $G\alpha_S$, $G\alpha_o$ and β/γ from $G\alpha_i$. Our previous study showed that $G\alpha_{12}$ and $G\alpha_{13}$, members of the fourth family of G protein subunits, are not linked to D_1 receptors, but are linked to D_5 receptors.[8] As with the D_3 receptor,[9–11] $G\alpha_{12}$ and $G\alpha_{13}$ are expressed in the kidney, especially in the renal proximal tubules (RPTs).[8] However, it is not known whether or not $G\alpha_{12}$ and/or $G\alpha_{13}$ are also involved in the mechanisms by which the D_3 receptor promotes sodium excretion. Therefore, we studied the effect of the D_3 receptor on sodium excretion in normotensive Wistar–Kyoto (WKY) rats, and investigated the effect of the D_3 receptor on the linkage between D_3 receptor and the members of the fourth family of G protein subunits ($G\alpha_{12}$ and $G\alpha_{13}$) in kidney and RPT cells from WKY rats.

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METHODS

Blood pressure and renal function studies in rats

Nine- to 16-week-old WKY rats (n=6) (Taconic Farms, Germantown, NY, USA) were maintained on rat chow (6% NaCl) until one day before the experiment; water was given *ad libitum*. The rats were anesthetized with pentobarbital (50 mg per kg body wt i.p.), placed on a heated board to maintain their body temperature at about 37 °C and then tracheotomized. Anesthesia was maintained by infusion of pentobarbital at 0.8 mg per 100 g body wt per hour. Catheters (PE-50) were placed into the external jugular and femoral veins and femoral artery. Systemic arterial pressure was monitored electronically (Cardiomax II, Columbus Instruments, Columbus, OH, USA). Laparotomy was performed and both the right and left ureters were catheterized (PE-10). The right renal artery was exposed; the right suprarenal artery, which originates from the right renal artery, was catheterized (PE-10 heat stretched to 180 μm); and the vehicle (saline) or PD128907 ($1\,\mu g\,kg^{-1}\,min^{-1}$)[12] was infused at a rate of $40\,\mu l\,h^{-1}$.[13] The duration of the surgical procedures was about 60 min. Fluid losses during surgery were replaced with 5% albumin at 1% body weight over 30 min. Glomerular filtration rate was determined by the clearance of [^{14}C]-inulin (NEN, Boston, MA, USA) in normal saline infused at 5 ml per 100 g body wt for 30 min, followed by a rate of 0.8 ml per 100 g body wt per h until the end of the experiment, as previously reported.[10] After an equilibration period of 120 min, urine was collected every 40 min for clearance measurements.[13]

Preparation of renal brush border membranes (BBMs)

Kidneys were obtained from WKY rats. Renal BBMs were prepared by $MnCl_2$ precipitation and differential centrifugation and studied under approved protocols with institutional guidelines.[10] The BBMs have no immunoblottable sodium-hydrogen exchanger 1 (NHE1) and Na^+–K^+–ATPase (markers for basolateral membranes), but express immunoreactive NHE3, γ-glutamyl transpeptidase and alkaline phosphatase (markers for BBMs), indicating minimal contamination with basolateral membranes.[14,15] Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

Cell culture

Immortalized RPT cells from 4- to 8-week-old WKY rats were cultured at 37 °C in 95% air/5% CO_2 atmosphere in DMEM/F-12.[13,16] The cells (80% confluence) were extracted in ice-cold lysis buffer, sonicated, kept on ice for 1 h and centrifuged at 16 000 *g* for 30 min. All samples were stored at −70 °C until use.

Co-localization of D_3 receptor and $G\alpha_{12}$, $G\alpha_{13}$ in RPT cells

RPT cells grown on coverslips were treated with PD128907 (10 nM) for 15 min, fixed with 4% paraformaldehyde, permeabilized with 0.05% Triton X-100 in PBS and double immunostained as follows: D_3 receptor was probed using a polyclonal rabbit anti-D_3 receptor antibody (1:200; Abcam, Cambridgeshire, UK) followed by Alexa Fluor 488-donkey anti-rabbit IgG antibody (Molecular Probes, Eugene, OR, USA), while the $G\alpha_{12}$ or $G\alpha_{13}$ was visualized using an IgG affinity-purified goat anti-$G\alpha_{12}$ or anti-$G\alpha_{13}$ antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by Alexa Fluor 555-donkey anti-goat IgG antibody (Molecular Probes). The cover slips were mounted on microscope slides using Fluoro-Gel mounting medium (Electron Microscopy Sciences, Hatfield, PA, USA). Confocal and differential interference contrast images were obtained using Carl Zeiss LSM 510 META with an ×63/1.4 NA oil immersion objective and processed using Zeiss 510 META with Physiology Software ver. 3.5 and Multiple Time Series Software ver. 3.5 (Carl Zeiss International, Dublin, CA, USA).

Immunoprecipitation studies

BBMs or RPT cells were lysed with lysis buffer (50 mM Tris-Cl, pH, 7.4, 1% NP-40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, $10\,\mu g\,ml^{-1}$ aprotinin and $10\,\mu g\,ml^{-1}$ leupeptin) on ice for about 1 h, and centrifuged at 16 000 *g* for 30 min. The lysates (supernatant, 300 μg protein ml^{-1}) were then incubated with affinity-purified anti-D_3 receptor antibodies ($1\,\mu g\,ml^{-1}$) at 4 °C for 1 h and protein-G agarose at 4 °C for 2 h. The immunoprecipitates were pelleted and washed four times with lysis buffer. After the sample buffer was added, the samples were boiled for 10 min and subjected to immunoblotting with the $G\alpha_{12}$ or $G\alpha_{13}$ antibody. To determine the specificity of the bands found on the immunoblots, IgG (negative control) and $G\alpha_{12}$ or $G\alpha_{13}$ antibodies (positive control) were used as the immunoprecipitants instead of the D_3 receptor antibodies (data not shown).[8,13]

Na^+–K^+–ATPase activity assay

Rat RPT cells were treated with vehicle (dH_2O), or a D_3 receptor agonist (PD128907, Sigma, St Louis, MO, USA), at the indicated concentrations and durations of incubation. Na^+–K^+–ATPase activity was determined as the rate of inorganic phosphate released in the presence or absence of ouabain.[17] To prepare membranes for Na^+–K^+–ATPase activity assay, RPT cells cultured in 21 cm^2 plastic culture dishes were collected and centrifuged at 3000 *g* for 10 min. The cells were then placed on ice and lysed in 2 ml of lysis buffer (1 mM $NaHCO_3$, 2 mM $CaCl_2$ and 5 mM $MgCl_2$). Cellular lysates were centrifuged at 3000 *g* for 2 min to remove intact cells, debris and nuclei. The resulting supernatant was suspended in an equal volume of 1 M sodium iodide, and the mixture was centrifuged at 48 000 *g* for 25 min. The pellet (membrane fraction) was washed twice and then suspended in 10 mM Tris containing 1 mM EDTA (pH 7.4). Protein concentrations were determined by the Bradford assay (Bio-Rad Laboratories) and adjusted to $1\,mg\,ml^{-1}$. The membranes were stored at −70 °C until further use. To measure Na^+–K^+–ATPase activity, 100 μl aliquots of membrane fraction were added to an 800 μl reaction mixture (75 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 6 mM sodium azide, 1 mM Na_4EGTA, 37.5 mM imidazole, 75 mM Tris-HCl and 30 mM histidine; pH 7.4) with or without 1 mM ouabain (final volume=1 ml) and pre-incubated for 5 min in a water bath at 37 °C. Reactions were initiated by adding Tris-ATP (4 mM) and terminated after 15 min of incubation at 37 °C by adding 50 μl of 50% trichloroacetate. For determination of ouabain-insensitive ATPase activity, NaCl and KCl were omitted from the reaction mixtures containing ouabain. To quantify the amount of phosphate produced, 1 ml of coloring reagent (10% ammonium molybdate in 10 N sulfuric acid + ferrous sulfate) was added to the reaction mixture. The mixture was then mixed thoroughly and centrifuged at 3000 *g* for 10 min. Formation of phosphomolybdate was determined spectrophotometrically at 740 nm, against a standard curve prepared from K_2HPO_4. Na^+–K^+–ATPase activity was estimated as the difference between total and ouabain-insensitive ATPase activity and expressed as percent change of control.

To eliminate the effect of proteases and phosphatases, protease inhibitors (1 mM phenylmethylsulfonyl fluoride, $10\,\mu g\,ml^{-1}$ each leupeptin and aprotinin) and a phosphatase inhibitor (50 μM sodium orthovanadate) were added in all solutions used after drug/vehicle incubations.[18]

Statistical analysis

The data are expressed as mean ± s.e.m. Comparison within groups was made by repeated-measures analysis of variance, and comparison among groups was made by factorial analysis of variance and Duncan's test; *t*-test was used when only two groups were compared. A value of $P<0.05$ was considered significant.

RESULTS

Stimulation of renal D_3 receptors increases sodium excretion in WKY rats

To determine the effect of D_3 receptors on sodium excretion, the D_3 receptor agonist PD128907 ($1.0\,\mu g\,kg^{-1}\,min^{-1}$ for four periods, each period lasting 40 min) was infused into the right renal artery in WKY rats (n=6) maintained on high sodium diet (6% NaCl), using a protocol reported previously.[13] The intrarenal arterial infusion of the vehicle into the right kidney had no effect on blood pressure, urine flow (V), fractional sodium excretion (FE_{Na}), absolute sodium excretion ($U_{Na}V$), potassium excretion (U_KV) or glomerular filtration rate (data not shown). PD128907 had no effect on blood pressure (Figure 1a), but increased glomerular filtration rate, V, $U_{Na}V$ and FE_{Na} in WKY rats (Figures 1b–e).

Stimulation of D_3 receptors inhibits Na^+–K^+–ATPase activity in RPT cells

To determine whether or not the natriuretic effect of D_3 receptor is related to inhibition of Na^+–K^+–ATPase activity, the effect of PD128907, on Na^+–K^+–ATPase activity was measured in RPT cells.

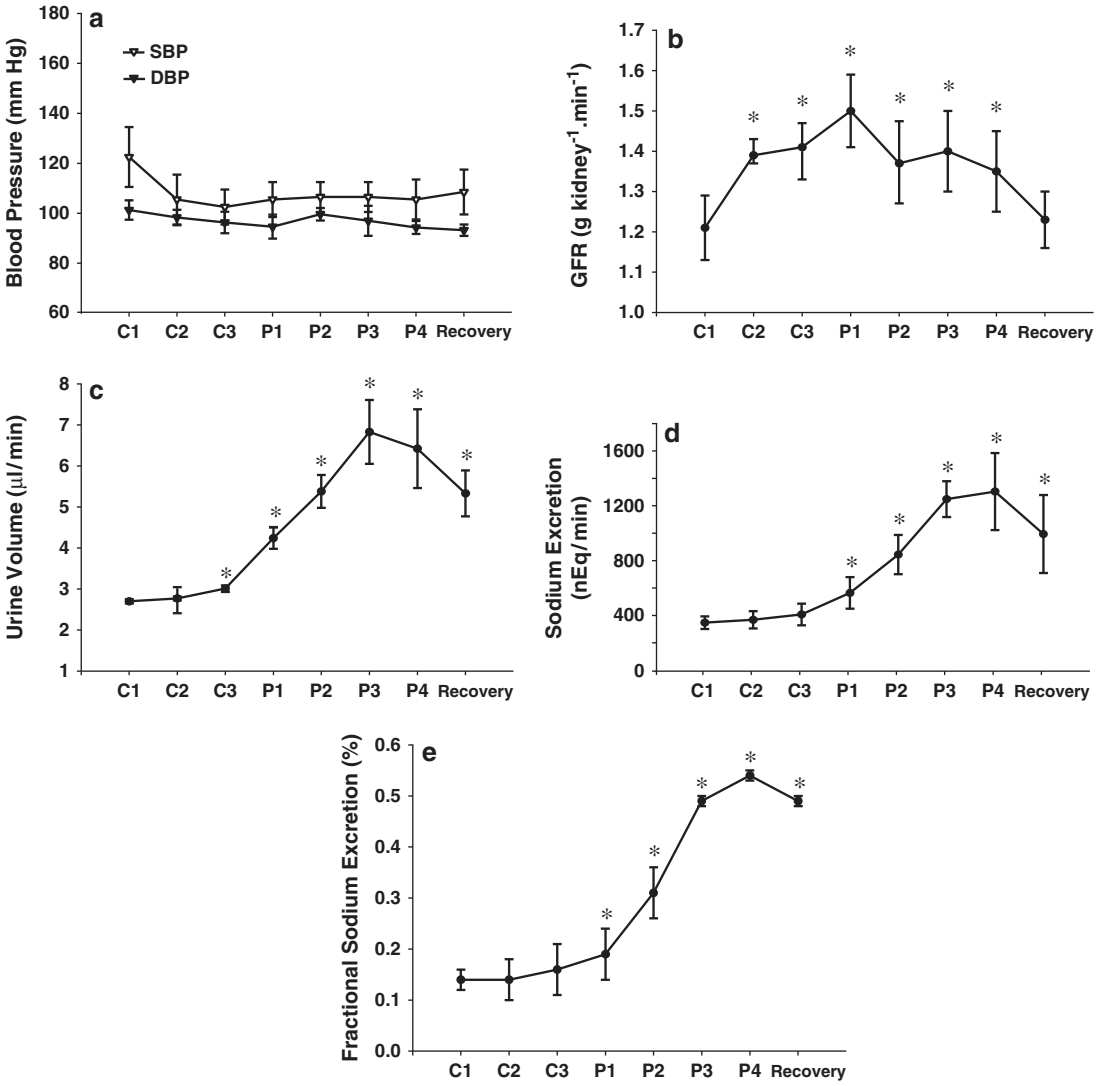

Figure 1 Effect of the intrarenal infusion of a D_3 receptor agonist (PD128907) on blood pressure and renal function in WKY rats. The D_3 receptor agonist, PD128907 ($1.0\,\mu g\,kg^{-1}\,min^{-1}$), was infused into the right renal artery of WKY rats. Blood pressure (**a**), glomerular filtration rate (**b**), urine volume (**c**), absolute sodium excretion (**d**) and fractional sodium excretion (**e**) were measured (n=6). During the control (C1–C3) and recovery periods, only the vehicle was infused; during periods 3–6 (P1–P4, respectively), PD128907 was infused. Each period lasted for 40 min. *$P<0.05$ *vs.* control, ANOVA, Duncan's test.

We found that PD128907 inhibited Na^+–K^+–ATPase activity in a concentration-dependent manner. The inhibitory effect was evident at 10^{-8} M (Figure 2).

D_3 receptor colocalizes with $G\alpha_{12}$ or $G\alpha_{13}$ in RPT cells

We next evaluated the colocalization of these proteins via laser-scanning confocal microscopy to determine the capacity of receptor and Gα subunits to interact in RPT cells. Under basal condition, both the D_3 receptor and $G\alpha_{12}$ are localized at the plasma membrane and the cytoplasm, where they partially colocalize. D_3 receptor stimulation with PD128907 promoted the endocytosis of both the receptor and $G\alpha_{12}$ and enhanced the extent of colocalization at the perinuclear area (Figure 3a). Similarly, D_3 receptor and $G\alpha_{13}$ are basally distributed and colocalized at the plasma membrane and cytoplasm. Receptor activation promoted the internalization of both proteins and markedly increased the colocalization between D_3 receptor and $G\alpha_{13}$ (Figure 3b).

Immunoprecipitation of $G\alpha_{12}$, $G\alpha_{13}$ and D_3 receptors in RPT cells

To confirm the apparent interaction between $G\alpha_{12}$ or $G\alpha_{13}$ with D_3 receptors noted in the laser confocal microscopic studies, we determined whether or not $G\alpha_{12}$ or $G\alpha_{13}$ co-immunoprecipitated with D_3 receptors in renal BBMs and RPT cells. $G\alpha_{12}$ and $G\alpha_{13}$ co-immunoprecipitated with D_3 receptors in BBMs (Figures 4a and b) and this co-immunoprecipitation was increased following D_3 receptor agonist stimulation (PD128907, $1\,\mu g\,kg^{-1}\,min^{-1}$) in WKY rats. There was negligible co-immunoprecipitation when the immunoprecipitant was IgG instead of anti-D_3 receptor antibodies (data not shown).

Consistent with the results of the *in-vivo* study, we found that in RPT cells the co-immunoprecipitation between D_3 receptor and $G\alpha_{12}$ or $G\alpha_{13}$ was increased by PD128907 (10^{-8} M/30 min) (Figures 4c and d).

DISCUSSION

In the current report, we confirmed that stimulation of D_3 receptors with the D_3 receptor selective agonist, PD128907, increases sodium

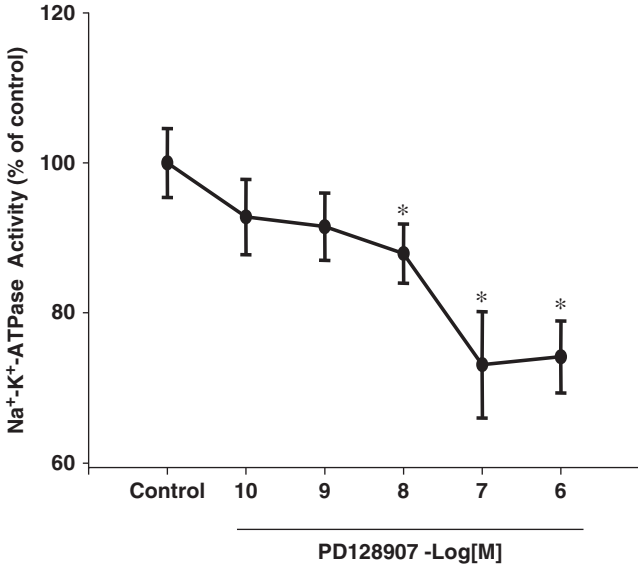

Figure 2 Effect of a D_3 receptor agonist (PD128907) on Na^+–K^+–ATPase activity in RPT cells. Concentration–response of Na^+–K^+–ATPase activity in RPT cells incubated with the D_3 receptor agonist, PD128907, for 15 min. Results are shown as % of control (n=6, $^*P<0.01$ *vs.* control, ANOVA, Duncan's test).

excretion in WKY rats. The natriuretic effect may be, in part, via Na^+–K^+–ATPase, as activation of the D_3 receptor inhibits Na^+–K^+–ATPase activity in RPT cells. We now report that there is a linkage between D_3 receptors and $G\alpha_{12}$, and D_3 receptors and $G\alpha_{13}$ in RPTs in native kidneys. We also report that the dose of PD128907 that increases sodium excretion in WKY rats increases the co-immunoprecipitation of D_3 receptors with $G\alpha_{12}$ or $G\alpha_{13}$ in renal BBMs, which was confirmed in RPT cells from WKY rats.

Dopamine receptors are classified into D_1- and D_2-like subtypes based on their structure and pharmacology.[1–3] Under euvolemic conditions or volume expansion, dopamine receptors act to increase sodium excretion and normalize blood pressure.[1–3,19] Most *in vivo* studies have shown that the natriuretic effect of dopamine is exerted via D_1-like receptors.[15,20] The effect of D_2-like receptors, independent of D_1-like receptors, on sodium excretion has ranged from anti-natriuresis, to no effect, to natriuresis. It is possible that these discrepant effects are related to the use of drugs that have poor selectivity to the D_2-like receptor subtypes. Thus, bromocriptine, a drug that has a similar affinity to the D_2 receptor and D_3 receptor, stimulates sodium transport.[21,22] In contrast, 7-OH-DPAT and PD128907, which are D_3 receptor agonists with preferential selectivity for D_3 over D_2 and D_4 receptors, decrease sodium transport and increase sodium excretion.[13,23] Acute intravenous administration of 7-OH-DPAT in Dahl salt-resistant rats increases glomerular filtration rate and sodium and water excretion without affecting blood pres-

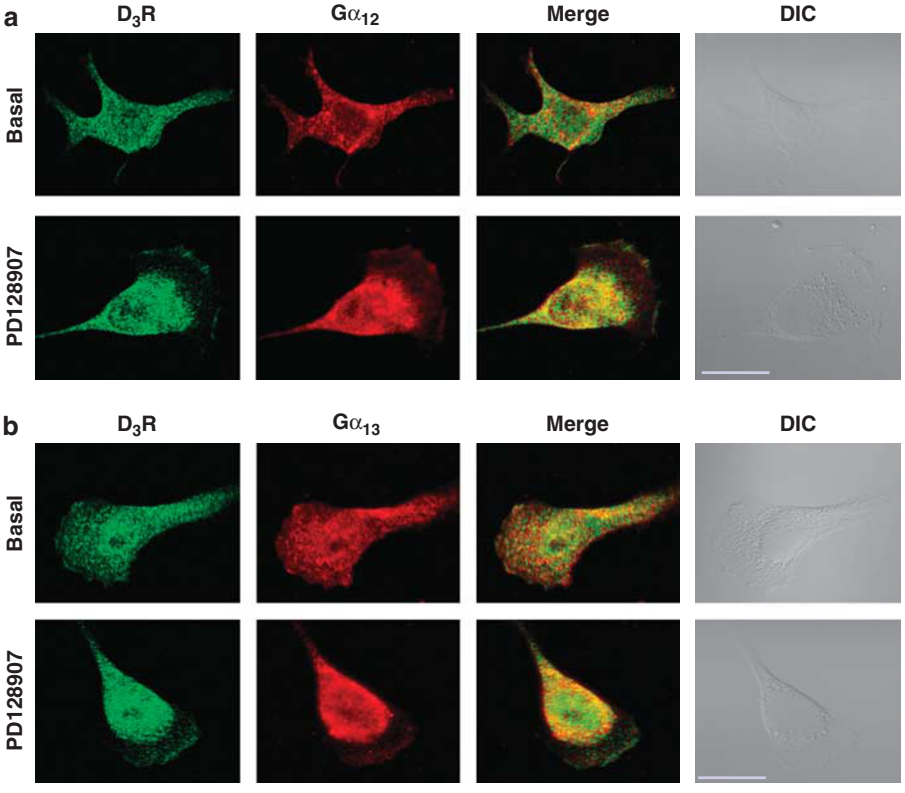

Figure 3 Co-localization of $G\alpha_{12}$, $G\alpha_{13}$ and D_3 receptors in RPT cells. The cells grown on coverslips were serum-starved before being treated with PD128907 (10^{-8} M/15 min). The cells were then fixed and double-immunostained for D_3 receptor and $G\alpha_{12}$ or $G\alpha_{13}$, as described in Methods. Colocalization was evaluated via laser-scanning confocal microscopy and appears as yellow punctate areas in merge images between D_3R and $G\alpha_{12}$ (**a**) or $G\alpha_{13}$ (**b**). Differential interference contrast (DIC) images were obtained to indicate the cellular confines of the cells. Magnification ×600, scale bar=10 μm.

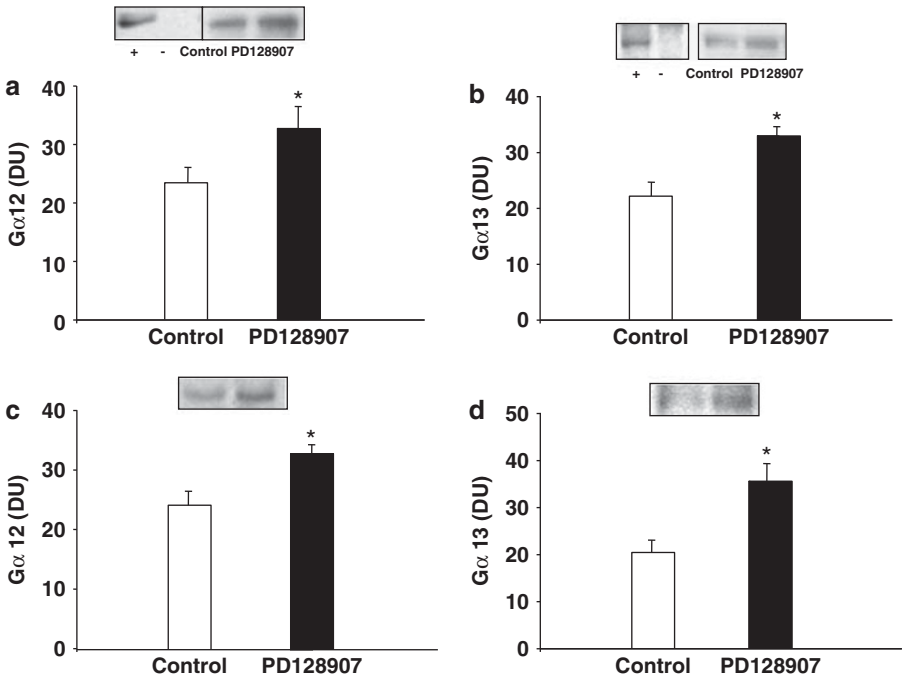

Figure 4 Co-immunoprecipitation of $G\alpha_{12}$ or $G\alpha_{13}$ and D_3 receptors in kidney and RPT cells. (**a**, **b**) Co-immunoprecipitation of $G\alpha_{12}$ or $G\alpha_{13}$ and D_3 receptors in rat kidney. BBMs were prepared from kidneys of WKY rats treated with vehicle or PD128907 ($1.0\,\mu g\,kg^{-1}\,min^{-1}$). The same amounts of protein were then immunoprecipitated with D_3 receptor antibody and immunoblotted for $G\alpha_{12}$ (**a**) or $G\alpha_{13}$ (**b**). For positive controls, anti-$G\alpha_{12}$ and $G\alpha_{13}$ antibodies ($1\,\mu g\,ml^{-1}$) were used as the immunoprecipitants; for a negative control, normal rabbit IgG ($1\,\mu g\,ml^{-1}$) was used as the immunoprecipitant instead of the anti-D_3 antibodies and immunoblotted with anti-$G\alpha_{12}$ or $G\alpha_{13}$ antibodies as above. $^*P<0.05$ *vs.* control ($n=6$), ANOVA, Duncan's test, DU=density units. (**c**, **d**) Co-immunoprecipitation of $G\alpha_{12}$ or $G\alpha_{13}$ and D_3 receptors in RPT cells. RPT cells from WKY rats were treated with vehicle or PD128907 (10^{-8} M every 30 min). The same amounts of protein were then immunoprecipitated with D_3 receptor antibody and immunoblotted for $G\alpha_{12}$ (**c**) or $G\alpha_{13}$ (**d**). For positive controls, anti-$G\alpha_{12}$ and $G\alpha_{13}$ antibodies ($1\,\mu g\,ml^{-1}$) were used as the immunoprecipitants; for a negative control, normal rabbit IgG ($1\,\mu g\,ml^{-1}$) was used as the immunoprecipitant instead of the anti-D_3 antibodies and immunoblotted with anti-$G\alpha_{12}$ or $G\alpha_{13}$ antibodies as above. $^*P<0.05$ *vs.* control ($n=7$), ANOVA, Duncan's test, DU=density units.

sure.[23] That D_3 receptors can mediate natriuresis is supported by the decreased ability of D_3 receptor-deficient mice to excrete an acute saline load.[24] Consistent with our previous study,[13] the intrarenal infusion of PD128907, a D_3 receptor agonist, increases sodium excretion in salt-loaded WKY rats.

We now report a linkage between the D_3 receptor and $G\alpha_{12}$ and the D_3 receptor and $G\alpha_{13}$. The D_3 receptor negatively regulates renin secretion.[24] $G\alpha_{12}$ and $G\alpha_{13}$ can increase intracellular calcium, and calcium can decrease renin secretion.[25,26] It is tempting to speculate that $G\alpha_{12}$ or $G\alpha_{13}$ may be important in the D_3 receptor-mediated negative regulation of renin secretion, as well as sodium transport in normotensive rats. $G\alpha_{12}$, $G\alpha_{13}$ and D_3 receptor influence sodium transport by regulating the activities of the Na^+–K^+–ATPase and sodium–hydrogen exchanger 1. $G\alpha_{12}$ and $G\alpha_{13}$ stimulate sodium–hydrogen exchanger 1,[27] whereas D_3 receptor inhibits Na^+–K^+-ATPase and NHE3 activities[9,28,29] in adult WKY rats. The fact that a D_3 receptor agonist increases the interaction between the D_3 receptor with either $G\alpha_{12}$ or $G\alpha_{13}$ in renal BBMs and RPT cells from WKY rats suggests that the D_3 receptor may participate in the regulation of sodium transport/pump activity by hampering $G\alpha_{12}$ or $G\alpha_{13}$ action, similar to that suggested for the D_5 receptor.[8] It is possible that the increase in co-immunoprecipitation following agonist stimulation between the D_3 receptor, $G\alpha_{12}$ and $G\alpha_{13}$, and therefore, reduction of 'free' $G\alpha_{12}$ and $G\alpha_{13}$, may explain the inhibitory effect of D_3 receptor on other sodium transporters (for example, Na/Pi, Na^+/HCO_3^-, Cl^-/HCO_3^-), which needs to be confirmed in the future. However, the negative regulation of renin secretion by the D_3 receptor is probably independent of $G\alpha_{12}$ as it is not expressed in juxtaglomerular cells[8] or $G\alpha_{13}$, but by its ability to decrease cAMP production.[26,30]

In summary, we found that stimulation of the D_3 receptor increases sodium excretion in WKY rats and the effect is, in part, via inhibition of Na^+–K^+–ATPase activity. There is linkage between D_3 receptors, $G\alpha_{12}$ and $G\alpha_{13}$ in RPTs, which is increased by stimulation of D_3 receptor in WKY rats, suggesting that the D_3 receptor may participate in the regulation of sodium transport by hampering $G\alpha_{12}$ and/or $G\alpha_{13}$ action.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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